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Valorisation of the Peptidic Fraction of Cheese Whey

Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação de
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Julho de 2007

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Título da tese

Valorização da fracção peptídica do soro de queijo

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Ano de conclusão 2007

Doutoramento em Engenharia Química e Biológica

Universidade do Minho, 31 de Julho de 2007

Acknowledgements

A PhD thesis is not possible without the contribution of several people and institutions. Thus I would like to acknowledge:

My supervisors, Prof. José Teixeira and Prof. Pilar Gonçalves, for believing in me, for their scientific guidance and for their help in solving research problems when they were popping up. I would like to thank Prof. Teixeira for his inconditional optimism and for having set the basis for my future scientific work. To Prof. Pilar Gonçalves I would like to thank her close supervision during my stay at FEUP, precise guidelines and scientific accuracy;

All my colleagues at Minho University, particullarly to the "Lipinhos" for their friendship, co-operation and good humour;

All staff of DEB, particularly to "Sr. Santos" for being able to solve most of my "operational" problems and Engª Madalena Vieira for always being available when she was needed, even though it would mean working late or out of hours, and for all the technical help in the analytical questions (HPLC, chloride and sodium analysis, ...);

Maria do Carmo for the discussions about peptide analysis and for her availability in trying electrophoresis 2-D;

Lilla Dücso for all the help in the initial establishment of the immobilization protocols;

Davi Neri for his gentle contribution with the production of POS-PVA and for the support with the SEM photos;

Prof. Victor Freitas and to Ms. Zélia Azevedo for allowing me to perform the RP-HPLC/MS analyses at FCUP;

Dr. Paula Sampaio from the IBMC for the availability, warmful reception and invaluable help during the gels microstructure analyses;

Fábio Larotonda, Loïc Hilliou and Margarida, for their pleasant collaboration during the year I spent at FEUP. To Loïc Hilliou I would like also to acknowledge all his help on the instrumentation questions and for initiating me in the meanders of rheology;

DEB/UM and DEQ/FEUP for providing the material conditions to do this work;

ESTG/IPVC for allowing me to do this thesis;

My parents, for always making me believe I could do anything I put myself into and for always arranging the means for that to happen;

My parents and parents-in-law for their caring support and for being always available to help in my "family duties", especially with the girls. This would have not been possible without the four of them;

Sofia and Sérgio for all the optimistic incentive through these four years;

António for his inconditional and caring support during all these years and, of course, for all his help in editing and revising the dissertation;

The girls, Sofia and Catarina, just for being there for better and for worse with all their energy and big smily or not so smily, but always beautiful, faces, allowing me to believe on all the gray days that all problems were minor and eventually solvable.

Projecto co-financiado pelo fundo Social Europeu no âmbito do concurso Público 1/5.3/PRODEP/2003, pedido de financiamento nº 1012.012, da medida 5/acção 5.3 – Formação Avançada de Docentes do Ensino Superior submetido pela Escola Superior de Tecnologia e Gestão do Instituto Politécnico de Viana do Castelo.

To Cátia

Summary

Cheese whey is a waste effluent with a high polluting content that cannot disposed directly into the environment and can become an environmental and economical problem for dairy industries. Cheese whey treatment for disposal can be expensive and laborious as it is highly putrescible and has a very low solid concentration. Valorisation of dairy by-products is thus of great interest for economic and environmental reasons.

Bovine milk whey proteins are widely used in food formulations due to their nutritional and functional properties. In fact, whey proteins have a high nutritional capacity and balanced amino acid content, particularly essential amino acids. Furthermore, major whey proteins, α-lactalbumin and β-lactoglobulin, are an important source of bioactive peptides, compounds with a health promoting potential. The functional applications of whey proteins include emulsification, gelation, foaming and filler/water binder.

The wish of the food industry to convert waste products into value-added, high-priced commodities has inspired a growing interest in the development of processes for the enhancement of whey protein functionality. Thus, the modification of whey proteins to improve their functional properties in specific food systems has become a focus of current research. One market that is in huge expansion is that of health-promoting foods. However, such novel products have to meet consumer acceptance, in terms of efficacy, organoleptic properties and price. Therefore the development of health-promoting foods comprises a range of processes which need to be integrated, including optimisation of protein hydrolysis, peptide characterization, study of peptides' physical-chemical properties and interactions with other food components (lipids, polysaccharides, salts and others) and establishment of a standard methodology to determine biological activity *in vivo*.

Based on the premises described in the previous paragraphs, the work presented in this document is the result of a plan that aimed at studying the hydrolysis of whey proteins for food applications. In particular, the research undertaken was directed to the hydrolysis of whey proteins (aiming at changing their functional properties) and to the study of rheological interactions between whey proteins/hydrolysates and galactomannans, with the final goal of obtaining new textures, with high protein content or with interesting (e.g. bioactive) peptides that can be used in existing food formulations or in the development of new food products. The hydrolysis of whey proteins was performed with the aid of enzymes, both free and immobilized in different carriers. A comparison was established for the various conditions tested based on the enzyme's activity and specificity, kinetic parameters and peptide profile of the hydrolysates produced. The gelling properties of the hydrolysates were tested and the hydrolysates were combined with a polysaccharide (locust bean gum), in order to evaluate the interaction of those components in terms of possible new functional properties.

The work performed allowed to conclude that the choice of the hydrolysis enzyme is particularly important in determining the properties of the resulting hydrolysates. Besides choosing the type of enzyme it is also important to select an adequate form of the chosen enzyme with the adequate purity and treatment (for instance a treated trypsin with low chymotryptic activity) for the desired application, as different hydrolysates are achieved with different forms of the enzyme. The selection of the adequate operational conditions (time, pH and temperature) also determines the composition of the resulting hydrolysate. Higher reaction times lead obviously to higher degrees of hydrolysis and smaller peptides (usually more hydrophobic) and pH and temperature determine the resistance of whey proteins to the hydrolysis as well as the activity of the enzyme.

Considering the enzyme immobilization procedures, the activity recovery was low with all carriers except for trypsin crosslinked on zeolites, where it was satisfactory. However, when a more purified enzyme from bovine pancreas was used with glyoxyl-spent grain or POS-PVA with glutaraldehyde, the activity retention was of 46 % and 73 % against 11 % and 9 % with crude enzyme.

Thus it can be stated that trypsin was successfully immobilized on spent grains by multipoint covalent attachment using glycidol and on POS-PVA functionalized with glutaraldehyde. Even so, the immobilized trypsin with the highest activity was achieved with covalent binding through glutaraldehyde to silanized zeolite followed by crosslinking with glutaraldehyde, probably due to a positive effect of the zeolite on the enzyme activity.

Only trypsin immobilized on spent grain showed significant activity towards whey proteins. Although trypsin immobilized on cross-linked zeolite NaY and trypsin covalently immobilized on POS-PVA and glutaraldehyde have shown a high activity towards a small substrate (e.g. BAPNA), this did not happen when whey proteins were used as substrate. Peptide profile of hydrolysates from whey protein isolate with both free and immobilized on spent grain enzymes were similar, which indicates that spent grains can be used as carriers for trypsin to produce hydrolysates similar to those obtained with the free enzyme. The control of the extent of the hydrolytic reaction is extremely important to ensure that a hydrolysate with the intended properties is obtained. The immobilization allows such control by simply withdrawing the enzyme from the reaction medium, without the need of using high temperatures or considerable pH shifts. Further, immobilization also allows the reuse of the enzyme, with obvious advantages from the economical point of view.

The gelling ability of whey proteins can be changed by limited hydrolysis. Depending on the environmental conditions it can either be improved or impaired. At WPC concentrations close to the gelling point, stronger gels with lower gelation temperatures can be achieved with limited hydrolysis of whey proteins. However, at higher protein concentrations this effect is impaired. There is an increase of the gel strength with the increase of the protein concentration, as expected, but this increase is smaller for the hydrolysates than for the intact proteins. In fact, a similar increase in the protein concentration corresponds to a lower increase in the amount of protein with effective gelation ability in the case of the hydrolysates. The relative importance of non-covalent interactions in the structure of whey protein gels seems to increase with the degree of hydroslysis.

LBG alters the microstructure of whey protein gels by modifying the equilibrium between aggregation and segregation. The gelation time is also decreased. The volume of the protein-enriched phase decreases with the increase of the LBG concentration and the protein concentration probably increases within that phase. The final structure of the gels is a result of the equilibrium between aggregation and segregation and of the increase of the protein concentration on the protein-enriched phase. The behaviour of gels from whey proteins or whey protein hydrolysates towards the presence of LBG is very similar. For whey proteins and for whey protein hydrolysates a small amount of LBG in the presence of salt leads to a big enhancement in the gel strength.

The gelation process is very sensible to environmental conditions and to processing and often leads to quite coarse data. The factorial planning used in this work allowed validating conclusions using fewer experiments than those needed if no planning had been used, while still getting statistical significance out of the results. However, as many factors are involved, the modelling of the process was not straightforward. A simple linear or quadratic function was generally not enough to accurately describe the system behaviour.

In short, hydrolysates with many different functional, nutritional and biological properties can be produced by manipulating the hydrolysis conditions and the source of the enzyme (alone or in combination; free or immobilized; pure or impure; ...). The introduction of a polyssacharide allows an even bigger range of functional properties and can be used to adjust the desired property to the desired application.

Resumo

O soro de queijo é um efluente das indústrias de lacticínios, muitas vezes rejeitado directamente para rios e lagos sem qualquer tratamento, que pode tornar-se um problema ambiental e económico para as indústrias de lacticínios. Regra geral, o processamento convencional do soro pode ser caro e trabalhoso já que é altamente putrescível e tem um baixo teor de sólidos. Assim, a valorisação de sub-produtos do soro é muito interessante tanto por razões económicas como por razões ambientais.

As proteínas contidas no leite de vaca são muito utilizadas na formulação de produtos alimentares devido às suas propriedades nutricionais e funcionais. De facto, as proteínas do soro têm uma capacidade nutritiva elevada e um conteúdo em aminoácidos muito equilibrado, em particular no que se refere a aminoácidos essenciais. Por outro lado, as principais proteínas do soro (α-lactalbumina e β-lactoglobulina) são uma fonte importante de péptidos bioactivos, que são compostos potencialmente benéficos para a saúde. As aplicações funcionais das proteínas do soro incluem a emulsificação, gelificação, formação de espuma e como ligantes de água.

O desejo da indústria alimentar em converter resíduos em produtos de elevado valor acrescentado levou a um interesse crescente no desenvolvimento de processos para o aumento da funcionalidade das proteínas do soro. Assim sendo, a modificação das proteínas do soro tendo em vista a melhoria das suas propriedades funcionais em sistemas alimentares específicos tornou-se um foco de atenção para os investigadores da área. O Mercado dos alimentos benéficos para a saúde, por exemplo, está em franca expansão. No entanto, esses novos produtos têm que ir ao encontro dos desejos dos consumidores em termos de eficiência, propriedades organolépticas e preço. Isto significa que o desenvolvimento de alimentos que promovem a saúde implica a integração de vários processos, incluindo a optimização da hidrólise proteica, a caracterização dos péptidos formados e o estudo das suas propriedades fisico-químicas e das interacções com outros componentes dos alimentos (lípidos, polissacarídeos, sais, entre outros), e o estabelecimento de uma metodologia-padrão para determinar a actividade biológica *in vivo*.

Baseado nas premissas descritas nos parágrafos anteriores, o trabalho aqui apresentado é o resultado de um plano cujo objectivo principal foi o de estudar a hidrólise de proteínas do soro para aplicações alimentares. Em particular, a investigação efectuada dirigiu-se à hidrólise de proteínas do soro (no sentido de mudar as suas propriedades funcionais) e ao estudo das interacções reológicas entre as proteínas do soro/seus hidrolisados e galactomananos, tendo por objectivo final a obtenção de novas texturas, com um elevado conteúdo proteico ou contendo péptidos bioactivos que possam utilizar-se em formulações alimentares já existentes ou no desenvolvimento de novos produtos alimentares. A hidrólise das proteínas do soro realizou-se com o auxílio de enzimas, quer livres quer imobilizadas em diferentes suportes. Estabeleceu-se uma comparação entre as várias condições testadas baseada na actividade e especificidade enzimática, em parâmetros cinéticos e no perfil peptídico dos hidrolisados obtidos. Testaram-se as propriedades gelificantes dos hidrolisados e combinaram-se estes com um polissacarídeo (goma de semente de alfarroba), por forma a avaliar a interacção destes components no sentido de obter novas propriedades funcionais.

O trabalho desenvolvido permitiu concluir que a escolha da enzima para hidrólise é determinante para as propriedades dos hidrolisados resultantes. Além disso, é também importante seleccionar a forma adequada da enzima escolhida, com a pureza e o tratamento adequados para a aplicação desejada, uma vez que se obtêm hidrolisados diferentes com diferentes formas da enzima. A selecção das condições operacionais adequadas (tempo de hidrólise, pH e temperatura) também é determinante na composição do hidrolisado resultante. Maiores tempos de hidrólise conduzem a maiores graus de hidrólise e a péptidos de menor tamanho (normalmente mais hidrofóbicos), e o pH e a temperatura são determinantes quer na resistência das proteínas do soro à hidrólise quer na actividade enzimática.

Relativamente ao procedimentos de imobilização, a recuperação de actividade foi baixa para todos os suportes testados, excepto para tripsina reticulada em zeólitos, onde se obteve um valor satisfatório. No entanto, quando se imobilizou uma enzima mais pura de pancreas bovino em *drêche* ou POS-PVA com glutaraldeído, a retenção de actividade obtida foi de 46 % e

73 % contra 11 % e 9 %, obtidos com a enzima menos purificada. Pode, portanto, afirmar-se que a tripsina foi imobilizada em *drêche* com sucesso por ligação covalente múltipla utilizando glicidol e em POS-PVA funcionalizado com glutaraldeído. Ainda assim, a maior actividade enzimática obteve-se para tripsina imobilizada em zeólito silanizado por ligação covalente com glutaraldeído seguida de reticulação com glutaraldeído, eventualmente devido a um efeito positivo do zeólito sobre a actividade enzimática.

Apenas a tripsina imobilizada em *drêche* mostrou uma actividade significativa sobre proteínas do soro. Apesar de se terem obtido valores elevados de actividade para tripsina imobilizada em zeólito NaY reticulado e para tripsina imobilizada covalentemente em POS-PVA e glutaraldeído actuando sobre um substrato de pequena dimensão (BAPNA), o mesmo não aconteceu quando se utilizaram proteínas do soro. O perfil peptídico dos hidrolisados de isolado de proteína do soro quer com enzima livre quer com enzima imobilizada em *drêche* foi semelhante, indicando que a *drêche* se pode utilizar como suporte para tripsina para produção de hidrolisados semelhantes aos que se produzem com a enzima livre. O controlo da extensão da reacção de hidrólise é extremamente importante para garantir que se obtém um hidrolisado com as propriedades pretendidas. A imobilização da enzima permite esse controlo simplesmente retirando a enzima do meio reaccional, sem necessidade de recorrer a temperaturas elevadas ou variações de pH consideráveis. Além disso, a imobilização também permite a reutilização da enzima, com vantagens óbvias sob o ponto de vista económico.

A capacidade gelificante das proteínas do soro pode ser modificada por hidrólise limitada. Dependendo das condições ambientais pode ser melhorada ou diminuída. A concentrações de proteínas de soro próximas do ponto de gelificação a hidrólise limitada conduz a geis mais fortes com menores temperaturas de gelificação. Contudo, a concentrações de proteína mais elevadas este efeito é invertido. Há um aumento da força do gel com o aumento da concentração de proteína, como esperado, mas esse aumento é menor no caso dos hidrolisados do que com proteínas intactas. De facto, no caso dos hidrolisados, um aumento semelhante na concentração de proteína corresponde a um aumento menor na concentração de proteína com capacidade gelificante efectiva. A importância relativa das interacções não covalentes na estrurura dos geis parece também aumentar com o grau de hidrólise.

A presença de goma de alfarroba altera a micro-estrutura dos geis de proteínas de soro, modificando o equilíbrio entre a agregação e a segregação. O tempo de gelificação diminuiu. O volume da fase rica em proteína diminuiu com o aumento da concentração de goma e a concentração de proteína aumentou provavelmente dentro dessa fase. A estrutura final dos geis é o resultado não só do equilíbrio entre agregação e segregação mas também do aumento da concentração de proteína na fase rica em proteína. O comportamento dos geis de proteínas de soro e de hidrolisados de proteína de soro na presença de goma de alfarroba é semelhante. Em ambos os casos, uma quantidade pequena de goma em presença de sal conduz a um grande aumento da força do gel resultante.

O processo de gelificação é muito sensível às condições ambientais e ao processamento e conduz frequentemente a dados muito dispersos. A planificação factorial usada permitiu validar conclusões usando menos experiências que as necessárias se nenhuma planificação tivesse sido realizada, obtendo-se resultados com significância estatística. Contudo, como estão envolvidos muitos factores, a modelação do processo não foi fácil. Uma função simples linear ou quadrática não foi genericamente suficiente para descrever o comportamento do sistema de forma precisa.

Resumindo, podem ser produzidos hidrolisados com propriedades funcionais, nutricionais e biológicas muito diferentes por manipulação das condições de hidrólise e da fonte escolhida para a enzima (só ou combinada, livre ou imobilizada, pura ou impura, por exemplo). A introdução de um polissacarídeo permite uma gama ainda maior de propriedades funcionais e pode ser usada para ajustar as propriedades desejadas à aplicação pretendida.

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List of general nomenclature

Symbol

AN_1 – amino nitrogen content of the protein substrate before hydrolysis [mg $g_{protein}^{-1}$]

AN_2 – amino nitrogen content of the protein substrate after hydrolysis [mg $g_{protein}^{-1}$]

B – base consumption [mL]

C – concentration [g/L]

C_0 – critical concentration [$mol.L^{-1}$]

DH – degree of hydrolysis [-]

f – frequency [Hz]

G' – storage modulus [Pa]

G'' – loss modulus [Pa]

G^* – complex modulus [Pa]

h – number of cleaved peptide bonds [-]

h_{tot} – number of peptide bonds in the intact protein [$mequivalent_{peptide\ bonds}.g_{protein}^{-1}$]

IC_{50} – concentration of substance needed to inhibit 50 % of the original ACE activity [$mol.L^{-1}$]

K_M – apparent Michaelis constant [$equivalent_{peptide\ bonds}.L^{-1}$]

k' – Huggins' coefficient [-]

k'' – Kramers' coefficient [-]

M/G – mannose to galactose ratio [-]

$\overline{M}_v$ – viscosity average molecular weight [-]

m – average relative molar mass [-]

m_P – mass of protein being hydrolysed [g]

N_b – normality of the base [$mol_{base\ equivalents}.L^{-1}$]

Npb – nitrogen content of the peptide bonds in the protein substrate [mg $g_{protein}^{-1}$]

pK – average dissociation value for the α-amino groups liberated during hydrolysis [-]

RT – eluition time [min]

T - temperature [°C]

Tg – gelation temperature when a ramp of 2 °C/min is applied [°C]

t – time [min]

t_c – critical gelation time [min]

tg – gelation time at 80 °C after a ramp of 2 °C/min during 30 min [s]

v_0 – rate of protein denaturation [$mol.min^{-1}.L^{-1}$]

v_i – rate of hydrolysis [$mol.min^{-1}.L^{-1}$]

ν_{max} – maximum hydrolysis rate [min^{-1}]

z – average ion electric charge

Greek symbols

α – (average) degree of dissociation of the α-NH_2 groups [-]

δ – loss angle [-]

γ – strain [-]

γc – critical strain [-]

γr – rupture strain [-]

η – viscosity [Pa.s]

η' – dynamic viscosity [Pa.s]

η'' – out-of-fase component of the complex viscosity [Pa.s]

η^* – complex viscosity [Pa.s]

η_s – solvent viscosity [Pa.s]

η_{sp} – specific viscosity [-]

η_{rel} – relative viscosity [-]

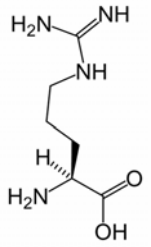
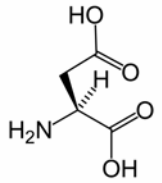
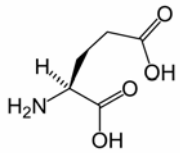
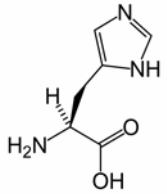
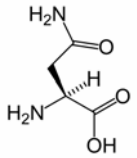
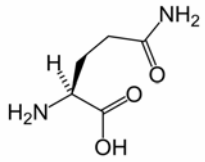
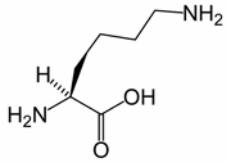
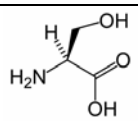
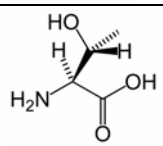
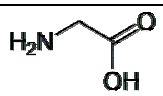
$[\eta]$ – intrinsic viscosity [dL/g]

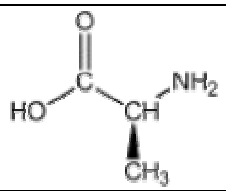
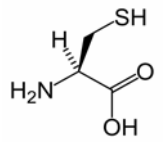
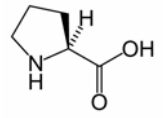
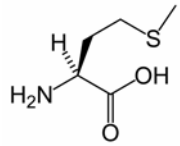
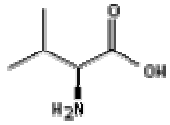
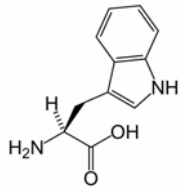
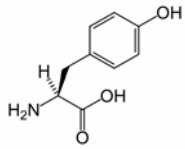
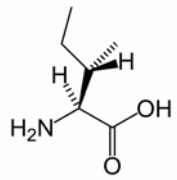
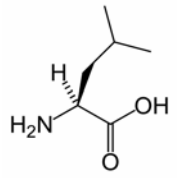
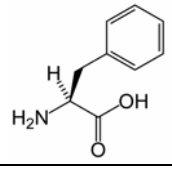
ρ – density [kg/m^3]

σ – shear stress [Pa]

ω – oscillatory frequency [rad/s]

Amino acids nomenclature (ordered by increasing hydrofobicity)

Name / chemical formula	1 letter code	3 letters code	Molecular weight (Da)	Structure
Arginine $C_6H_{14}N_4O_2$	R	Arg	174.2	
Aspartic acid $C_4H_7NO_4$	D	Asp	133.10	
Glutamic acid $C_5H_9NO_4$	E	Glu	147.13	
Histidine $C_6H_9N_3O_2$	H	His	155.16	
Aspargine $C_4H_8N_2O_3$	N	Asn	132.118	
Glutamine $C_5H_{10}N_2O_3$	Q	Gln	146.15	
Lysine $C_6H_{14}N_2O_2$	K	Lys	146.19	
Serine $C_3H_7NO_3$	S	Ser	105.09	
Treonine $C_4H_9NO_3$	T	Thr	119.12	
Glycine $C_2H_5NO_2$	G	Gly	75.07	

Alanine $C_3H_7NO_2$	A	Ala	89.1	
Cysteine $C_3H_7NO_2S$	C	Cys	121.16	
Proline $C_5H_9NO_2$	P	Pro	115.13	
Metionine $C_5H_{11}NO_2S$	M	Met	149.21	
Valine $C_5H_{11}NO_2$	V	Val	117.15	
Tryptophan $C_{11}H_{12}N_2O_2$	W	Trp	204.23	
Tyrosine $C_9H_{11}NO_3$	Y	Tyr	181.19	
Isoleucine $C_6H_{13}NO_2$	I	Ile	131.18	
Leucine $C_6H_{13}NO_2$	L	Leu	131.18	
Phenylalanine $C_9H_{11}NO_2$	F	Phe	165.19	

List of abbreviations

ACE – Angiotensin I-converting enzyme

BAEE – N-α-benzoyl-L-arginine ethyl ester

BAPNA – N-α-benzoyl-DL-arginine-p-nitroanilide

bh – Sodium borohydride

BLP – Protease from *Bacillus licheniformis* (Alcalase®)

BOD – Biological oxygen demand

BSA – Bovine serum albumin

CM – Carboxymethyl

COD – Chemical oxygen demand

DEAE – Diethylaminoethyl

DMSO – Dimethyl sulphoxyde

FTIR – Fourier transform infrared spectroscopy

GMP – Glycomacropeptide

HPLC – High performance liquid chromatography

Ig – Immunoglobulin

LBG – Locust bean gum

LBG20 – Fraction of the locust bean gum soluble at 20 °C

LBG80 – Fraction of the locust bean gum soluble between 20 and 80 °C

LBGP – Purified locust bean gum

OPA – o-phthaldialdehyde

P1.5 – Whey protein hydrolysate from pepsin with a degree of hydrolysis of 1.5 %

P2.5 – Whey protein hydrolysate from pepsin with a degree of hydrolysis of 2.5 %

P4.9 – Whey protein hydrolysate from pepsin with a degree of hydrolysis of 4.9 %

PEG – Polyethyleneglycol

POS-PVA – polysiloxane-polyvinyl alcohol composite

PP3 – Proteose peptone component 3

PP5 – Proteose peptone component 5

PP8 fast – Proteose peptone component 8 (fast)

PP8 slow – Proteose peptone component 8 (slow)

PVA – Polyvinyl alcohol

RBITC – Rhodamine B isothiocyanate

RP-HPLC – Reverse phase high performance liquid chromatography

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SHR – Spontaneously hypertensive rats

T1.0 – Whey protein hydrolysate from trypsin with a degree of hydrolysis of 1.0 %

T3.5 – Whey protein hydrolysate from trypsin with a degree of hydrolysis of 3.5 %

TCA – Tri-chloroacetic acid

TEOS – Tetraethylorthosilicate, Tetraethoxysilane

TFA – Tri-fluoroacetic acid

TG – Transglutaminase

TIM – Tube inversion method

TNBS – trinitrobenzenesulphonic acid

TPP – Total proteose peptone

TRIS – tris(hydroxymethyl)aminomethane

WP – Whey proteins

WPC – Whey protein concentrate

WPC35 – Whey protein concentrate with 35 % protein

WPH – Whey protein hydrolysate

WPI – Whey protein isolate

α-La – α-lactalbumin

β-Lg – β-lactoglobulin

List of tables

Chapter 1 Thesis outline

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Food technology is a multidisciplinary subject that involves knowledge from areas such as chemistry, biochemistry, termodynamics, physics and engineering.

Food markets are commercially very interesting but also very competitive. In search of added-value, companies are eager for attractive innovations that can easily be communicated to the target group of clients in order to wake their will to consume even if that means to pay a higher price. These innovations are usually introduced through new scientific and technological achievements that bring e.g. more pleasant textures, more nutritional benefits or a better overall appearance to the foods.

One market that is in huge expansion is that of health-promoting foods. Advances in food technology and scientific evidences linking diet to health and/or disease opened the way for improved "healthy" foods that are supposedly able to promote health and reduce the risk of diseases, instead of merely correcting nutritional deficiencies or achieving the need for basic nourishment (de Wit and Moulin, 2001; Korhonen, 2002; Saxelin and others, 2003; Menrad, 2003; Clydesdale, 2004). Prebiotics, probiotics, omega-3, fitosterols or bioactive compounds are terms that are growingly entering Europeans', Americans' and Japaneses' fridges.

The addition of bioactive peptides to food products that are targeted at particular consumer groups, for example specific health risk groups, aged people or athletes, becomes a strong marketing tool. However, such novel products have to meet consumer acceptance, in terms of efficacy, organoleptic properties and price. Thus, the development of health-promoting foods comprises a range of processes which need to be integrated, including optimisation of protein hydrolysis, peptide characterization, study of peptides' physical-chemical properties and interactions with other food components (lipids, polyssacharides, salts and others) and establishment of a standard methodology to determine biological activity *in vivo*.

The recycling of whey from cheese production industries has the double advantage of reducing the polluting load of the effluents (with all the economic benefits, both direct and indirect, that can be obtained from this action) and of obtaining added-value products.

Although the production of whey proteins is not the main aim of the dairy industry at the moment, whey proteins are widely used in food formulations due to their nutritional and functional properties. Besides their classical nutritional benefits, they are interesting for their excelent functional properties (such as emulsifying, gelling or foaming abilities) as well as for being a potential source of many bioactive peptides. Whey protein hydrolysis can release these bioactive sequences, reducing at the same time the allerginicity

of some whey proteins. Hydrolysis under limited conditions can also improve whey protein functional properties. The use of immobilized proteins in the hydrolysis process allows for a better control of the end of the process, leading to a hydrolysate with the desired properties (either functional, nutritional or health promoting).

The functionality of whey proteins can also be changed by the presence of other components. Proteins and polysaccharides are present in many systems and have a fundamental importance in defining their structure, texture and stability, mainly due to their thickening or/and gelling properties. Though there is a deep knowledge about functional properties of proteins and polyssacharides individually, and some knowledge about the role of protein-polyssacharide interactions in the functionality of complex multiphasic systems, not much is known about the interaction of whey protein hydrolysates and polyssacharides.

The aim of this thesis is to study the hydrolysis of whey proteins for food applications. Key areas are a) the hydrolysis of whey proteins to change their functional properties and b) rheological interactions between whey proteins/hydrolysates and galactomannans. The final objective is to obtain new textures, with high protein content or with bioactive peptides that can be used in existing food formulations or in the development of new food products.

Figure 1-1 resumes the motivation for this thesis.

The text is organised in eight chapters. Each of the chapters containing experimental results (Chapters 3 to 7) is provided with a specific introduction and a specific list of references.

Chapter 2 presents a short overview on whey proteins, their properties and applications. Hydrolysis of whey proteins is then deeply reviewed and some basic concepts of enzyme immobilization are also provided (such concepts are needed for the immobilization of trypsin for whey protein hydrolysis). A “state-of-the-art” of whey proteins heat-set gelation is presented. Finally some basic concepts on the gelation of polysaccharide/protein mixed systems are explained.

Hydrolysis of whey proteins is introduced in Chapter 3. Trypsin, alcalase and pepsin are studied as possible proteolytic enzymes. The degree of hydrolysis and the peptide profile obtained by RP-HPLC/UV was monitored for different hydrolysis conditions; under such conditions, the optimal operational parameters were determined.

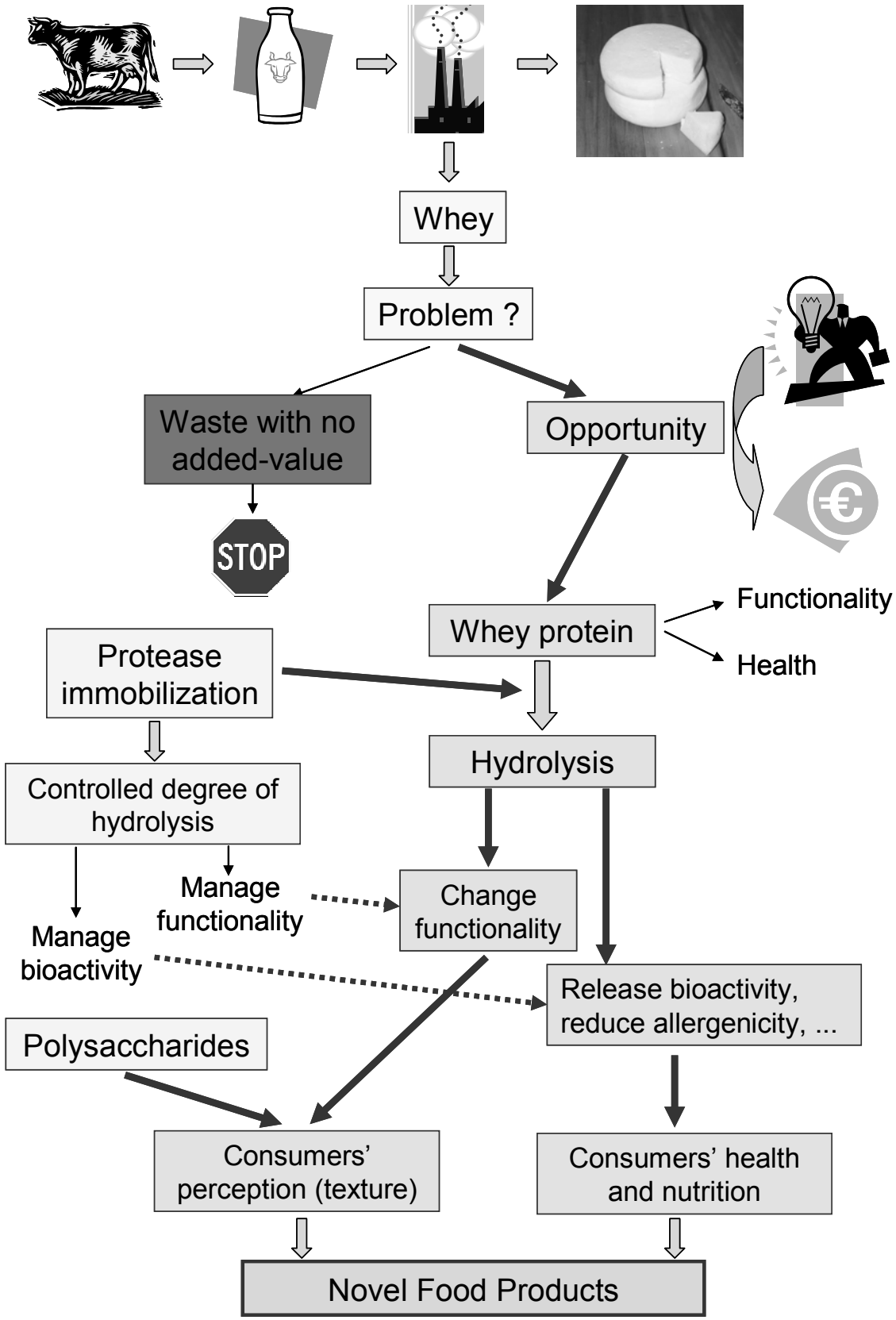

Figure 1-1: Schematic representation of the motivation for the work presented in this Thesis

In Chapter 4 several methods for trypsin immobilization are compared (trypsin is the main enzyme used to hydrolyse whey proteins in the subsequent chapters). Four different enzyme carriers are evaluated: silica, spent grains, POS-PVA and zeolites. Parameters such as the immobilization efficiency, activity retention, operational stability and storage stability of each immobilized enzyme are studied.

Chapter 5 assesses the influence of the immobilization process (optimized in Chapter 4) on the whey protein hydrolysis. In this chapter a comparison is made between the enzyme activity and specificity, kinetic parameters and peptide profile of the hydrolysates produced with free and immobilized trypsin.

Chapter 6 is dedicated to whey protein and whey protein hydrolysates gelling ability. The heat set gelling properties of whey protein concentrate, two whey protein hydrolysates from trypsin and three whey protein hydrolysates from pepsin with different degrees of hydrolysis are studied through small deformation oscillatory tests.

The behaviour of whey protein hydrolysates and locust bean gum mixtures is analysed in Chapter 7. Different fractions of locust bean gum are compared. The gel structure of the mixed systems is characterized based on confocal microscopy photographs.

Overall conclusions and suggestions for future work are presented in Chapter 8.

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Chapter 2 General introduction

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Whey is the milk serum (yellow-green liquid) that separates from the curd during casein coagulation. It can be produced either by acid precipitation (pH < 5) of caseins (acid or sour whey) or by rennet curdling (rennet or sweet whey).

The composition of whey has two important features particularly significant to its disposal: high content on lactose (fairly 100 % of the total milk lactose) and high protein content (about 20 % of the total milk proteins). These components are responsible for the high putrescibility and biological oxygen demand (BOD) of whey (Smithers and others, 1996). Whey has total solid content ranging between 6.0 – 7.0 % (w/v), a biological oxygen demand (BOD_5) of 30000 - 50000 ppm and a chemical oxygen demand (COD) of 60000 - 80000 ppm (Mawson, 1994; Siso, 1996; Durham and others, 1997, Pintado and others, 2001). Traditionally cheese whey was a waste effluent directly disposed into rivers and other "water resources". As the environmental restritions grew and due to its high polluting content, cheese whey could not be disposed directly into the environment anymore and became an environmental and economical problem for dairy industries. The use of whey as a by-product has two main drawbacks: its content in solids is very low, which makes the recovery of the whey components more expensive with high capital and energy costs due to the high volumes to be treated (Neville, 2006); and its perishable nature that imposes its stabilization right after its "production", for instance by pasteurization and refrigeration, or immediate processing (Durham and others, 1997). Bioconversion of whey lactose to ethanol, yeast biomass or methane (biogas) has been used to reduce the organic load by more than 75 % while producing saleable products but in most cases the resulting effluent still needs further treatment before disposal (Mawson, 1994).

In 1997, 83 000 000 ton of cheese whey were produced in the EU. In Portugal, 55 713 ton of cheese were produced in 2006 (INE, 2007) and, in the 25 countries of the European Union, 8 346 000 ton were produced in 2005. As ca. 9 kg are produced per kg of cheese one can predict that 500 000 ton of cheese whey were produced in 2006 in Portugal, and 75 000 000 ton in 2005 inside the EU. From these, only a small part is reused in Portugal (in 2005, only 22400 ton were further transformed), mainly by concentration/drying or directly in whey cheese manufacture (such as Portuguese *requeijão*) and animal feeding (drinking waters, for example), though 60 % of the produced whey is available for further use (Frazão, 2001). Concentrated whey or whey powder (mostly) and lactose are the main whey products used by the Portuguese industry. About 50 % of the produced whey powder is used for animal feed, while lactose is mostly employed for the cookies, chocolates, icecreams and confectionary industries (Figure 2-1).

Conventional whey processing involves high energy processes, such as evaporation and spray drying that are only economically viable when conducted on a very large scale. On the other hand, transport costs are high as water content in whey is also very high (high volumes are produced with low solid content that can be valorised). Thus, membrane pre-concentration to 20 % solids is sometimes employed to enable the whey to be transported to a central whey processing facility (Durham and others, 1997).

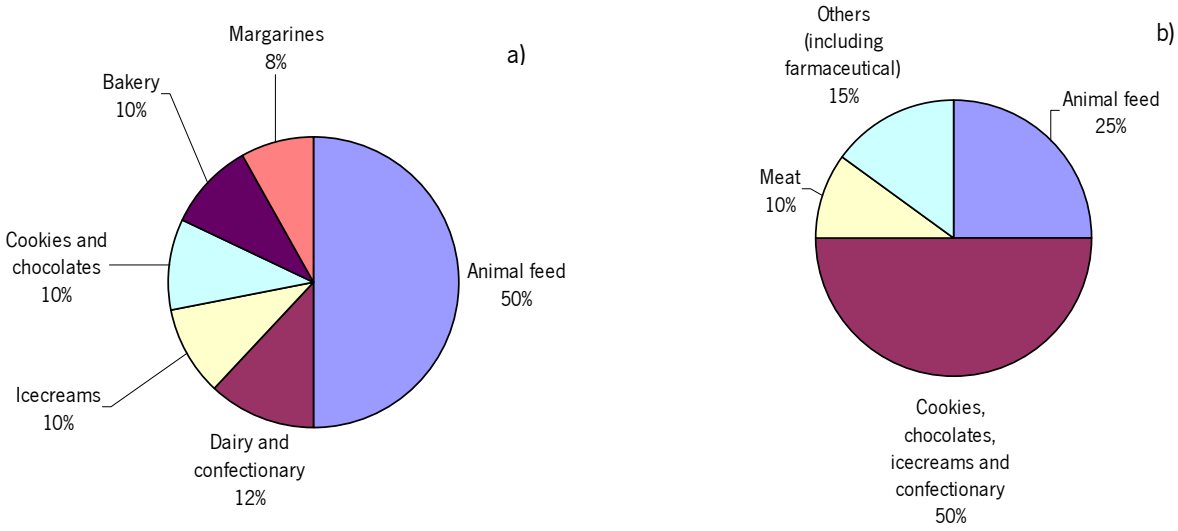


Figure 2-1 Application of whey products in the Portuguese industry (data from Frazão, 2001): a) dried whey; b) lactose

Valorization of dairy by-produtcs is thus of great interest for economic and environmental reasons (Brulé, 1995). The same reasons that make whey "difficult" to dispose of also make it interesting as a potential value added product. In fact, both lactose and whey proteins have a number of functional, physiological and nutritional properties that make them potentially useful in a wide range of applications (Table 2-1).

Table 2-1 Major features of using whey (adapted from Alais, 1984)

Strong	Weak
Protein fractioning: with high nutritional value (Lys, Thr, Leu, Ser)	High dilution – dehydration necessary – high energy costs
Major milk components recovery	High salt content (ca 10 % of dry matter)
Lactose production	High protein/sugar content – delactosation needed
Reduce pollution	Highly putrescible raw material (protection; celerity)
	Highly dispersed cheese production facilities
	Technical innovation needed (ultrafiltration, diafiltration, ...)

In Mesopotamia, ca. 5,000 B.C., Kanana discovered that warm milk stored in a bag made of fresh stomach skin of sheep (or goat) produced curds and, concomitantly, whey. Later, nomad shepherds started boiling whey in copper kettles and eventually obtained a nourishing solid food – a whey cheese (Pintado and others, 2001). Therapeutic applications were advocated already in Ancient Greece by Hippocrates (Jelen, 2002). Whey drinking cures have been used in antiquity, the Middle Ages and modern time (Barth and Behnke, 1997).

The unbalanced composition of whey solids has limited whey use in human food products (Table 2-2). On the one hand, high concentration in mineral salts leads to an excessive saline taste making it unable to use in dietetic or baby food, for instance. Nowadays several demineralization procedures have been developed to obviate this problem. On the other hand, its high content in lactose and lactose's low sweetening power (only 40 % when compared to sucrose) limits its use as a sweetner. A high proportion of whey would have to be added to sweeten the product to the required flavour and, as its protein content is low when compared to the lactose content, the protein/sugar ratio would be unbalanced and could fall out of that allowed by law. Besides, due to lactose low solubility (18 % in water at room temperature), this high proportion of whey could lead to a poorer texture of the product due to the formation of lactose crystals (critical for instance in icecream production). Both the sweetening power (up to 70 % when compared to sucrose) and the solubility of lactose are increased by hydrolysis into glucose and galactose. In this way, the use of cheese whey as a sweetener is facilitated (Siso, 1996).

Table 2-2 Composition of cow milk and whey

	Milk (%)	Cheese whey (%)
Proteins	3.2-3-5	0.6-1.0
Whey proteins	0.5-0.7	0.7
Caseins (α_{s1}; α_{s2}; β; κ; γ)	2.5-2.8	0.0
Lactose	4.8-4.9	4.5-5.0
Fat	3.7-4.5	0.05-0.5
Ash	0.7	0.59-0.7
Non-protein nitrogen	0.19	-
Total solids	12.7	5.8-6.6

Data compiled from Siso (1996); Smithers and others (1996); Fox and McSweeney (1998); Durham and others (1997)

Whey has also useful characteristics to several fermentation processes due to its composition (4.0-4.5 % lactose, 0.8-0.9 % soluble proteins, vitamins and minerals). When used as substrate in a fermentation process, proteins are removed from whey by ultrafiltration to remove soluble proteins with high nutritional value and, at the same time, to avoid problems with foam (Perea and others, 1993). This allows the production of a wide range of valuable products from the lactose such as biomass, β-galactosidase, ethanol, organic acids (acetic, propionic, lactic, citric, among others), fermented beverages, galactose, glycerol, xanthan gum, flavours and carotenoids.

Bovine milk whey proteins (WP) are widely used in food formulation due to their nutritional and functional properties (de Wit, 1998; Turgeon and Beaulieu, 2001). In fact, whey proteins have a high nutritional capacity and balanced aminoacid content, particularly essential aminoacids. Their biological value exceeds even that of whole egg protein. The sulphur amino acids content of whey proteins is higher than that of whole-milk proteins (1.35 % versus 0.36 %) and their lysine content is also higher in whey than in total milk-proteins (10.5 % versus 7.75 %), making it fit for special diets deficient in those aminoacids (Siso, 1996). Sulphur-containing amino acids also support antioxidant functions (Sinha and others, 2007). Furthermore, major whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), are an important source of bioactive peptides, compounds with a health promoting potential (Zhao and others, 1994; Gill and others, 1996; Mullally and others, 1997b; Pihlanto-Leppälä and others, 1997; Pihlanto-Leppala and others, 1998). Enzymatic digestion is a common way to produce these peptides (Madsen and others, 1997; Otte and others, 1997; Chen and others, 1994).

The functional applications of whey proteins include emulsification, gelation, foaming and filler/water binder. However, one of the main problems with whey proteins is their instability during heat treatment, such as pasteurization (Doucet and others, 2001). The wish of the food industry to convert waste products into value-added, high-priced commodities has inspired a growing interest in the development of processes for the enhancement of whey protein functionality (Hudson and others, 2000). Thus, the modification of whey proteins to improve their functional properties in specific food systems has become a focus of current research (Wilcox and Swaisgood, 2002).

The main products of industrial separation of the protein fraction from whey are whey protein concentrate (WPC) and whey protein isolate (WPI). WPC's are usually defined as whey protein products having a protein content between 34 and 85 % (de Wit, 2001; Huffman and Harper, 1999) while WPI's have at least 90 % (de Wit and Moulin, 2001).

Table 2-3 Whey ingredients in food products

Ingredients	Food product	Function
Whey powder	Sports nutrition specialties; dairy products (chocodrinks, yoghurt, icecream); bakery (bread, biscuits, cakes); meat and fish products (hams, surimi, comminuted); confectionery (chocolates, candies, aerated confections)	Nutrition (protein supplement); low cost milk solids; emulsification, foaming, gelation (egg albumin replacer); filler/ water binder; thickner
Demineralised whey powder	Infant formula (term, pre-term, follow-on)	Nutrition
WPC (35-80)	Infant formula; sports nutrition specialties; dairy products (chocodrinks, yoghurt, icecream); bakery (bread, biscuits, cakes); beat and fish products (hams, surimi, comminuted); confectionery; sports nutrition	Skim milk replacer (WPC35); gelation, emulsification; foaming; adhesion; nutrition;
WPI	Sports nutrition (sports beverages and powdered beverages), nutricional products; infant formula	Nutrition
Edible grade lactose	Infant formula; confectionary; meat products	Sweetner; flavour enhancer; texture enhancer, colour fixation
Pharmaceutical grade lactose	Pharmaceuticals (nutritional drugs, inhalers, tablets)	Tableting excipient; raw material for lactose derivatives (e.g. oligossacharydes or lactulose - prebiotics)
α-Lactalbumin	Infant formula (baby formula)	Nutrition
β-Lactoglobulin	Meat and fish products; fortified beverages; bakery; sports beverages)	Nutrition; gelling agent; replacement of egg white;
Lactoferrin	Infant formula; meat	Iron-binding; antimicrobial
Lactoperoxidase	Milk; pharmaceuticals; cosmetics	Bactericide, antioxidant; anticaries
Immunoglobulins	Nutraceuticals; dietetic foods (for AIDS patients, e.g.)	Immunological; anticancer
Whey protein hydrolysates	Infant formula; sports food (nutritional bars and drinks); dietetic foods (clinical foods, slimming foods, elderly foods)	Nutrition; reduce allergenicity; foaming; emulsification (alternativly to egg white)
Bioactive peptides	Dairy; nutraceuticals (probiotics, prebiotics, bioactive proteins); dietetic foods	Health promoter and nutrition

Data compiled from Barth and Behnke (1997); Durham and others (1997); de Wit (1998); Huffman and Harper (1999), de Wit (2001); Fox (2001)

In short, several products are made from liquid whey, including whey powder, lactose, demineralised whey powder, delactosied whey powder, milk salts, whey protein concentrates, whey protein isolates, lactalbumin (contains all of the heat precipitable whey proteins, is insoluble in water and heat stable and is used mainly to fortify foods (Huffman and Harper, 1999)), β-Lg, α-La, lactoferrin, lactoperoxidase, immunoglobulins, edible grade lactose, pharmaceutical grade lactose, whey protein hydrolysates, lactulose, lactitol, hydrolysed lactose, oligosaccharides, growth factors, bioactive peptides. Some applications of a few of these whey derivatives are summarized in Table 2-3.

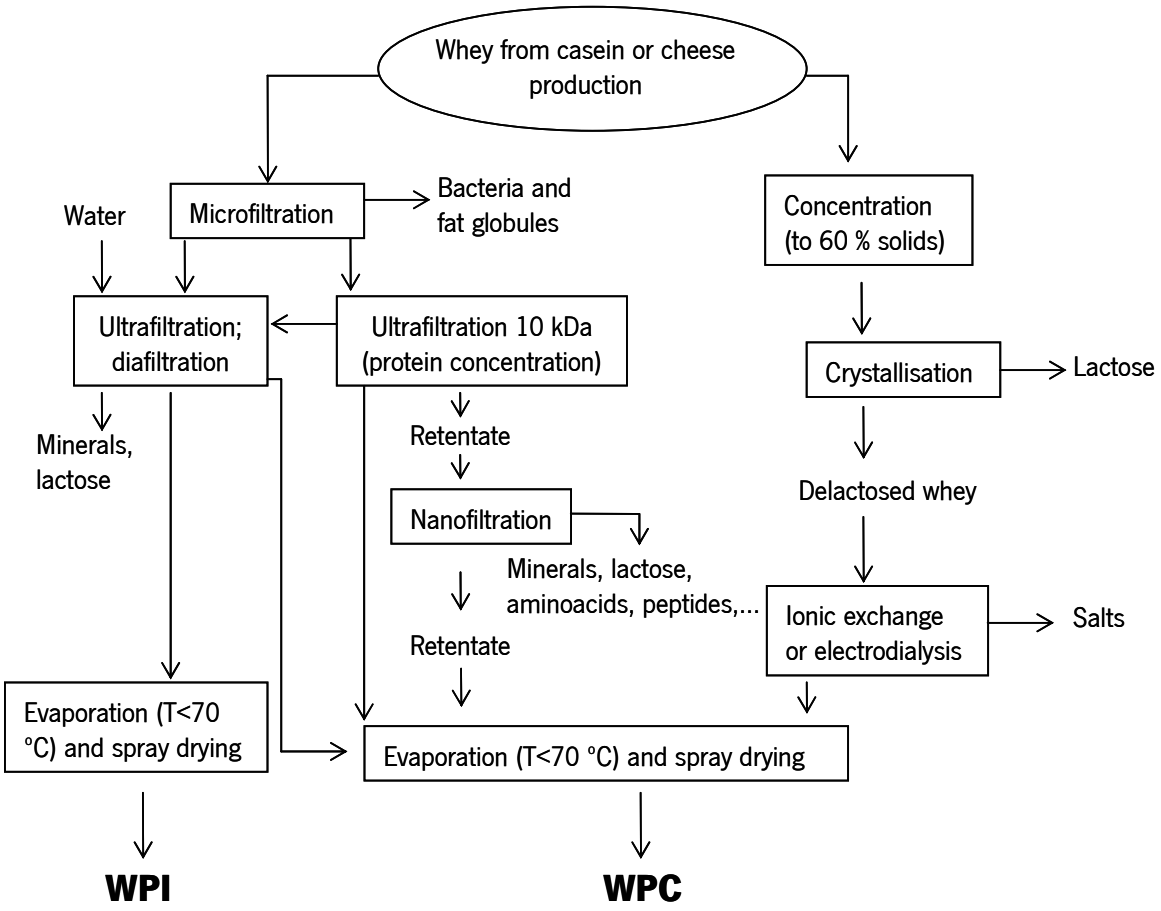


Figure 2-2 WPC and WPI production (Durham and others, 1997; de Wit, 2001; de Wit and Moulin, 2001)

Whey is usually processed by various methods such as pasteurization, vacuum evaporation, ultrafiltration, reverse osmosis, ion exchange, gel filtration, electrodialysis, crystallization and spray-drying (Ji and Haque, 2003) to produce low value comodities such as whey powder, whey protein concentrate with 35 % protein (WPC35) and edible lactose. The production of high value comodities, such as whey protein isolates (WPI), protein fractions, bioactive proteins, growth factors, pharmaceutical grade lactose and lactose by-products

such as lactulose is done only by a few big dairy companies in the world and is not yet widespread. Unlike the first group, these products are used mainly for human consumption.

A possible route to produce WPC or WPI is presented on Figure 2-2.

Commercial uses of WPCs have some drawbacks because of large variations in their functional properties, due to variations in the composition of ashes and proteins (Morr and Foegeding, 1990; Havea and others, 2002; Ji and Haque, 2003) that depend on the source, on the recovery method and on the industrial process used to produce the WPC or WPI. Whey protein concentrates' and isolates' typical composition is presented in Table **2-4**.

Table 2-4 Composition (%) of whey protein concentrate and isolate

Product	Protein	Lactose	Ash	Fat	Moisture
Skim milk	35	50	8	1	4
WPC 35	30-34	49-50	7-8	1-3	3-5
WPC 80	76	5-6	3-4	0.3-7	4-5
WPI	89-96	0.1-1	2-4	0.2-1	4

Data from Holt and others (1999); Huffman and Harper (1999); de Wit (2001); de Wit and Moulin (2001)

2.1 Whey proteins: nutritional and physiological properties

Whey proteins are the group of milk proteins that remain soluble in "milk serum" or whey after precipitation of caseins at pH 4.6 and 20 °C (Fox, 2001). They represent about 20 % of the cow's milk protein, are resistant to the action of chymosin and are rich in essential amino acids such as lysine, tryptophan, cystine, and methionine.

The WP's possess high levels of secondary, tertiary and, in most cases, quaternary structures. Most of them are globular proteins (feature responsible for many of their functional properties) and are denatured on heating (e.g. completely at 90 °C for 10 min). They are not phosphorylated and are insensitive to Ca^{2+}. All whey proteins contain intramolecular disulphide bonds that stabilize their structure (Fox, 2001).

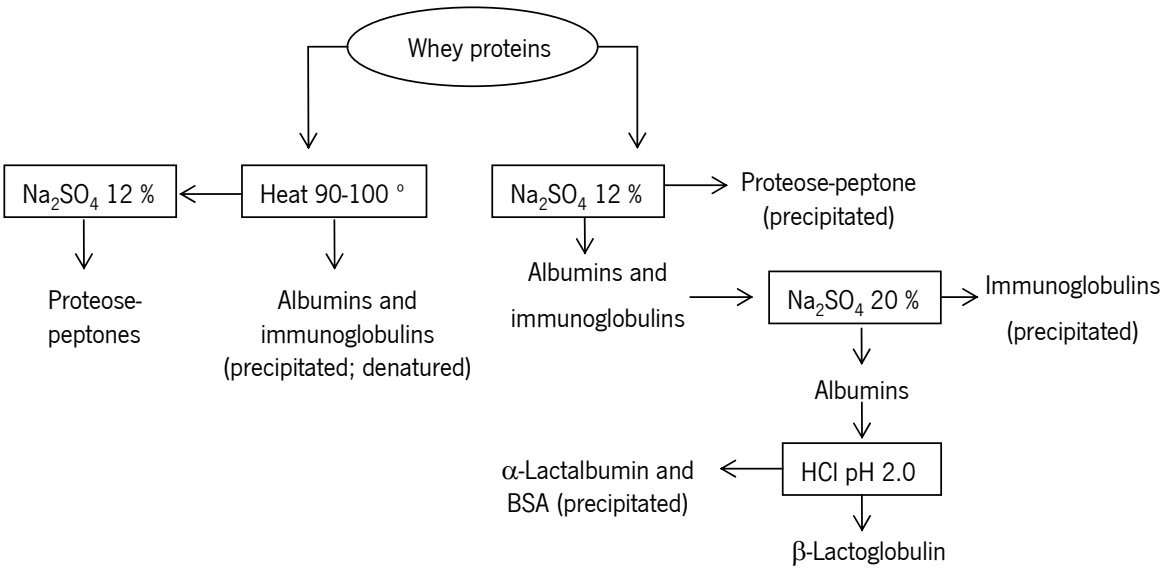


Figure 2-3 Whey proteins (adapted from Veisseyre, 1975 and Alais, 1984)

Heating whey is the most ancient method for separating whey proteins but they are highly denatured by this procedure. This protein fraction was usually called "albumin" and included all whey proteins except proteose-peptones. A rough (and traditional) way to separate and identify whey proteins is described in Figure 2-3. Three main groups are considered: albumins, immunoglobulins (formely included in the albumins group) and proteose-peptones. Albumins represent about 75 % of the total whey proteins and includes β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and serum albumin (BSA). More recent fractionating techniques allow more precise sorting and quantification of individual proteins.

The major whey proteins in cow's milk are β-Lg (50 %), α–La (12 %), immunoglobulins (10 %) and BSA (5 %). Acid and rennet wheys also contain casein-derived peptides; both contain proteose-peptones (that do not exist in human milk), produced by plasmin, mainly from β-casein, and the latter also contains glycomacropeptides produced by rennets from κ-casein (Fox and McSweeney, 1998). Although whey proteins concentration changes with many factors, typical values are given in Table 2-5.

2.1.1 β-lactoglobulin

β-lactoglobulin corresponds to ca 50 % of the bovine whey proteins and is absent in human milk. In its native form, bovine β-Lg is a globular protein with a monomer molecular weight of 18.3 kDa, with two disulphide bonds and one free thiol group, which exhibits an increased reactivity above pH 7 (Caessens and others, 1997). It has five cysteine residues (see Figure 2-4) and four of them are involved in the two disulphide bonds (66-160 and 106-119 or 106-121) that sustain the protein tertiary structure.

The secondary structure of β-Lg contains 43 % β-sheet, 10 % α-helix, and 47 % unordered structure, including β-turns (Papiz and others, 1986).

Table 2-5: Whey proteins

Protein	Concentration (g/L)	MW (kDa)	Isoelectric point	Biological function
β-lactoglobulin	2.7-3.0	18.36	5.2	Retinol carrier, binding fatty acids, possible antioxydant
α-lactalbumin	0.7-1.2	14.15	4.5-4.8	Lactose synthesis
Bovine serum albumin	0.3-0.4	69	4.7-4.9	Fatty acid transfer
Immunoglobulins	0.6-0.65	150-1000	5.5-8.3	Immunity
Lysozime	0.06-0.18	15	-	Antimicrobial, synergistic effect with immunoglobulins and lactoferrin
GMP [a)]	1.2-1.5 (sweet whey)	7-8	-	Antiviral, bifidogenic
Lactoferrin	0.05-0.1	78	9.0	Antimicrobial, antioxidative, immunomodulation, iron absorption, anticarcinogenic
Lactoperoxidase	0.02-0.03	89	9.5	Antimicrobial
Proteose peptones	> 0.6	3.6-22		Opioid activity

a) only in rennet whey;
Data from Zydney (1998); Durham and others (1997); Shah (2000)

Native β-Lg is a predominantly β-sheet protein consisting of a β-barrel of eight continuous antiparallel β-strands folded into two antiparallel β-sheets shaped into a flattened cone or calyx and an additional β-strand, one major α-helix and four short helices attached to this calyx (Kuwata and others, 1999). One side of sheet 1 is hydrophobic and the other side is hydrophilic. Sheet 2 is also hydrophobic on one side which faces the hydrophobic side of sheet 1, thus creating a very hydrophobic cavity, which is nevertheless filled with water. Small hydrophobic molecules may bind to this central cavity (the β-barrel). There is also another hydrophobic region on the side of sheet 2, where a three-turn helix lies above. This

α-helix covers the CysH residue, providing it remains packed against the exterior of the calyx (Considine and others, 2007).

There are ca. ten identified genetic variants from bovine β-Lg. Variants A and B are the most common. Although they only differ on two amino acids (amino acid 64 of variant B is Gly instead of Asp and aminoacid 118 is Ala instead of Val), they have significant differences on their properties (Huang and others, 1994; Sawyer and others, 1999).

Even though it is abundant in the whey fraction of milk, its function is still not clear. It has been reported that β-Lg plays an important role as a carrier of retinol, a provitamin A (Papiz and others, 1986; Godovac-Zimmermann, 1988; Perez and Calvo, 1995). As its globular structure is remarkably stable against the acids and proteolytic enzymes present in the stomach (de Wit, 1998), retinol would be able to reach the intestinal tract of the young calf where β-Lg would facilitate its uptake. The biological role of β-Lg in ruminants (but not in other mammals whose milk has β-Lg) could also be to aid milk fat digestion in the newborn animal by promoting pregastric lipase activity (Perez and others, 1992; Perez and Calvo, 1995).

β-Lg associated form changes with pH, temperature, ionic strength and protein concentration. Between 5.2 (the isoelectric point) and 7.5, native β-Lg occurs as a dimer in solution. Between pH 3.5 and 5.2 β-Lg reversibly forms tetramers/octamers, whereas below 3.5 and above 7.5 it dissociates into monomers due to electrostatic repulsions. At temperatures higher than 30 °C the dimeric form of β-Lg dissociates to monomers and at temperatures higher than 55 °C unfolding of the molecule starts to occur, which results in an increased activity and oxidation of the thiol group (Caessens and others, 1997).

β-Lg's sulphydryl group is buried within the molecule in the native protein but becomes exposed and active on denaturation of the protein by various agents (including heat, pressure and urea) and can then undergo sulphydryl– disulphide interactions with itself or other proteins (Fox, 2001). Thus, a slight denaturation of the globular β-Lg molecule can have a great impact on its surface-active behaviour (Caessens and others, 1997). This property is responsible for many technological features of whey proteins.

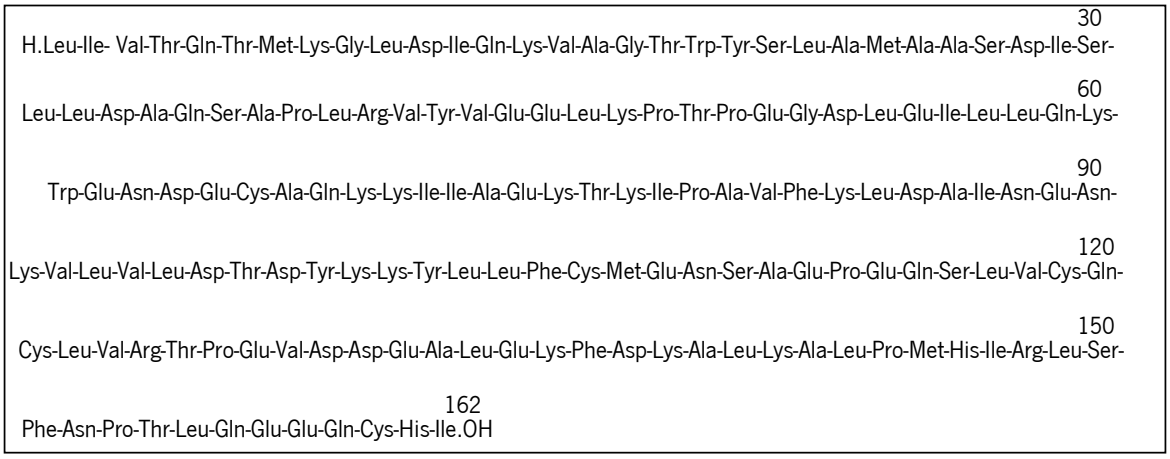


Figure 2-4 Primary structure of bovine β-lactoglubulin variant A (Alais, 1984)

2.1.2 α-lactalbumin

The second most abundant protein in cow's milk is α-lactalbumin. α-La has a molecular weight of 14.2 kDa and it is remarkably rich in tryptophan (Figure 2-5). The eight cysteine residues form four disulphide bridges (6-120, 26-111, 61-77 and 73-91) that stabilize its tertiary structure. The protein has an ellipsoid shape with two distinct lobes divided by a gap; one lobe is comprised of four helices and the other lobe is comprised of two β-strands with a loop-like chain.

At pH 4.0, α-La unfolds and is susceptible to digestion by pepsin in the stomach (de Wit, 1998). There are two known genetic variants, A and B, although only variant B is found in European bovines. Variant A differs from variant B in the amino acid 10 (glycine instead of arginine).

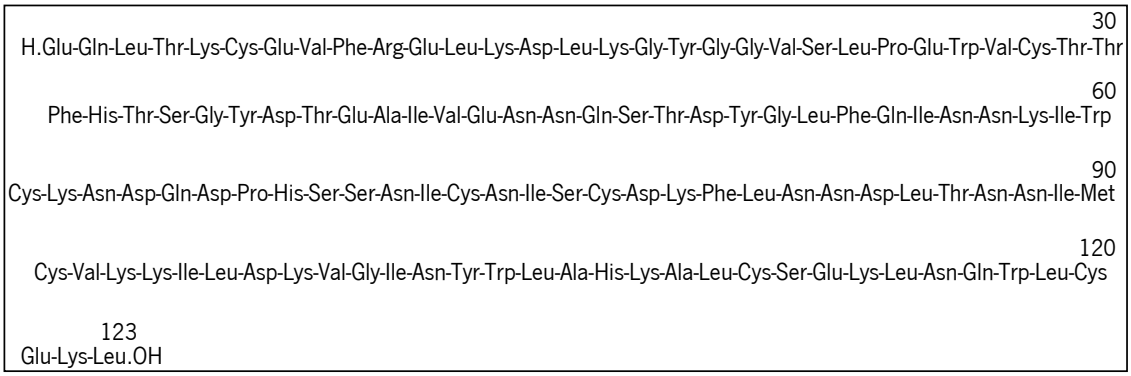


Figure 2-5 Primary structure of bovine α-lactalbumin variant B (Alais, 1984)

α-La is present in all mammals' milk that secrete lactose. This protein is one of the two components of lactose synthase and its main biological function is thus to support the biosynthesis of lactose, which is an important source of energy for the newborn.

One of the most interesting features of α-La is its ability to bind metal cations. It has one strong calcium binding site and also several zinc binding sites. The binding of Ca^{2+} to α-La causes pronounced changes in its tertiary structure and function and can increase its stability. Zinc or other cation binding might induce α-La aggregation to forms that have anticancer activity and perform various transport functions with apolar, lipophilic vitamins and metabolites (Permyakov and Berliner, 2000).

2.1.3 Bovine serum albumin

Bovine serum albumin is very similar to the human blood serum albumin. It has 582 amino acids and a molecular weight of ca. 69 kDa. Seventeen disulphide bridges stabilize its tertiary structure and it has one remaining free sulphydryl group. BSA is probably involved in the transport of insoluble free fatty acids in the blood.

2.1.4 Glycomacropeptide

Glycomacropeptide (GMP) or caseinomacropeptide corresponds to a heterogeneous group of peptides having the same peptide chain but variable carbohydrate and phosphorus contents (Elsalam and others, 1996). GMP peptide chain is composed by the 64 C-terminal amino acids of κ-casein, released by chymosin (or pepsin) cleavage of κ-casein during the manufacture of cheese (Thoma-Worringer and others, 2006) and has an average molecular weight of 8000 Da. Although it constitutes 15-20 % of the total renneted cheese whey proteins, it is probably the least well known of its components. Possible reasons for this can be the absence of aromatic amino acids which makes it invisible at 280 nm (the common protein detection wavelength), its negative charge, even at pH 3 (it is not collected on cation exchangers, nor does it move with the rest of the proteins in native polyacrylamide gel electrophoresis) and its low molecular weight that makes it difficult to visualize with Coomassie Blue stain in sodium dodecyl sulphate (SDS)-PAGE (Brody, 2000). It is resistant to the enzymatic action of several rennets (including chymosin) and to pepsin.

From the nutritional point of view GMP is not interesting because it lacks several essential amino acids (arginine, cysteine, histidine, tryptophan and tyrosine). However, its unique aminoacid composition makes

it handy for special diets. For instance, it also lacks all aromatic acids (Phe, Try, Tyr) which makes it fit for phenylketonuria patients' diet. GMP is rich in branched-chain amino acids (valine and isoleucine) and low in Met, which makes it a useful ingredient in diets for patients suffering from hepatic diseases (Elsalam and others, 1996).

From the biological point of view, GMP interacts with toxins, viruses and bacteria, exerting health promoting activities that are strongly mediated by the carbohydrate fraction (Lopez-Fandino and others, 2006). These interactions includes growing-promoting activity for *Bifidobacteria*, inhibition of the binding of cholera toxin to its receptor, enhancement of resistence to influenza viruses, potential release of bioactive peptides by tryptic proteolysis (Elsalam and others, 1996), modulation of the composition of the dental plaque microbiota, ability to nourish healthy gut microflora (prebiotic) and immunomodulatory effects (Lopez-Fandino and others, 2006).

2.1.5 Lysozyme

Lysozyme is a 15 kDa single chained protein secreted in milk and structurally related to α-lactalbumin, with the proteins sharing 40 % identity (Simpson and Nicholas, 2002). It is an antimicrobial enzyme with bacteriolytic action. It is thought to hydrolyze polymeric sugar residues present in bacterial cell walls, resulting in cell lysis, potentially providing protection against enteropathogens in the gut of the neonate (Shah, 2000).

2.1.6 Immunoglobins

Bovine serum contains three major classes of immunoglobins (Igs): IgG, IgM and IgA. The basic structure of all Igs is similar, and is composed of two identical light chains (23 kDa) and two identical heavy chains (53 kDa). These four chains are joined together with disulphide bonds. The complete Ig or 'antibody' molecule has a molecular weight of about 180 kDa (Korhonen and others, 2000). Igs bind to the "invasor agent" (antigen) and activates bacteriolytic reactions, increase the recognition and phagocytosis of bacteria by leucocytes, prevent the adhesion of microbes to surfaces, inhibit bacterial metabolism, agglutinate bacteria and neutralise toxins and viruses. Igs are partially resistant to proteolytic enzymes and are not inactivated by gastric acid (Korhonen and others, 2000).

2.1.7 Lactoferrin

Lactoferrins are single-chain polypeptides of about 80 kDa, containing 1-4 glycans, depending on the species. Bovine and human lactoferrins consist of 989 and 691 amino acids, respectively (Steijns, 2001).

Lactoferrin is an iron-binding glycoprotein thought to play a role in iron transport and absorption in the gut of the young. It has also been suggested that it has a role in the non-specific defence against pathogens, being important in antimicrobial defense of the mammary gland and mucosal surfaces, modulating the inflammatory response, and inhibiting both gram-positive and gram-negative bacteria (Simpson and Nicholas, 2002). It can also act as an antioxidant.

2.1.8 Lactoperoxidase

Lactoperoxidase is a glycoprotein of 608 aminoacids and an approximate molecular weight of 78 kDa. It has a broad biocidal and biostatic activity. The enzyme, in the presence of H_2O_2 catalyses the oxidation of thiocyanate (SCN^-) and produces an intermediate product with antimicrobial properties (Shah, 2000; Boots and Floris, 2006).

2.1.9 Proteose-peptones

The total proteose peptone (TPP) fraction of bovine milk represents about 10 % of total whey protein. It corresponds to the whey protein fraction soluble after heating at 95 °C during 30 min followed by acidification to pH 4.6 (Alais, 1984).

TPP fraction is often divided in two main groups:

- The first one includes proteose-peptones originary from casein hydrolysis; its principal components have been designated as components 5 (PP5), 8 fast (PP8 fast) and 8 slow (PP8 slow) according to their electrophoretic mobilities (Alais, 1984; Innocente and others, 1999); PP8 slow contains peptides with opioid activity and PP8 fast is a phosphopeptide which may enhance the gastrointestinal absorption of calcium (de Wit, 1998);
- PP3 constitutes the second group and is not derived from casein (it is found only in whey); it is extremely hydrophobic and particularly interesting because of its functional properties, such as its emulsifying power, strong affinity for oil-water interface, strong foaming properties and biochemical role (Innocente and others, 1999; Rodrigues and others, 2003).

2.2 Operational functional properties of whey proteins

The functional properties are defined as those properties, which determine the overall physicochemical behaviour of proteins in foods during production, processing, storage and consumption.

Whey proteins are well known for their versatile functional properties and this functionality can be exploited commercially in the manufacture of numerous foods (Innocente and others, 1998). These functional

properties include water solubility, water absorption, viscosity, gelation, emulsion properties, fat absorption, foaming properties, flavour and mineral binding abilities (de Wit, 2001).

Most applications of whey proteins in food products require specific functional attributes to obtain the desired performance. Each application requires one or several functional properties (Foegeding and others, 2002). It is very difficult to predict the behaviour of whey proteins in food systems. This behaviour is influenced not only by the proteins intrinsic properties (composition, structure, net charge, hydrophobicity) but also by extrinsic factors (such as temperature, pH, salts, concentration), by food components (lipids, sugars, minerals, proteins) and by effects of processing such as homogeneization, heating or freezing, storage (de Wit, 1998).

One of the most important handicaps of whey proteins is that they are unstable during a heat treatment, thus reducing its applicability to pasteurized food (Doucet and others, 2001). However, this heat sensitiveness can be used to tailor whey proteins functional properties such as foaming and emulsifying abilities (Galani and Apenten, 1999).

The water solubility of WP can influence the other functional properties such as gelling, foaming or emulsifying. Whey proteins are generally heat sensitive (except proteose-peptone) and their solubility decreases when temperature increases above their denaturation point. Immunoglobulins and α-La are the most heat sensitive, followed by β-Lg and BSA. WP solubility at both neutral and acid pH values is important for its use as nutritional fortifiers of soft beverages, such as fruit juices or sport drinks.

Whey proteins absortion capacity is important to bind water to some food products. They can be used in meat and bakery products to enhance texture and water binding properties (Barbut, 2006). Fat holding capacity and gelling at low temperatures are also important attributes for structuring meat and fish products.

In infant formulae, whey proteins are useful due to the thermostable emulsifying properties and sufficient emulsion stability during storage (de Wit, 2001).

Formulated foods often need whey proteins to assist in binding and releasing flavours under appropriate conditions (de Wit, 2001; Guichard, 2006) and, in nutritional foods, whey proteins can bind to minerals allowing their bioavailability (de Wit, 2001).

Foam is a dispersion of gas bubbles within a liquid or solid continuous phase. This material class is important to the structure and texture of many food products, including various cakes, confections, ice-creams, desserts, meringues and whipped toppings (Visser and Paulsson, 2001; Davis and Foegeding,

2007). Whey protein concentrate (> 60 %) or isolate may be used as an egg white replacer. Proteins work as natural surfactants in many applications that involve foam production, lowering the interfacial tension and allowing the formation of the gas bubbles. In this case, it is important that the presence of (unsaturated) lipids is reduced to the minimum (or absent) for foam stabilisation.

In soups, gravies and desserts whey proteins are used because of their heat-induced thickening and stabilizing properties that ensure a pleasant mouth feel. They can also be used as fat replacers in low fat products.

The ability of β-lactoglobulin to bind fatty acids has been exploited by its use as an emulsifying agent in food technology (Perez and Calvo, 1995).

Emulsifying properties of food proteins are usually described by the emulsion capacity or emulsion activity, which reflects the ability of the proteins to aid formation and stabilisation of the newly created emulsion (or reflects the amount of oil that can be emulsified, by certain quantity of protein prior to phase inversion or collapse of the emulsion), and by the emulsion stability, which reflects the ability of the proteins to impart strength to emulsion for resistance to stress (Patel and Kilara, 1990). The surface properties of the whey proteins make them good emulsifying agents as they facilitate the formation of small oil droplets during homogenization by lowering the interfacial tension and increasing the stability of the droplets formed preventing aggregation through the increase of the repulsive colloidal interactions between them (Surh and others, 2006). They are used as emulsifiers in a wide variety of emulsion-based food products, including beverages, frozen desserts, icecreams, sport supplements, infant formula and salad dressings (Surh and others, 2006). However caseins, for instance, have better emulsifying and foaming characteristics than whey proteins.

Whey protein operational functionallity can be enhanced by altering the protein/non-protein composition and/or by modifying the proteins. Direct modification of the proteins can be achieved by: (1) covalently attaching other compounds such as carbohydrates; (2) causing non-covalent and/or covalent interactions among proteins to produce aggregates or polymers or (3) hydrolyzing proteins to various degrees (Foegeding and others, 2002). For instance, it has been reported that binding of some ligands to different proteins can stabilize their structure and increase their resistance to heat denaturation: fatty acids binding to bovine serum albumin (BSA) or β-Lg, calcium (Ca^{2+}) binding to α-La or iron (Fe^{3+}) binding to lactoferrin stabilize these proteins against heat (Barbeau and others, 1996).

2.3 Enzymatic hydrolysis of whey proteins

Hydrolysis of food proteins is widely used to improve functional properties of foods such as modification of the gelation behaviour, improvement of thermal stability, alteration of solubility, improvement of foamability and foam stability; improvement of water and fat holding capacities, improvement of emulsifying capacity and/or tailoring of the functionality of the protein to meet specific needs (Gauthier and others, 1993; Singh and Dalgleish, 1998; Huang and others, 1999; Doucet and others, 2001; Doucet and others, 2003b; Panyam and Kilara, 2004; Aluko and McIntosh, 2005; Davis and others, 2005; Guan and others, 2007; Kong and others, 2007). The desired degree of hydrolysis and the adequate source of protein depend on the desired functionality and on the type of product in which they will be incorporated. A way of improving even more whey protein hydrolysates functionality is by membrane separation to obtain fractions with various degrees and types of functionalities (Foegeding and others, 2002).

During recent decades, interest has grown in the nutritional efficacy of whey proteins in infant formula and in dietetic and health foods, using either native or predigested proteins (Korhonen and others, 1998).

From a dietary point of view, the use of whey protein hydrolysates (WPH) instead of the intact protein isolates is interesting e.g. to reduce allergenicity (particularly useful in food for hypersensitive children) or to improve digestability of foodstuffs (Silvestre, 1997). Gastrointestinal absorption of the hydrolysates is more effective when compared to the absorption of the intact protein and they can be used in the nutrition of people that can not digest intact proteins. Besides providing essential amino acids, milk and whey proteins' oligopeptides have been shown to possess biological functions (Pihlanto-Leppälä and others, 1997). Thus, whey protein hydrolysates (WPH) are also interesting as a source of bioactive peptides (compounds with a health promoting potential).

Another possible feature of whey protein hydrolysates is due to the fact that resulting peptides may be more easily assimilated by microorganisms with favorable results in fermentation processes (Perea and others, 1993). Recently it has been suggested that hydrolysates from α-La can be used to create self-assembling nanotubes with both food and non-food applications (Graveland-Bikker and de Kruif, 2006).

Proteins hydrolysis can be carried out by enzymes, acids or alkali. Acid or alkaline hydrolysis tends to be a difficult process to control and yields products with reduced nutritional qualities. Besides, chemical hydrolysis can destroy L-form amino acids, produce D-form amino acids and form toxic substances like lysino-alanine (Clemente, 2000). To increase whey proteins susceptibility to hydrolysis, treatments such as heat, sulfitolysis or high pressure can be used (Foegeding and others, 2002).

During protein hydrolysis, amide bonds are cleaved and, after the addition of a water molecule, peptides and/or free amino acids are released: R_1-CO-NH-R_2 + H_2O ⟶ R_1-COOH + R_2-NH_2

The newly formed peptides can be new substrates for enzymatic hydrolysis.

The extent of the hydrolysis can be measured through the percentage of peptide bonds cleaved. Thus, if h_{tot} is the number of peptide bonds in the intact protein (calculated from the protein amino acid composition) and h is the number of cleaved peptide bonds, the degree of hydrolysis (*DH*) is calculated according to Equation 2.1:

$$DH = \frac{h}{h_{tot}} \times 100 \qquad \text{(Eq. 2-1)}$$

The degree of ionisation of the free carboxyl and free amino groups formed after hydrolysis depends on the pH at which the hydrolysis reaction is conducted. Once the pK values of –COOH and $^{+}H_3N$- in polypeptides range between 3.1-3.6 and 7.5-7.8, respectively, it is expected that the carboxyl group is a) undissociated at pH values below 2; b) partially dissociated at pH values between 2 and 5 and c) fully dissociated at pH values above 5, while the amino group will be d) fully protonated at pH values below 6; b) partially protonated at pH values between 6 and 9.5 and c) unprotonated at pH values above 9.5. The hydrolysis of protein is therefore either producing or consuming H^+ ions, meaning that the pH will change during the reaction, exception made for pH values between 5 and 6, where the production and consumption of H^+ ions cancel each other. If the reaction is progressing below pH values of 3.1-3.6, the amino group will be fully protonated and the carboxyl group will be less than half dissociated, leading to a net uptake of 0.5 to 1 equivalent H^+ for each equivalent peptide bond which is cleaved; this will be noticed by a fast increase of the pH of the medium if no control is applied. On the other hand, if the reaction is progressing at pH values above 7.5-7.8 (at 25 °C), the amino group will be less than half protonated while the carboxyl group will be fully dissociated; in this case, a net release of 0.5 to 1 equivalent of H^+ will occur for each peptide bond cleaved and a fast decrease of the pH of the medium will be observed if no control is applied (Adler-Nissen, 1986).

The result of a proteolytic process depends on the chosen enzyme, on the protein substrate and on the hydrolysis conditions. Thus, to fully describe a hydrolysis experiment, the hydrolysis parameters have to be specified. These are: substrate concentration, enzyme-substrate ratio, pH and temperature (Adler-Nissen, 1986). These parameters will determine the rate of the hydrolysis reaction as well as other characteristics of the hydrolysis process.

Secondary and tertiary structures of globular proteins can partially or totally block the acess of the enzyme to the cleavable peptide bonds. Thus it has been suggested by Linderstrøm-Lang in the early 50's that reversible denaturation might be the initial reaction in protein hydrolysis (Adler-Nissen, 1976; Adler-Nissen, 1986):

$$Native \underset{v_{-0}}{\overset{v_{+0}}{\rightleftarrows}} 'Denatured' \xrightarrow{enzyme;v_I} 'Intermediate' \xrightarrow{enzyme;v_{II}} 'End\_product'$$

In the beginning one or a few peptide bonds will be broken destabilizing the molecule structure and causing an irreversible unfolding by which more peptide bonds will be exposed (Adler-Nissen, 1976). When the rate of protein denaturation ($v_0 = (v_{+0}) - (v_{-0})$) is much higher than the rate of hydrolysis (v_i), the native protein molecules will rapidly be degraded to an intermediary form which will be slowly degraded to end products. The produced hydrolysates will contain mainly intermediate size peptides and this type of reaction is called "zipper" reaction. On the other side, if $v_0 << v_i$, the initial denaturation step will be the rate limiting step for hydrolysis and each denatured protein molecule will be quickly hydrolysed to end products. The resulting hydrolysate will contain both intact proteins and end products, but no appreciable amounts of intermediate size peptides. This type of reaction is designated as a 'one-by-one' reaction, indicating that one enzyme molecule degrades one substrate molecule at a time (Adler-Nissen, 1976; Adler-Nissen, 1986; van der Ven, 2002).

Both mechanisms are involved in most proteolytic reactions and the real situation is usually between these two extreme cases; however, if an irreversible denaturation of the protein occurs before the hydrolysis, there is a significant increase in the number of accessible peptide bonds and the degradation of the protein will expectedly proceed according to a zipper-type reaction. In this latter case, there are other factors (e.g. decreased solubility) which might affect the initial reaction rate (Adler-Nissen, 1986).

2.3.1 Proteolitic enzymes

The choice of the enzyme used will determine which peptides will be formed because of differences in enzyme specificities. As a result, hydrolysates that have been formed by various enzymes may have different functionalities (Caessens and others, 1999).

Proteolytic enzymes can be classified according to their mechanism of hydrolysis into endopeptidases (or proteinases) and exopeptidases. The enzymes from the first group cut the polypeptide chain internally, at

specific amino acids, to produce large fragments. Enzymes from the second group act near an end of the polypeptide chain to liberate products with one or a few amino acid residues; they can act at the C-terminal residue, liberating a single residue (carboxypeptidases) or a dipeptide, or they can act at the free N-terminus liberating a single aminoacid (aminopeptidases), a dipeptide or a tripeptide (Barrett, 2000).

Peptidases have also been divided according to their origin (animal, plant or microbial) or according to its catalytic residues: serine, cysteine, aspartic, metallo and, more recently, threonine proteases (Dunn, 2000).

Whey protein hydrolysis designed for nutritional applications should have a high degree of hydrolysis and, thus, be rich in small peptides because they are less antigenic and more heat stable. On the other hand, they should have a low content in free amino acids to be more easily absorbed, which suggests the use of endopeptidases (Foegeding and others, 2002). Positive correlations between surface activity and peptide chain length and between hydrophobicity and peptide functionality have been reported and it has since been generally accepted that a peptide should have a minimum length (> 20 residues) to possess good functional properties, namely emulsifying and interfacial properties (Gauthier and others, 1993). Thus, the application target of the hydrolysates should be beared in mind when choosing the hydrolysis enzyme and the desired degree of hydrolysis.

Also, enzyme specificity is important to peptide functionality because it strongly influences the molecular size and hydrophobicity of the peptides produced. For example, tryptic peptides from whey proteins have better emulsifying and interfacial properties than chymotryptic peptides (Gauthier and others, 1993). Enzymatic hydrolysis of whey protein concentrate (WPC) with prolase, pronase, or pepsin resulted in improved foaming properties but caused decreased emulsifying properties compared to the nonhydrolyzed WPC (Caessens and others, 1999).

Pepsin

Pepsin is an aspartic proteolytic enzyme secreted in all mammals' stomach. It is an endopeptidase with a low specificity. Pepsin's activity is high at very low pH (1.5 to 4) allowing its use with a low risk of microbial contamination. It also allows a high degree of hydrolysis of denatured proteins that have been precipitated with acid. It hydrolyses preferencially C-terminal and N-terminal aromatic amino acids. In spite of having some affinity for hydrophobic amino acids and due to the attack in narrow ranges of amino acid residues, freeing of bitter peptides is many times relevant (Godfrey, 1996a). Hydrolysis rate is not as high

as trypsin's but it is more heat tolerant. Optima acting temperature and pH are ca. 60 °C and 1.8 – 2.0, respectivly.

Trypsin

Trypsin is a serine endopeptidase from animal origin (pancreas) that preferencially catalyses the hydrolysis of peptide bonds between the carboxyl group of two basic amino acids (arginine and lysine) and the amine group of any other adjacent amino acid (Margot and others, 1997). Tryptic hydrolysates generally have a bitter taste which limits its use for food applications unless careful control of the hydrolysis degree and/or the application of debittering enzymes are included in the processing. Trypsin does not have any affinity for breaking hydrophobic amino acid bond pairs, and this is the reason for the bitter peptide creation (Godfrey, 1996b). Optimal operational temperature is ca 50 °C (though the concept of optimum here is relative since the activity of the enzyme also depends e.g. on the time during which it has been subjected to a determined temperature) and optimal pH is ca. 8-8.5. It has a molecular weight around 23.5 kDa though slight variations may occur depending on the trypsin's origin (Johnson and others, 2002; Hau and Benjakul, 2006). Trypsin is a monomeric enzyme, it consists of a single folded polypeptide chain (by contrast with oligomeric enzymes). Many trypsin preparations are contaminated with chymotrypsin because the two enzymes are difficult to split as they are very similar in size and isoelectric point. Chymotrypsin cleaves preferencially at Phe, Tyr and Trp or Leu amino acids, depending on the variant of chymotrypsin being used (Yamamoto and Takano, 1999a).

Protease from *Bacillus licheniformis* (Alcalase®)

This alkaline protease of microbial origin is essentially subtilisin. It is a serine protease (with the active serine at the amino acid 221), with an optimal operational temperature of 60 °C and an optimum pH of 8-9 (though activity is high between 6 and 12). Alcalase® has a broad specificity but mainly breaks peptide chains at hydrophobic amino acids (His-Leu, Ala-Phe, Gly-Phe). Alkaline bacterial proteases hydrolyse almost any proteic substrate.

2.3.2 Bioactive peptides

From the food industry point of view, a bioactive peptide is a peptide that exerts one or several specific beneficial functions in the organism, beyhond the benefit from the traditional ingredients that the food product contains. Amoung the main biological functions of bioactive peptides one can include protective functions, regulation of digestion and nutrient uptake, and metabolic or physiological regulation of the

body. In the cardiovascular system the most commonly found roles include inhibition of the angiotensin converting enzyme (ACE) with an hypotensive effect, inhibition of the fibrinogen binding to a specific receptor region on the blood platelet surface inhibiting platelets aggregation (antythrombotyc activity (Meisel, 2005)), antioxidant and hypocolesteremic effects. Examples of milk derived bioactive peptides that act on the cardiovascular system are very common in scientific literature (see, for instance, Mullally and others, 1997b; Meisel and others, 1997; Yamamoto and Takano, 1999b; Groziak and Miller, 2000; Pihlanto-Leppala and others, 2000; Hernandez-Ledesma and others, 2002; Vermeirssen and others, 2003; Gobbetti and others, 2004; Hernandez-Ledesma and others, 2005; Didelot and others, 2006; Lopez-Fandino and others, 2006; Pihlanto, 2006; Chen and others, 2007; Otte and others, 2007). They can bind to opioid receptors with an agonist or antagonist effect affecting the nervous system (Brantl and others, 1979; Henschen and others, 1979; Meisel, 1986). In the gastrointestinal system they can act as mineral fixator (bind to mineral ions, facilitating their transport and increasing their bioavailability) or have anti-apetite or antimicrobial tasks (Vegarud and others, 2000; Gobbetti and others, 2004; Kim and Lim, 2004; Kim and others, 2007). Finally, in the immunitary system they can play immunomodulatory and anticancer or cytomodulatory functions and eliminate sensitive microorganisms (for examples see Kayser and Meisel, 1996; McIntosh and others, 1998; Pihlanto-Leppala and others, 1999; Gill and others, 2000; van Hooijdonk and others, 2000; Mercier and others, 2004; Exposito and Recio, 2006; Gauthier and others, 2006; Mehra and others, 2006; Pan and others, 2006; Rydlo and others, 2006).

Bioactive peptides are inactive within the sequence of the parent protein and can be released in three ways: by enzymatic hydrolysis with digestive enzymes; by fermentation of the intact protein with proteolytic starters and through the action of enzymes derived from proteolytic microorganisms (Chen and others, 1994; Foegeding and others, 2002). Another via for bioactive peptides production is by synthesis if the peptide structure is known. This synthetic peptides can be produced chemically, enzymaticaly or using recombinant DNA technology (Gill and others, 1996).

Although animal as well as plant proteins contain potential bioactive sequencies, milk proteins, especially caseins, are the most important source of bioactive peptides at present (Yamamoto and Takano, 1999c). Many reviews on bioactive peptides derived from milk can be found in literature (Schlimme and Meisel, 1995; Meisel, 1997a; Meisel, 1997b; Clare and Swaisgood, 2000; Shah, 2000; Kitts and Weiler, 2003; Korhonen and Pihlanto, 2003b; Korhonen and Pihlanto, 2003a; Meisel, 2004; Meisel, 2005; Severin and Xia, 2005; Korhonen and Pihlanto, 2006; and many others). Throughout recent years, the major whey protein components, α-La and β-Lg, were also shown to contain bioactive sequences. Peptides showing

opioid and angiotensin I-converting enzyme (ACE) inhibitory activity both *in vitro* and *in vivo* were found in α-lactalbumin and β-lactoglobulin. Opioid peptides, α-lactorphin and β-lactorphin, were liberated during in vitro proteolysis of bovine whey proteins, and pharmacological activity was observed at micromolar concentrations. Whey hydrolysates showed ACE-inhibitory activity after proteolysis with different digestive enzymes, and several active peptides were identified (Pihlanto-Leppälä and others, 1998, Clare and Swaisgood, 2000; FitzGerald and Meisel, 2000; Shah, 2000; among others). Moreover, and in contrast to endogenous bioactive peptides, many milk-derived peptides reveal multifunctional properties, i.e. specific peptide sequences having two or more different biological activities have been reported. For example, some regions in the primary structure of caseins contain overlapping peptide sequences that exert different biological effects. These regions have been considered as "strategic zones", which are partially protected from proteolytic breakdown (Meisel, 1997a). So far, the most common way to produce bioactive peptides has been through enzymatic digestion (Korhonen and Pihlanto, 2006). Pancreatic enzymes – preferably trypsin – have been used for identification of many known bioactive peptides. However, other enzymes including Alcalase® and pepsin have been used to generate bioactive peptides (Chen and others, 1994; Gill and others, 1996; Mullally and others, 1997b; Madsen and others, 1997; Otte and others, 1997; Pihlanto-Leppala and others, 1998; to cite a few).

Theoretical peptides that can be produced from tryptic hydrolysis of β-Lg are presented on Table 2-6, corresponding to the hydrolysis of the protein at arginine and lysine residues (see Figure 2-4). Tryptic hydrolysates of β-Lg commonly contain two more peptides linked by disulfide bonds: β-Lg 61-69 + 149-162 (2720-2778 Da; Groleau and others, 2003a) and β-Lg 61-70 + 149-162 (2849-2907 Da; Groleau and others, 2003a), which account for 7.3 % of all peptides produced (Groleau and others, 2002). Other bioactive peptides derived from whey proteins are presented in Table 2-7, Table 2-8 and Table 2-9.

ACE inhibition is measured by the concentration of substance needed to inhibit 50 % of the original ACE activity (IC_{50}). A lower IC_{50} value indicates higher efficacy. Published research studies on ACE inhibitory activity of various whey-derived peptides show results at a level of 42.6–1062 μM (Mullally and others, 1997a; Pihlanto-Leppala and others, 2000; Ferreira and others, 2007).

Opioid activity is measured through the peptide concentration required to displace ^{3}H-ligand (^{3}H-naloxone) binding by 50 % (Meisel and FitzGerald, 2000). Antithrombotic activity is measured through the peptide concentration required to inhibit by 50 % thrombin induced platelet aggregation (Rutherfurd and Gill, 2000).

Table 2-6: β-Lg tryptic peptides and their bioactivity (source of molecular weights: Groleau, 2003)

Peptide	Sequence	Mw (Da)	Bioactivity (IC_{50} in μmol/L)	Reference
f(1-8)	LIVTGTMK	933.2	-	-
f(9-14)	GLDIQK (lactokinin)	672.8	ACE-inhibition (580)	Pihlanto-Leppala and others, 1998; FitzGerald and Murray, 2006
f(15-40)	VAGTWYSLAMAASDI SLLDAGSAPLR	2706.3	Several "sub"-peptides with bioactivity	See Table 2-8
f(41-60)	VYVEELKPTPEGDLEI LLQK	2313.7	-	-
f(61-69)	WENDECAQK	1064.1	-	-
f(61-70)	WENDECAQKK	1192.3	-	-
f(71-75)	IIAEK	572.7	Hypocholesterolemic	Groleau and others, 2003b
f(76-77)	TK	247.3	-	-
f(78-83)	IPAVFK	673.8	Antimicrobial (bactericidal)	Pellegrini and others, 2001
f(84-91)	LDAINENK	916.0	-	-
f(92-100)	VLVLDTDYK (β-lactorphin)	1065.2	Antimicrobial (bactericidal)	Pellegrini and others, 2001 Groleau and others, 2003b
f(102-124)	YLLFCMENSAEPEQS LVCQCLVR	2648.0	Several "sub"-peptides with bioactivity	See Table 2-8
f(125-135)	TPEVDDEALEK	1245.3	-	-
f(136-138)	FDK	408.4	-	
f(139-141)	ALK	330.4	-	
f(142-148) (lactokinin)	ALPMHIR	837.0	ACE Inhibitory (42.6)	Mullally and others, 1997b
f(149-162)	LSFNPTLQEEQCHI	1715.0	-	

Table 2-7: α-La derived bioactive peptides

Protein source and fragment	Treatment	Sequence	Peptide name	Bioactivity (IC_{50} μmol/L)	Reference
f(1-5)	Trypsin	EQLTK		Antimicrobial	Pellegrini and others, 1999
f(17-31)S-S (109-114)	Trypsin	GYGGVSLPEWV- CTTF ALCSEK			Pellegrini and others, 1999
f(18-20)	Synthetic	YGG		Immunomodu-latory	Kayser and Meisel, 1996
f(50-52)	Pepsin + trypsin + chymotrypsin	YGL		ACE-inhibitory (409)	Pihlanto-Leppala and others, 2000
f(50-53)	Synthetic; pepsin	YGLF	-lactorphin	Opioid agonist (67), ACE-inhibitory (733.3)	Gill and others, 1996; Meisel and FitzGerald, 2000; Pihlanto-Leppala, 2000
f(52-53)	Synthetic	LF		ACE-inhibitory (349.1)	Chatterton and others, 2006
f(50-51); f(18-19)	Synthetic	YG		Immunomodul atory; ACE inhibitory (1522.6)	Kayser and Meisel, 1996; Pihlanto-Leppala, 2000
f(61-68)S-S (75-80)	Chymotrypsin	CKDDQNPH ISCDKF		Antimicrobial	Pellegrini and others, 1999
f(99-108)	Trypsin	VGINYWLAHK		ACE-inhibitory (327)	Pihlanto-Leppala and others, 2000
f(104-108)	Trypsin	WLAHK		ACE-inhibitory (77)	Pihlanto-Leppala and others, 2000
f(105-110)	Pepsin	LAHKAL		ACE-inhibitory (621)	Pihlanto-Leppala and others, 1998

Table 2-8: Other β-Lg derived bioactive peptides

Protein source and fragment	Treatment	Sequence	Peptide name	Bioactivity (IC_{50}μmol/L)	Reference
f(7-9)		MKG		ACE-inhibitory (71.8)	Hernandez-Ledesma and others, 2006
f(9-14)	Fermentation+ pepsin+trypsin	GLDIQK		ACE-inhibitory (580)	Pihlanto-Leppala, 2000
f(10-14)		LDIQK		ACE-inhibitory (27.6)	Hernandez-Ledesma and others, 2006
f(15-19)	Pepsin+trypsin+ chymotrypsin	VAGTW		ACE-inhibitory (1054 μg/mL)	Pihlanto-Leppala and others, 2000
f(15-20)	Yogurt starter+ trypsin+pepsin; trypsin + contaminant	VAGTWY		ACE-inhibitory (1682); Antimicrobial (bactericidal)	Pihlanto-Leppala and others, 1998; Pellegrini and others, 2001 FitzGerald and Murray, 2006
f(17-19)	Fermentation with lactic acid bacteria + prozyme 6	GTW		ACE-inhibitory (464.4)	Chen and others, 2007
f(19-29)	Corolase PP	WYSLAMAASDI		Antioxidant	Hernandez-Ledesma and others, 2005; Hartmann and Meisel, 2007
f(22-25)	Trypsin+ contaminant	LAMA		ACE-inhibitory (1062)	Pihlanto-Leppala and others, 2000
f(25-40)	Trypsin + contaminant	AASDISLLDAQSAPLR		Antimicrobial	Pellegrini and others, 2001
f(32-40)	Trypsin+conta-minant	LDAQSAPLR		ACE-inhibitory (635)	Pihlanto-Leppala and others, 2000
f(42-46)		YVEEL		Antioxidant	Hartmann and Meisel, 2007
f(58-61)		LQKW		ACE-inhibitory (34.7)	Hernandez-Ledesma and others, 2006
f(78-80)	Proteinase K	IPA	β-lactosin A	ACE-inhibitory (141)	Abubakar and others, 1998
f(81-83)	Trypsin+conta-minant	VFK		ACE-inhibitory (1029)	Pihlanto-Leppala and others, 2000
f(94-100)	Trypsin+ chymotrypsin; Pepsin+trypsin+ chymotrypsin	VLDTDYK	Lactokinin	ACE-inhibitory (946)	Roufik and others, 2007; Pihlanto-Leppala and others, 2000
f(102-103)		YL	Lactokinin	ACE-inhibitory	Meisel, 2005
f(102-105)	Trypsin+conta-minant; synthetic; or pepsin+trypsin	YLLF	β-lactorphin	ACE-inhibitory (172); opioid (38)	Groleau and others, 2003b; Meisel and FitzGerald, 2000; Pihlanto-Leppala, 2000
f(106-111)	Pepsin+trypsin+ chymotrypsin	CMENSA		ACE-inhibitory (788)	Pihlanto-Leppala and others, 2000
f(142-145)		ALPM	β-lactosin B	Anti-hypertensive (928)	Murakami and others, 2004
f(142-146)	Pepsin+trypsin + chymotrypsin	ALPMH		ACE-inhibitory (521); hypocholesterolemic	Pihlanto-Leppala and others, 2000; Murakami and others, 2004
f(145-149)		MHIRL		Antioxidant	Hartmann and Meisel, 2007
f(146-149)	Trypsin + contaminant; chymotrypsin	HIRL	β-lactotensin	Opioid agonist	Pihlanto-Leppala, 2000

Table 2-9: Other whey derived bioactive peptides

Protein source and fragment	Treatment	Sequence	Peptide name	Bioactivity (IC_{50}µmol/L)	Reference
BSA					
f(208-216)		ALKAWSVAR	albutensin A; Serokinin	ACE-inhibitory (3.0)	FitzGerald and others, 2004
f(221-222)	Proteinase K	Phe-Pro		ACE-inhibitory (315)	Abubakar and others, 1998
f(399-404)	Pepsin	YGFQNA	serophin	Opioid (85)	Meisel and FitzGerald, 2000
Lactoferrin					
f(17-41)	Pepsin	FKCRRWEWRMKKLGAPSIPC VRRAF	Lactoferricin B	Antimicrobial; immuno-modulatory	Bellamy and others, 1992; Meisel, 2005
f(318-323)	Pepsin	YLGSGY (OCH3)	Lactoferroxin A	Opioid (antagonist)	Shah, 2000; Gobbetti and others, 2004
GMP					
f(106-116)	Synthetic			Antithrombotic (10)	Rutherfurd and Gill, 2000
f(112-116)	Trypsin			Antithrombotic	Rutherfurd and Gill, 2000

Many other studies have been made on peptides with possible bioactivity but many of them do not identify the peptide responsible for the health benefit. For example, Mercier and others (2004) have reported immunomodulatory effects of whey proteins and short chain neutral/basic whey peptides (with less than 5 kDa) but without identifying the peptides' sequence; antimicrobial activity from whey protein hydrolysates has also been reported (Pihlanto-Leppälä and others, 1999); ACE-inhibitory activity of hydrolysates from whey proteins produced with several proteases was also described by Mullally and others, 1997a). Some of these authors have fractionated the hydrolysates and identified the ranges of molecular weights on which the activity is higher. ACE-inhibitory activity was also described to be present in the product of β-Lg treated with Proteinase K of *Tritirachium album* or fermented with *Kluyveromyces marxianus var. marxianus*.

In order to exert their physiological effects in vivo, orally administered bioactive peptides have to escape the action of digestive enzymes. Although β-Lg f142-148 was reported to be transported intact across Caco-2b cell monolayers and to be one of the most potent ACE inhibitory whey peptides, recent studies have shown that this peptide is degraded during simulated gastrointestinal digestion because it is totally

hydrolysed by chymotrypsin (Roufik and others, 2007). Moreover, the peptide failed to act as a hypotensive agent following ingestion by two human volunteers, although this result is not conclusive due to the small size of the tested group (Walsh and others, 2004). These results suggest that lactokinin β-Lg f(142-148) and other bioactive peptides may need protection against gastric or intestinal enzymatic degradation in order to exert their physiological effects *in vivo*. For instance, it has been shown that the interaction of lactokinin β-Lg f(142-148) with β-Lg A produces a conformational change in the protein that provides some resistance against degradation of the complexes by chymotrypsin. The delay observed during *in vitro* chymotryptic hydrolysis of the β-Lg A:peptide complexes suggests that their hydrolysis during gastrointestinal digestion could be delayed, thus allowing the protein to deliver intact lactokinin β-Lg f(142-148) closer to the sites of the intestinal absorption (Roufik and others, 2007).

Although *in vivo* studies are important, there have been few such studies on bioactive peptides, and many of the peptides that had strong *in vitro* activity do not show high *in vivo* activity. Whey-derived peptides β-Lg f(78-80) (Ile-Pro-Ala), β-Lg f(42-45) (Ala-Leu-Pro-Met) and β-Lg f(17-19) (GTW) have been found to have anti-hypertensive effect in spontaneously hypertensive rats (SHR) (Pihlanto-Leppälä and others, 2000; Chen and others, 2007; Murakami and others, 2004). The opioid peptides α-lactorphin and β-lactorphin improved vascular function and α-lactorphin lowered blood pressure in SHR (Sipola and others, 2001; Nurminen and others, 2000).

Most of the available research data concerning the bioactivity of milk proteins and the peptides arising from its hydrolysis are the results of *in vitro* and animal studies. To commercialize milk protein and peptide derived products with bioactivity claims, the efficacy of these peptides *in vivo* in humans has to be proved (Manso and Lopez-Fandino, 2004).

The occurrence of many biologically active peptides in milk proteins is now well-established. Numerous scientific, technological and regulatory issues have, however, to be resolved before these substances can be optimally exploited for human nutrition and health. Firstly, there is a need to develop novel technologies, e.g., chromatographic and membrane separation techniques by means of which active peptide fractions can be produced and enriched. Secondly, it is important to study the technological properties of the active peptide fractions and to develop model foods which contain these peptides and retain their activity for a certain period. It is recognized that peptides can be more reactive than proteins, due to their lower molecular weight, and the peptides that are present in the food matrix may react with other food components. The interaction of peptides with carbohydrates and lipids as well as the influence

of the processing conditions (especially heating) on peptide activity and bioavailability should also be investigated. In particular, possible formation of toxic, allergenic or carcinogenic substances, such as acrylamide or biogenic amines, warrants intensive research. To this end, modern methods need to be developed to study the safety of food stuffs containing biologically active peptides (Korhonen and Pihlanto, 2003b).

A recent review on present food applications of bioactive peptides is given by Hartmann and Meisel (2007).

2.4 Enzyme immobilization

Immobilized enzymes are defined as "enzymes which are physically confined or localized in a certain region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Katchalski-Katzir, 1st Enzyme Engineering Conference, New Hampshire, 1971, cited by Worsfold, 1995, Powell, 1996...).

The major disadvantages of conventional batch enzymatic hydrolysis are the utilization of large quantities of enzyme, the eventual production of off-flavors and bitterness in case of excessive hydrolysis, the low yields, and poor productivity due to uncomplete reaction inhibited by the product, the nonuniform composition of the end product which may contain several fractions of varying molecular weights and the need to inactivate enzymes at the end of the reaction (D'Alvise and others, 2000).

The conversion of enzymes into water-insoluble products possessing specific catalytic activity is of interest since such 'water-insoluble enzymes' may readily be removed from the reaction mixture, thus reducing downstream processing cost and allowing its reuse. If stable, they may be employed repeatedly to induce specific chemical changes in relatively large amounts of substrate (Bareli and Katchalski, 1960), especially useful for expensive enzymes. Other advantages include operation in continuous reactors such as packed bed columns with enzymic activity, easy handling, less waste and enhanced stability towards temperature, pH or organic solvents (important when substrates or products have poor water solubility). Decreased inhibition, in the presence of enzyme inhibitors or products, and/or altered selectivity is also often achieved. However, during the immobilization method severe losses of activity often occur. Furthermore, additional costs of carriers and other immobilization reagents are involved and there may be mass transfer limitations during reactor operation.

Only in 1967 immobilized enzymes were used for the first time in an industrial process: in Japan, immobilized *Aspergillus oryzae* aminoacylase was employed for the resolution of synthetic racemic DL-

amino acids. Around 1970, two other immobilized systems were launched on a pilot plant scale. In England, immobilized penicillin acylase was used to prepare 6-aminopenicillanic acid from penicillin G or V, and in the United States, immobilized glucose isomerase was used to convert glucose into fructose. The use of immobilized enzymes in industry is now well established (Katchalski-Katzir, 2005;Katchalski-Katzir and Kraemer, 2000).

Although immobilization is "relatively" old and widely used, only recently it has been revealed as a very powerful tool to improve almost all enzyme properties, if properly designed: e.g., stability, activity, specificity and selectivity, reduction of inhibition (Mateo and others, 2007). For an industrial application these properties are of huge importance as the enzyme has to be reused several times for the process to be economically feasible, thus the design of new protocols that may permit to improve the enzyme properties during immobilization is still an exciting goal.

2.4.1 Immobilization carriers

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in advance which support will be most suitable for a particular enzyme (Worsfold, 1995).

Important features of an immobilization carrier include mechanical stabitity, chemical stability (including compatibility with reaction medium, substrates, products and enzyme of the process in question), suitable geometric properties (shape, size, thickness and length) and good economical and ecological performance (low cost, without environmental restrictions, safe for use, easy disposal – biodegradable, for instance, low volume – low cost for solid handling). As a small amount of attrition or leaching can ccur, it is a requirement that for food applications the material is non-toxic or potentially carcinogenic. For that reason, biological or inorganic carriers are generally preferred.

Physical and chemical nature of the carriers (especially the microenvironment) also has to be considered. For example their hydrophilic or hydrophobic nature, the charges on the carriers, and the binding chemistry can strongly dictate the catalytic characteristics of the enzyme as activity, retention of activity and stability (Cao, 2005). It should have a high capacity to bind the enzyme, which is determined by the available surface area, pore size and particle size, the ease with which the support can be activated and the resulting density of enzyme binding sites (Worsfold, 1995).

Synthetic matrices can be designed to be resistant to pH, temperature, and biological degradation, their hydrophobic-hydrophillic properties can be easily altered by appropriate selection of co-monomers used

during the synthetic step, their morphology might be altered by selection of type and amounts of inert diluents in the polymerisation step and surface modifications are easily achieved and various reactive groups may be anchored on to the matrix in a defined concentration (Bryjak and Kolarz, 1998). Therefore they are often considered as potencial enzyme carriers.

Hence enzyme immobilization has been performed on a wide variety of support materials. These materials can be divided into:

- Organic polymers (lipophilic) such as polystyrene, polyisocyanate, polyisithiocyanate, nylon, teflon membranes, polyacrylamide, polyvinyl alcohol, polyurethane;
- Natural polymers and derivatives (mainly hydrophilic biopolymers), including cellulose, CM-cellulose (cationic exchanger) and DEAE-cellulose (anionic exchanger), Sephadex, Sepharose, polydextrans, starch, agarose, colagene; chitin, alginates, bone char;
- Inorganic materials such as glass, silica, carbon, stainless steel, metallic oxides (ceramics), diatomaceous earth (Celite™), sand, caolinite, clay.

2.4.2 Immobilization methods

Many techniques for the preparation of immobilized forms of enzymes and other proteins have been reported. These methods can be divided in two main groups (Figure 2-6): one in which the enzyme is entrapped in a limited space (the enzyme remains in the interior of the immobilization matrix) and other in which the enzyme bonds to an insoluble support or carrier material (the enzyme is on the surface of the support).

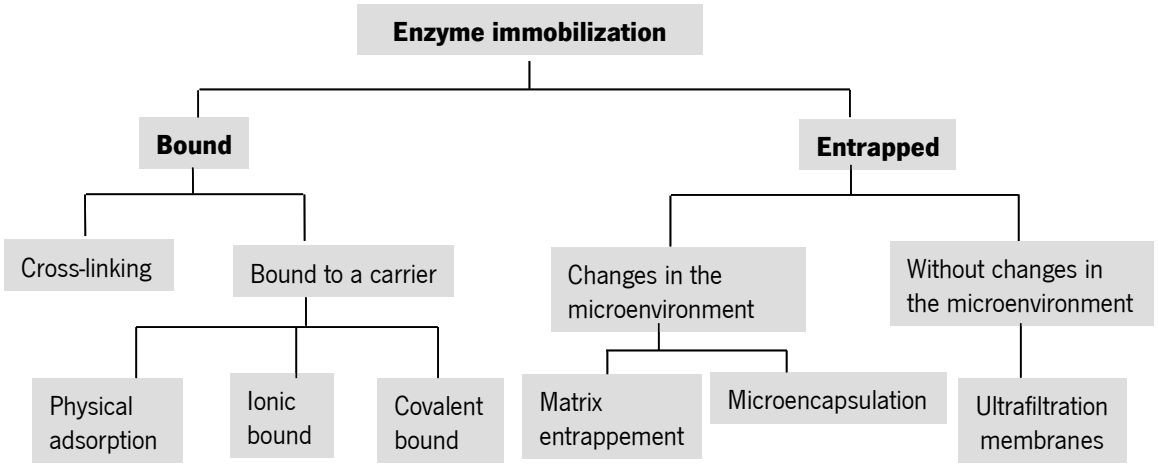


Figure 2-6 Immobilization techniques

Adsorption is the simplest and the oldest method of immobilizing an enzyme onto a water-insoluble carrier and has been widely referred in literature since mid-30's (e.g. Langmuir and Schaefer, 1938). The molecules of biocatalysts bind to the surface of the carriers by relatively weak physical forces (van der Waals forces). As a result the adsorbed enzyme can be easily desorbed by temperature fluctuations, and even more readily by changes in substrate and ionic concentrations. Some of the most common supports are alumina, amberlite CG-50, bentonite, calcium phosphate gels, activated carbon, collagen, glass, and silica gel.

Although in the 50's immobilization was still dominated by physical methods, i.e. non-specific physical adsorption of enzymes or proteins on solid carriers, carriers for specific ionic adsorption started to arise, such as phosphocellulose or DEAE–cellulose (Cao, 2005). Many ionic resins like DEAE-Sephadex, DEAE-cellulose, CM-Sephadex and CM-cellulose have been used as carriers since then. The enzyme will remain bond to the carrier provided that pH and ionic strength remain at adequate values.

In order to strengthen the linkage between enzyme and carrier their attachment by chemical covalent bonds seemed preferable. Such links should obviously be carried out by functional groups non-essential for enzymic activity (Bareli and Katchalski, 1960). As a result, in the early 60's covalent enzyme immobilization started being regularly studied, but those early-developed carriers were found to be less suitable for because of poor retention of activity (2–20 % of the native activity), probably attributable to the highly hydrophobic nature of the carriers used at that time or the unsuitable active functionality such as diazonium salt, which often affords an immobilized enzyme with lower retention of activity (Katchalski-Katzir, 2005). Covalent attachment can avoid leakage from the support but sometimes changes in both the conformational structure and the active center of the protein occurs resulting in a reduction of the biological activity.

A combination of the two above methods (physical adsorption and covalent linking) has also been used. An example involves the adsorption of protein as a monolayer onto the carrier (firstly colloidal silica particles were used), followed by intermolecular crosslinking with the bi-functional reagent glutaraldehyde, to give an enzyme "envelope" around each particle (Haynes and Walsh, 1969).

Covalent crosslinking of the protein through a bifunctional reagent is also possible, with or without a solid support.

Another method of immobilization is via entrapment of the enzyme of interest into polymers, gels and hollow fibres. In this case, the immobilized enzyme may exhibit altered properties as a result of changes in

its micro-environment and possible non-covalent interactions with the matrix. It may also have diffusional or leakage problems.

Several noncovalent linkages can be specifically promoted such as ionic and metal binding. Immobilization of an enzyme can also be made through disulphide bridges between accessible sulphydryl groups of an enzyme not essential to activity with sulphydryl groups on the carrier. Unlike covalent binding, this procedure is reversible and the enzyme can be dettached from the support under reducing conditions.

In affinity bindings, activation of the carrier is made with a specific ligand, such as an antibody or a metal ion. Carriers such as cellulose, glass and nylon if treated with salts of transition metals, such as titanium, vanadium or iron chlorides, can quelate enzymes; strong metal bridges are formed between hydroxyl oxygen atoms of the carrier and amino nitrogen atoms of the enzyme (Palmer, 1991). In this case little changes in the activity of the enzyme often occur. In recent years, several oriented immobilization techniques have been developed. Affinity ligands include nickel-nitrilotriacetic acid (Ni-NTA) to capture histidine-tagged proteins and avidin to capture biotinylated biomolecules (Palmer and Leung, 2007).

Strong noncovalent coupling of an enzyme to a solid support can be achieved through fusion proteins. These proteins are produced by genetic engineering through the fusion and expression of the gene responsible for the enzyme production with a second gene for an affinity domain for immobilization. A fusion protein of yeast α-glucosidase containing at its C-terminus a polycationic hexa-arginine peptide was produced by this method. The polycationic peptide extension allowed strong, noncovalent attachment of the fusion protein to a solid matrix containing polyanions as functional groups (Stempfer and others, 1996). The binding affinity between streptavidin and biotin is among the strongest noncovalent bonds known to exist. Once formed, it is highly resistant to denaturing reagents, extremes in pH and temperature, protease digestion, and denaturing reagents. Hybrid proteins, produced by the fusion and expression of streptavidin genes with a second gene of interest, provide an opportunity to design chimeric proteins containing both an affinity domain for immobilization and a second domain displaying bioactivity. This approach allows a one-step immobilization of streptavidin fusion proteins using a biotinylated affinity matrix. A trypsin-streptavidin (TRYPSA) fusion protein was designed by this method and its expression in *Escherichia coli* was evaluated (Clare and others, 2001). A review about this technique of producing immobilized enzymes is given by Boersma and others (2007).

Other alternative is to build a carrier and incorporate on it a mimic of the enzyme (for instance, zeozymes, which are zeolite-based enzyme mimics where the protein that usually surrounds the active site is replaced by an inorganic framework; thus the zeozyme is constituted by an enzyme active site and the

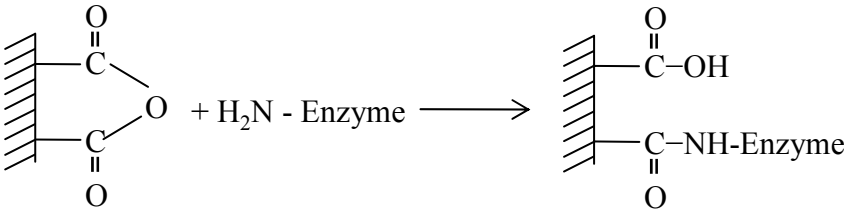
inorganic framework that imposes the geometric and steric constrains on the reaction of substrate molecules) or even to synthetise (mimic) an "ideal" enzyme active site for a desired application (Parton and others, 1994; Katchalski-Katzir and others, 2003).

2.3.2.1. Covalent binding to a solid support

This kind of immobilization can be done in organic, inorganic or biological carriers. The only requirement is a properly derivatization of the surface to produce a functional group that can be chemically activated to become reactive towards an aminoacid side chain. Some carriers do not need derivatization or activation as they have an activated functional group (such as an anhydride, a chloride or a bromide):

$$\text{Cellulose-OCO}{-}\text{CH}_2\text{Br} + \text{Enzyme} \longrightarrow \text{Cellulose-OCO}{-}\text{CH}_2{-}\text{Enzyme}$$

or



(Palmer, 1991)

Some polymers are even designed and "built" with activated functional groups (Li and others, 1998; Katchalski-Katzir and Kraemer, 2000, Rao and others, 2006, e.g.). Other supports only need the activation step as they already have functional groups as aromatic amino groups that can be activated by diazotization as describe below.

It is desirable that the immobilization procedure involves mild operating conditions, non toxic reagents, strong covalent binding and low cost.

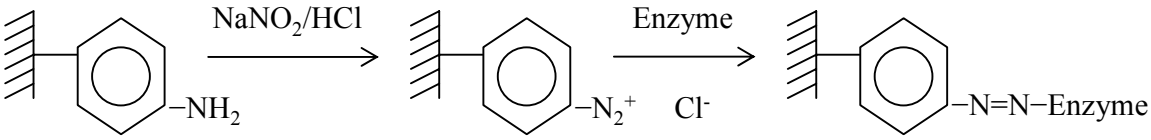
The enzyme functional groups most commonly linked by covalent bonds to a carrier are free α-amino groups from the aminoacid chain and free ε-amino from lysine and arginine. Phenolic (from tyrosine), hydroxyl (serine or treonine), imidazole (from hystidine), thyol (cysteine) or free carboxyl groups (terminal or from aspartic or glutamic acid) may also be involved (Wang and others, 1979; Palmer, 1991; Cao, 2005).

The immobilization methods to convalently bond an enzyme may be grouped accordingly to the reactive group on the unmodified support utilized (Wang and others, 1979; Worsfold, 1995). Some examples are given for each group.

- Activation of $-NH_2$

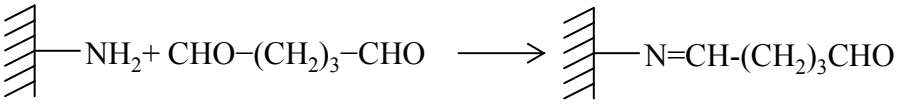
Carriers with $-NH_2$ can be activated as follows:

- Through diazotization – the enzyme binds to a diazonium derivative of the carrier using phenolic, imidazol or free amino groups from the enzyme through azo bonds; this method can be use to immobilize enzymes through a diazonium derivative on p-aminobenzyl-cellulose (Mitz and Summaria, 1961, e.g.) or polyaminopolystyrene, for instance.

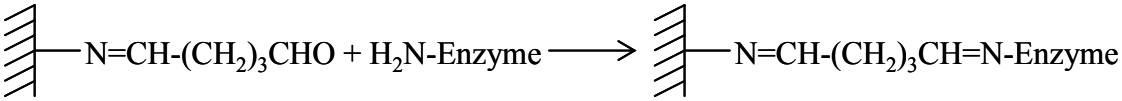


- With dialdehydes such as glutaraldehyde – it is a bi-funtional reagent and can also be used immobilize enzymes via crosslinking; it was used by many authors including Bryjak and Noworyta (1993); Carrara and Rubiolo (1994); Chellapandian and Sastry (1994); George and others (1996); Isgrove and others (2001); Moeschel and others (2003).

1st Activation step



2nd Coupling step



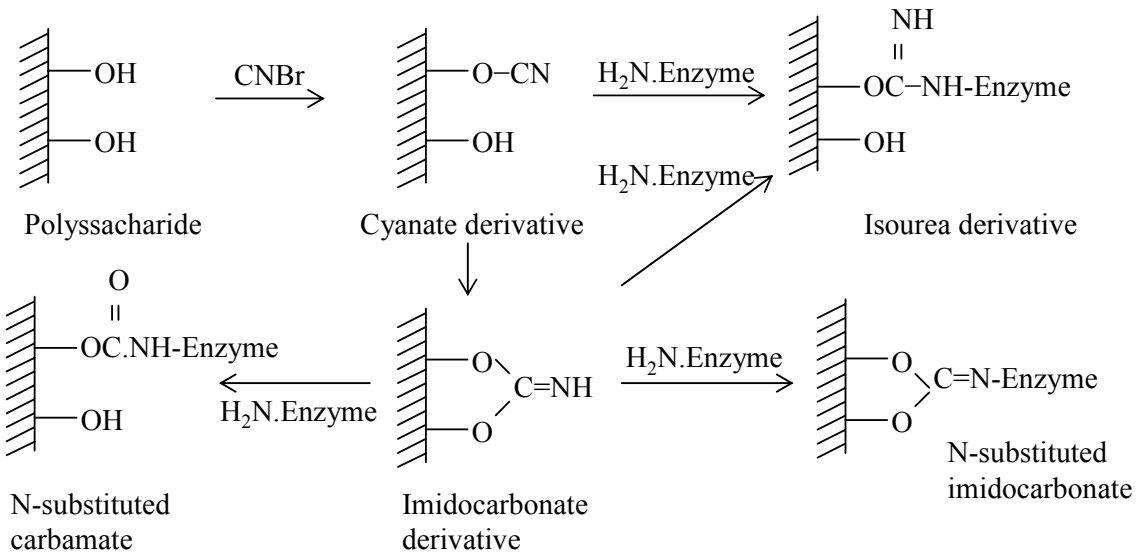
Sodium borohydride or sodium cyanoborohydride may be used to reduce unstable Schiff's bases (formed between aldehyde groups of the glutaraldehyde molecule and terminal amino groups of the enzyme) into stable secondary amines (e.g. Limbut and others, 2004);

Carriers with amine groups can be modified to have other terminal groups such as an epoxy (with e.g. 1,4-butanediol diglycidyl ether), imidazole (with carbonyl diimidazole) or carboxylic acid terminal group (Sousa and others, 2001; Moeschel and others, 2003).

- Activation of hydroxylic supports (with –OH):

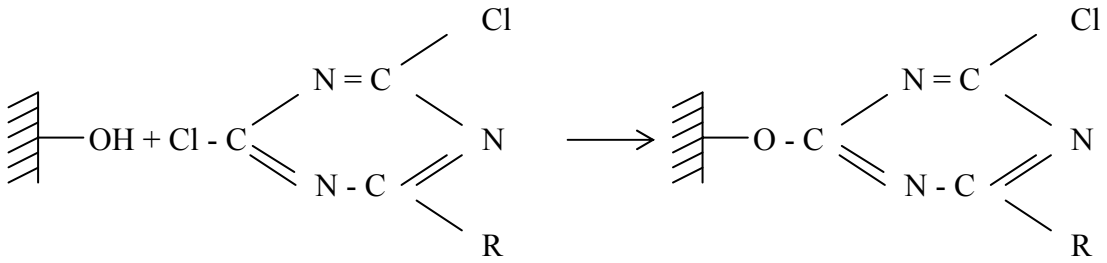
There are many immobilization protocols for supports with –OH groups. These include activation using:

- Cyanogen bromide – used for polyssacharides as celluloses (used by Axen and others, 1967; Wilchek and others, 1975; Wang and others, 1979; Ishikawa and others, 1987; Palmer, 1991; among others); results in a reactive cyanate or an imidocarbonate (among other non reactive products) that allows further binding of the enzyme through an amine group (peptidic bond); most significant reactions that occurs can be summarized by:

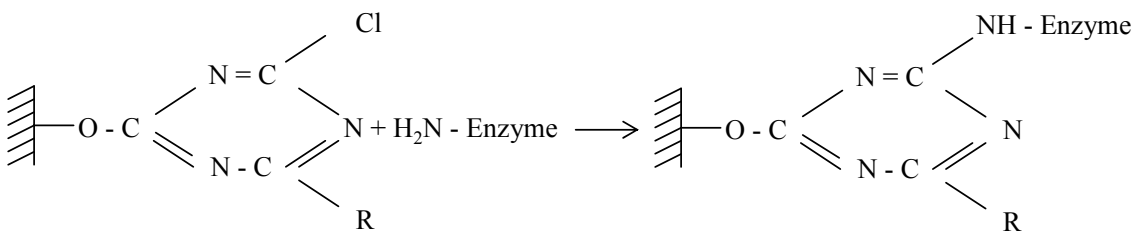


- S-triazine derivatives (e.g. cyanuric chloride) - used by Lenfeld and others (1995); Spagna and others (1995).

1st Activation step:

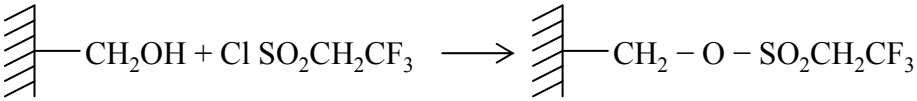


2nd Coupling step



- Sulfonyl chlorides, as tresyl chloride (2,2,2-trifluoroethanesulphonyl chloride) or tosyl chloride (p-toluenesulphonyl chloride) – form very stable secondary amine bonds with proteins; used in gels (such as Sepharose) or inorganic carriers as silicas by Nilsson and Mosbach (1981); Comfort and others (1989); Goetz and others (1991); George and others (1996) and Puleo (1996), e.g.

1st Activation step:



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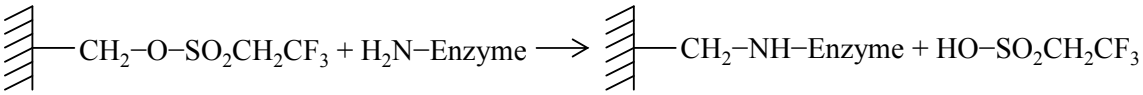
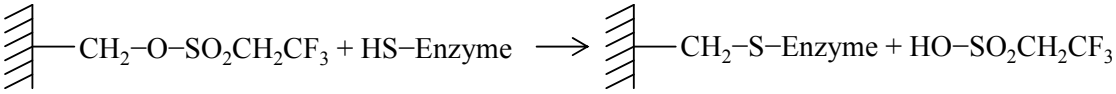
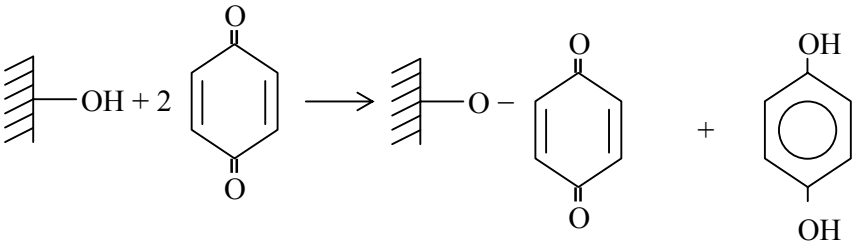


or



- Benzoquinone (Kalman and others, 1983; Vertesi and others, 1999);

1st Activation step:



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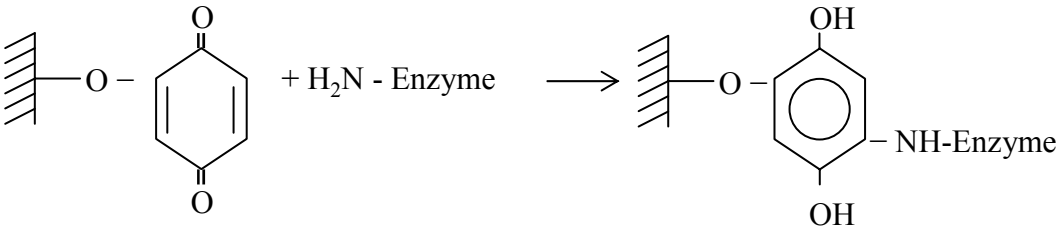
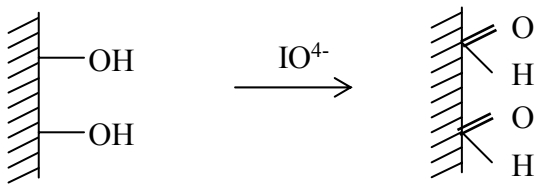
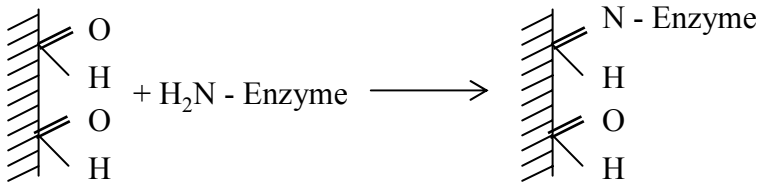


- Sodium periodate (Cavalcante and others, 2006; George and others, 1996); results in a Schiff base that needs to be reduced with sodium borohydride.

1st Activation step:

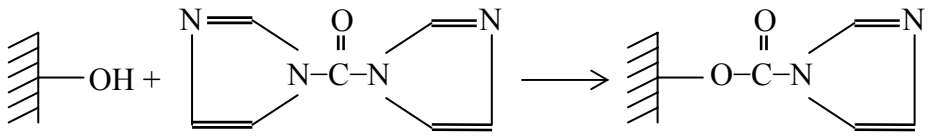


2nd Coupling step



- Carbonyldiimidazole (Ferreira and others, 2003; Akgol and others, 2001; Goetz and others, 1991) – results peptidic bonds between the carrier and the enzyme.

1st Activation step:



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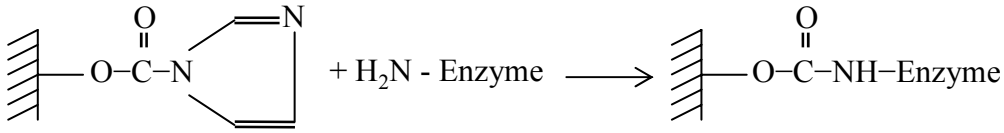
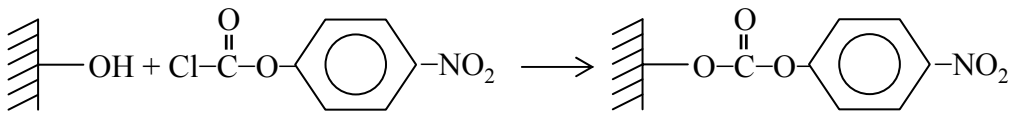
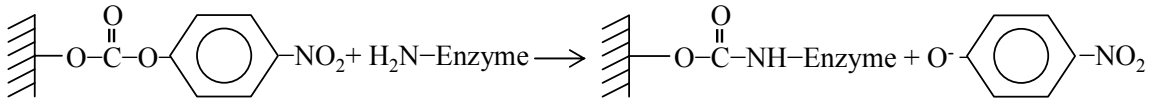


- p-Nitrophenyl chloroformate (used e.g. by Puleo, 1996; Scouten and Dvorak, 1995) or similar chloroformates; once again the enzyme binds to the carrier through a peptidic bond.

1st Activation step:

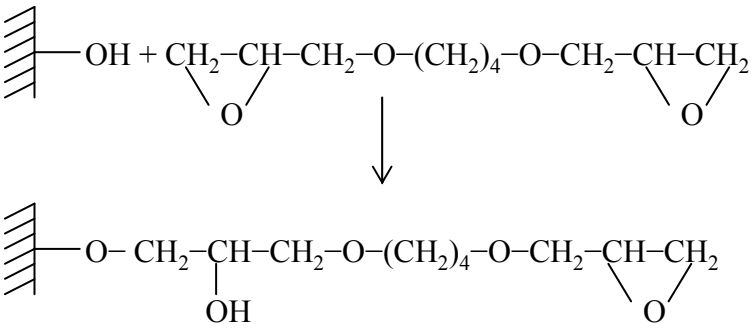


2nd Coupling step



- Epoxy reagents such as epichlorohydrin (1-chloro-2,3-epoxypropane or chloromethyloxirane or glycidyl chloride) or bifunctional oxiranes (1,4-butanedioldiglycidyl ether, for instance) react with an amino or hydroxyl groups on the support resulting in epoxy activated carriers (Sundberg and Porath, 1974; Murthy and Moudgal, 1986; Lorenzen and Schlimme, 1995; Mateo and others, 2000; Tumturk and others, 2000; Ragnitz and others, 2001b; Mateo and others, 2003; Brandi and others, 2006; Shewale and Pandit, 2007). This method allows the incorporation of an hydrophobic spacer arm with the desired length, e.g. 5 carbon atoms with epichlorohydrin and 12 carbon atoms with 1,4-butanedioldiglycidyl ether (Guo and Ruckenstein, 2001) that can bind to an enzyme through several functional groups including amine, thiol and phenolic. The carrier can directly bind to the enzyme or be further derivatized with diaminobenzene and activated by diazotization as described above (Ruckenstein and Guo, 2001). Epichlorohydrin binds to the support through the chloride while bifunctional oxiranes bind to the support through an epoxy group.

1st Activation step:



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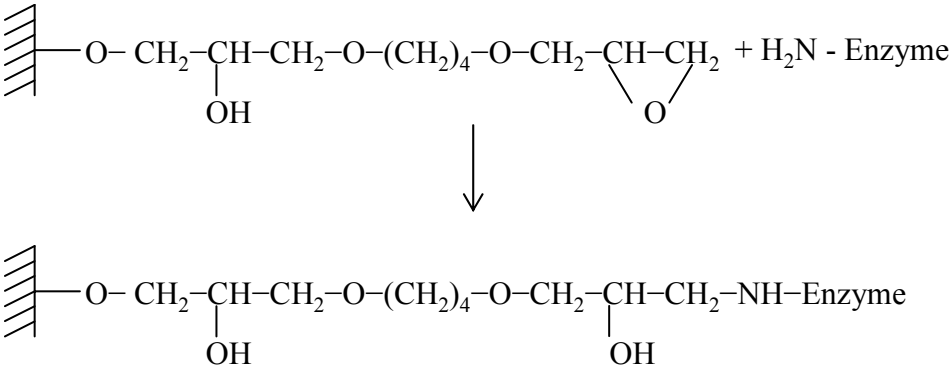
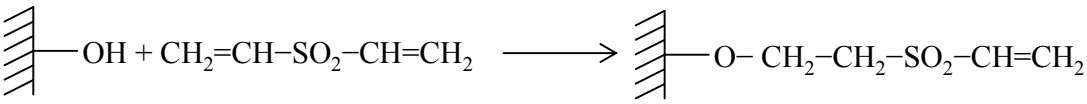


- Divinylsulphone - Lihme and others (1986); Noel and others (1996) - can bind to an $-NH_2$ group of the enzyme.

1st Activation step:



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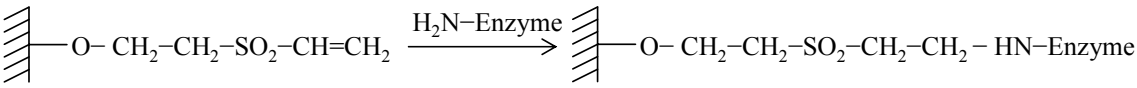
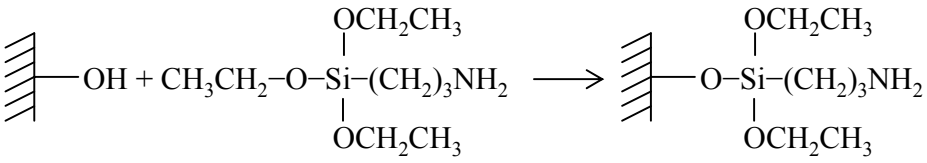


Organic and inorganic carriers with –OH groups can be derivatized to have amine or carboxylic groups.

The nonspecific adsorption of proteins constitutes the main disadvantage of some inorganic supports including glass and silica. Modifying its surface with organosilanes allows the introduction of more specific functional groups (Kuraoka and others, 2001).

Thus, inorganic supports are commonly derivatized through silanization (with aminopropyltriethoxysilane or a similar organosilane, e.g.) to have an amine group prior to the enzyme attachment:



The resulting aminopropyl-carrier may then be activated with glutaraldehyde as described above (Martino and others, 1996, Wilson and others, 1994; Subramanian and others, 1999; Costa and others, 2001; Ettalibi and Baratti, 2001; Ferreira and others, 2003; Limbut and others, 2004; Nam and Walsh, 2005).

Alternatively they can be silanized to have an epoxy group with 3-glycidoxypropyltrimethoxysilane and N,N-diisopropyltrimethoxysilane that can be directly bond to the enzyme (Subramanian and others, 1999; Felix and Descorps, 1999).

Inorganic surfaces with succinamidopropyl groups can be better for certain applications than surfaces with aminopropyl groups and are prepared by succinylanation of aminopropyl groups. The resulting carboxyl groups of the succinamidopropyl-carrier can be activated with carbodiimide (Janolino and Swaisgood, 1982) or with thionyl chloride (Stabel and others, 1992).

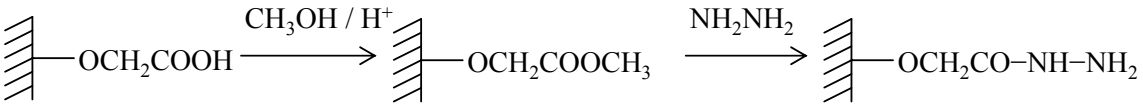
- Carrier with –COOH

The peptic bond between a free amino group from the enzyme and a carboxyl group of the support may be formed if the latter group is activated to an isocyanate, acid azide or other reactive derivative or via condensing agents such as carbodiimides. The activation can be made:

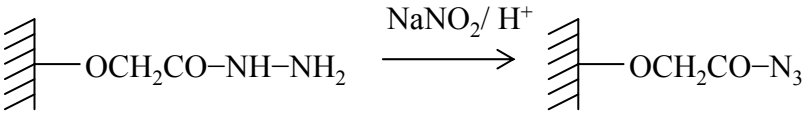
- Via azide derivative (Mitz and Summaria, 1961; Epstein and Anfinsen, 1962; Yodoya and others, 2003) - this method has been used for example to immobilize enzymes on carboxymethyl-cellulose (cellulose-OCH_2COOH).

1st Activation step

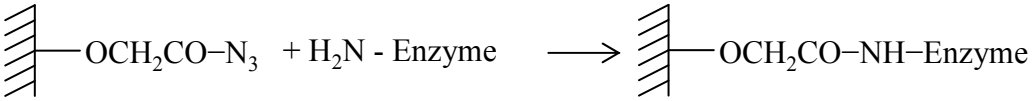
The 1st step of the support activation consists on a esterification with methanol and HCl, followed by treatment of the methyl ester with hydrazine resulting in a carrier-hydrazide:



During the 2nd step hydrazide is converted to azide with nitrous acid ($NaNO_2$ in HCl):

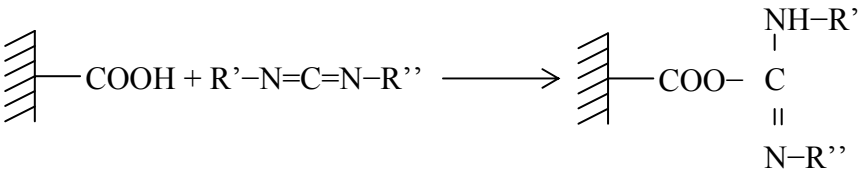


2nd Coupling step

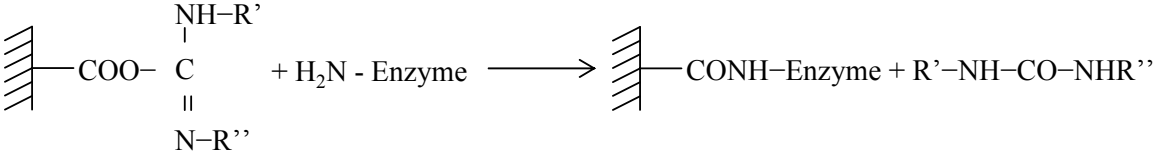


- With carbodiimide (CM cellulose) – under suitable conditions, carbodiimides can react with carboxylic, amino, phenolic and thiol groups; applied by, among many others, Spagna and others (1995); Ragnitz and others (2001a); Vertesi and others (1999); Yodoya and others (2003).

1st Activation step



2nd Coupling step



- With thionyl chloride (used by Spagna and others, 1995, e.g.).

- Other reactive groups on the support

2.4.3 Improvement of enzyme activity retention during an immobilization procedure

Methodologies that can increase enzyme activity retention include the presence of substrates, substrate-analogues or reversible inhibitors that often lead to higher activity retention, probably by protecting the active site of the enzyme from covalent attachments.

The use of spacer arms allows the enzyme to attach the solid support at a certain distance from the surface in order to keep biologically important sites accessible to the substrates. Usually it confers flexibility onto the enzyme molecule but they can also be used to shield the enzyme from the carrier surface when the hydrophobicity is high and harmful to enzyme stability (Nouaimi and others, 2001). The carrier itself can be designed to include spacer "chains"; for instance, the attachment of the enzyme to polymeric carriers can be designed to occur through flexible side-chains that act as spacers and ensure free movement of the catalyst molecules in the reaction mixture (Bareli and Katchalski, 1960). Long and flexible spacers with an hydrophilic character such as dextran can also be used to improve the activity of the enzyme when conventional small hydrophobic ''spacer arms'', usually with 6 to 12 carbon atoms, are unsuitable (Penzol and others, 1998; Manta and others, 2003; Betancor and others, 2004). This may happen, for instance, when the immobilized ligand is not a small molecule but a protein having a large surface area involved in the recognition of soluble macromolecular substrates such as other proteins. However care is needed when using very long spacers on highly activated supports because intense multipoint attachment of the spacer to the carrier may cause rigidity and, at the same time, make it behave as a short spacer arm, creating new steric hindrances. Polyethylenimine is also used sometimes (Rocha and others, 2006).

2.4.4 Enzyme stabilization by immobilization techniques

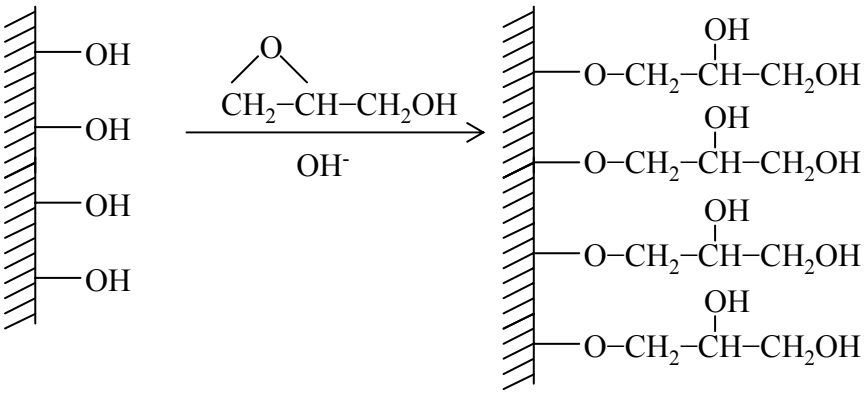
The development of methodologies that can increase enzyme stability is an important goal in enzyme technology. Stabilization may be achieved using protein engineering, adequate immobilization techniques, through stabilizing additives or chemical modification (Betancor and others, 2004). Thus, multicrosslinked enzyme derivatives, the multipoint covalent attachment of proteins to preexisting solids (in this case, we can consider the support to be the crosslinking reagent), and intramolecular crosslinkings by chemical modification are some of the physico-chemical methodologies that can be used to increase the intrinsic rigidity of the enzyme structure, and in this way, to increase their stability (Fernandez-Lafuente and others, 1995).

Minimal modification of the enzyme surface with a hydrophilic polymer could be a good strategy to form a shell around the enzyme surface and protect it against the interaction with hydrophobic interfaces when enzyme immobilization on solid supports is not feasible, e.g. in the use of proteases in hydrolysis of solid proteins, or the use of soluble enzymes in ultra filtration tanks (Betancor and others, 2004).

Operational stabilization of enzymes may be achieved by immobilization on porous supports. The enzyme will be immobilized inside a porous structure that maintains its molecules fully dispersed and without the possibility of interacting with any external interface. Thus, this immobilization will stabilize the enzyme against interaction with molecules from the enzymatic extract (preventing aggregation, autolysis or proteolysis by proteases from the extract), contact with any external hydrophobic interface (such as air bubbles) or contact with an organic solvent phase (Mateo and others, 2007).

Structural stabilization of the enzyme may be achieved through multipoint covalent immobilization, which allows the rigidification of the enzyme structure reducing its denaturation. In the case of multimeric enzymes, multisubunit immobilization may be needed (Mateo and others, 2007).

The use of glutaraldehyde (for stabilization purposes, it is usually used after pre-adsoption of proteins in supports with primary amino groups allowing the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support), aldehyde or epoxy solid carriers is often referred (Guisan, 1988; Blanco and others, 1989; Balcao and others, 2001, Tardioli and others, 2003, Lopez-Gallego and others, 2005, Mateo and others, 2006, Betancor and others, 2006, among others). Derivatization with glycidol and activation with sodium periodate to give a glyoxyl derivative of the support has the advantage of resulting in a more hydrophilic carrier than the epoxy derivative.



Thus, aldehyde groups, moderately separated from support surfaces with no steric hindrance for the amine-aldehyde chemical reaction are obtained. These carriers are very stable, even in moderately alkaline media (Guisan, 1988).

2.4.5 Immobilized enzyme characteristics - effects of immobilization

In order to access the immobilization procedure, several immobilized enzyme characteristics should be stated, in particular its activity and stability. Enzyme loading may also be important.

The percentage of enzyme immobilized is usually determined by measuring the amount of enzyme remaining in the supernatant after immobilization and subtracting this from the amount of enzyme originally present. The absolute enzyme activity remaining on the support after immobilization is more difficult to determine and an apparent activity is usually measured which takes into account mass transfer and diffusional restrictions in the experimental procedure (Worsfold, 1995). Thus, the retention of activity can be defined as the ratio of the activity of the immobilized enzyme to the activity of the same amount of free enzyme. When an enzyme is bound to a solid support enzymic activity may be lost in several ways (Wang and others, 1979):

- Some enzyme molecules may be immobilized relative to the support in a configuration that completely prevents substrate access to the active site;
- A reactive group in the active site may be involved in the binding to the support;
- Enzyme molecules on binding may be held in an inactive configuration;
- The reaction conditions for binding may cause denaturation or inactivation.

Storage stability (including the influence of storage conditions as temperature, pH, ionic strength and the influence of impurities incorporated during the immobilization step), operational stability and temperature stability are also important parameters.

Kinetic parameters are also relevant to describe the immobilization success. Apparent Michaelis constant (K_M) for appropriate substrates (which also give an indication of immobilized enzyme selectivity) often give an indication about possible difusional restriction or the affinity of the support to the substrate.

Optimum operating conditions (pH and temperature) of the free and the immobilized form of the enzyme may be different and should also be referred.

2.5 Gelation of whey proteins

The gelation of globular proteins is widely studied and can be induced by physical conditions, such as heat or pressure, or by chemical factors such as the adition of calcium or acid (Ju and Kilara, 1998; Britten and Giroux, 2001; Totosaus and others, 2002; Lee and others, 2006). In any case, the proteins have to be heat-denaturated prior to the addition of the gelling agent. Enzymatic gelation is also possible (Doucet and others, 2001; Totosaus and others, 2002). Cold-set or acid-set gelation of proteins could be very advantageous to the food industry because many products cannot be heated to the temperature needed for thermal gelation of whey proteins. However, heat-set gelation is still highly used and gels formed by this method are usually stronger than cold-set, acid-set or enzyme-induced gels (Ju and Kilara, 1998).

Heat-induced gelation is affected by many factors such as pH, protein concentration, ionic strength, type of ions, heating rate, cooling rate and heating temperature (Hines and Foegeding, 1993). Depending on these factors a precipitate, a gel or a dispersion of soluble polymers is formed (Britten and Giroux, 2001). It is generally accepted that heat-induced gelation of proteins involves the following three main steps: protein partial unfolding that exposes reactive groups and/or sites on the molecules that favour intermolecular interactions; formation of linear fibrils of denatured protein molecules via sulphydryl-disulphide interchange reactions; and setting of the fibrils into a gel network via noncovalent interactions (Boye and others, 2000). However gelling can also occur in the absence of sulphydryl/disulphide interchange reactions. In this case hydrogen bonding, electrostatic and hydrophobic interactions can be important in whey protein aggregation. The rate and the mechanism of the gelling process depend on the type of protein and on the environmental conditions and the mechanism of the gelation can be different when different conditions are used. Thus, depending on variations in aggregate size, shape, the rate and nature of crosslinking of aggregates and the dimensions of the void spaces within the crosslinks, gels with very different textural properties can be obtained (Boye and others, 2000)

When temperature rises above the proteins denaturation point (typically 50-80 °C), there is a thermally induced unfolding of the native protein, possibly after some degree of dissociation if a multisubunit is involved, and a change in conformation occurs. The protein secondary and tertiary structures modify on heating and the protein molecule becomes more reactive as internal hydrophobic groups of the protein become more exposed. The degree of protein modification is, of course, dependent on the temperature profile and the time during which the protein was subjected to that temperature profile. In spite of this, the size and shape of the macromolecule suffers little changes (< 20 %) and the resulting protein is often still

globular and is able to bind to other similarly unfolded species (Tobitani and RossMurphy, 1997; Clark and others, 2001).

In order to reduce the exposure of the hydrophobic groups to the aqueous environment, aggregation of protein globules arise. Agreggation of unfolded protein molecules is essential for gelling. Depending on the balance between attractive and repulsive forces among denatured molecules, two types of aggregates can appear: when intermolecular electrostatic repulsion is dominant nanometer thick strands are formed (Ikeda and Li-Chan, 2004); much coarser disordered particulate aggregates appear when the electrostatic repulsion is lower, that is when pH value is close to pI or when ionic strength is increased (Ikeda and Li-Chan, 2004; Foegeding, 2006). At neutral pH values, this aggregation can take place through sulphydryl-disulphide (SH/S-S) interchange reactions (Tobitani and RossMurphy, 1997). At these pH values the protein is highly charged (pH far away from pI) and if the ionic strength is low this aggregation step is limited in extent and the formed aggregates are predominantly linear (fibrils or small strands). Although much of the secondary structure of the protein remains, there is often an increased level of antiparallel β-sheet (Clark and others, 2001).

If the concentration is above a critical concentration (C_0) and the ionic strength is sufficiently high, gelling can occur after a critical gelation time (t_c). This third step involves the random association of aggregates (strands or coarse particules). Again, depending on the balance of protein-protein interaction, protein-water interactions and attractive and repulsive forces among adjacent polypeptide aggregates, two types of gels can appear (Avanza and others, 2005). The higher the ionic strength and the closer the pH is to the isoelectric point, the more unspecific the aggregation is and more turbid, syneresing, less elastic gels are formed (Tobitani and RossMurphy, 1997). The network of these gels is said to be particulate. They are composed of aggregates that can be as large as micrometers. When gelation is made under the opposite conditions (low ionic strength and pH >> pI or pH << pI) the intermolecular electrostatic repulsion is dominant. Repulsion forces and protein-water interactions help to keep polypeptide chains separated, favoring the formation of a homogeneous matrix (Avanza and others, 2005). In this case, fine stranded gels are formed that are transparent and have high water-holding capacity. They are composed of flexible strands or more rigid fibrils depending on pH and ionic strength. Strand diameters generally correspond to the diameter of one or several protein molecules (Ikeda and Li-Chan, 2004; Foegeding, 2006).

The following discussion will be focused on the gelation of β-Lg, as it is the main gelling protein in the WPC.

Protein unfolding of β-Lg seems to follow a first order kinetics while all protein aggregation steps are bimolecular and seem to follow a second order kinetics. The overall reaction of aggregation (including the unfolding step) is expected to be between 1 and 2 (de Wit, 1990; de Wit, 1998; Verheul and others, 1998a). In the aggregation step, the experimental conditions determine wheather chemical and/or physical bonds are formed between the molecules as well as the rate of aggregation reactions (Verheul and others, 1998a). Two limiting cases concerning the overall reaction kinetics can be distinguished: the unfolding reaction is rate limiting and the overall reaction order is 1 or the aggregation reactions are rate limiting and the overall reaction order is 2. At low temperatures, pH values close to the isoelectric point and at high NaCl concentrations the process is closer to the first situation. The higher the temperature, the lower the ionic strength and the farther pH is from the isoelectric point, the closer to the second situation is the overall kinetics. For instance, at temperatures higher than 70 °C, the protein unfolding near neutral pH values is not the rate limiting step (de Wit, 1990). For temperatures lower than 63 °C the concentration of unfolded protein controls the rate of aggregation (Elofsson and others, 1996).

On the other hand, chemical reactions are predominant at high pH values because of the increased reactivity of thiol groups. Non-covalent bonds are enhanced at pH values close to the isoelectric point and at high ionic strength as the intermolecular repulsion and solubility decrease (Verheul and others, 1998a; Galani and Apenten, 1999). At high ionic strength (high NaCl concentrations) two phases are observed in the aggregation step: the formation of primary "aggregates" and a secondary aggregation that starts at a critical concentration of primary aggregates, which is lower for higher salt concentrations and at pH values closer to the isoelectric point and dependent on the initial protein concentration (Verheul and others, 1998a). The influence of non-covalent interations on the overall aggregation mechanism also increases with temperature, becoming important at temperatures close to 90 °C (Galani and Apenten, 1999).

The rate of heating is also decisive in the gelling process. For instance, when a heating rate of 60 °C/min is used, aggregation of β-Lg does not occur below 83 °C, but at a heating rate of 1 °C/min, aggregation starts already at 73 °C. At both heating rates, aggregation (i.e., irreversible denaturation) appears to start when 60 % unfolding has occurred (de Wit, 1998).

Two types of β-Lg gels may appear depending on the environmental conditions: transparent fine-stranded gels or opaque particulate gels; this is a common behavior for globular proteins, which is the case of β-Lg (Figure 2-7).

When the ionic strength is high and/or pH is close to pI (5.3), electrostatic repulsion between proteins is weak due to charge screening, and a densely packed, opaque particulate gel is formed, as described above. In this case, no significant dissociation of the dimers (or other subunits, depending on the pH, concentration and temperature) occurs with the temperature raise due to the low protein-protein electrostatic repulsions near the pI. Protein aggregation is based on physical interactions more than on protein unfolding (that would promote hydrophobic and covalent interactions) and is promoted by the presence of salts due to the screening effect (Unterhaslberger and others, 2006). These gels are characterized by a previous random association into large and almost spherical ("primary") aggregates that subsquently link together to form the gel network (Lefevre and Subirade, 2000). In the case of particulate gels β-Lg molecules are less extensively unfolded and aggregate largely through non-specific hydrophobic or electrostatic interactions (Ikeda and Li-Chan, 2004). The β-sheet secondary structure appears to be well preserved (Euston and others, 2007).

At pH7.0 but with 0.3 M NaCl the formed gel is similar to the gel formed with low ionic strength near the isoelectric pH (Euston and others, 2007). The formation of a neutral particulate gel has appeared to result from accelerated aggregation of globules, while microscopic phase-separation, followed by heat induced gelation of phase-separated liquid droplets, may be involved in the formation of acidic particulate gels (Ikeda and Li-Chan, 2004).

Heat treatment of β-Lg at neutral pH causes the dissociation of the native dimers, followed by partial unfolding and denaturing. This critical change in the conformation of β-Lg occurs at 60–65 °C and exposes non polar groups and the buried -SH group of Cys121, initializing the sulfhydryl/disulphide (SH/S-S) interchange reactions and aggregation (Galani and Apenten, 1999). The protein is essentially in the monomeric form just before aggregation (Lefevre and Subirade, 2000). There is some evidence that disulphide bonded dimers primarily form in this process and that they can be important intermediates in the further aggregation of β-Lg (Havea and others, 2001). Fourier transform infrared (FTIR) spectroscopy analysis indicates that antiparallel intermolecular β-sheets result from aggregation (Lefevre and Subirade, 2000). The elementary unit of fine-strands at neutral pH is a primary small well defined aggregate ("cluster" or small fibril) of about 100 monomers, 15 nm length (Verheul and others, 1998a; Le Bon and others, 1999; Arnaudov and others, 2003; Ikeda and Li-Chan, 2004) that forms independently of concentration, temperature or ionic strength. In the second step these clusters aggregate and form structures with a broad size distribution. This step seems to be favoured by the presence of native protein

(Le Bon and others, 1999). It is also referred, based on FTIR spectroscopy analisys that this thermal aggregation is irreversible and that there is a strengthening of the intermolecular hydrogen bonds between β-sheets with decreasing temperature after the heat-gelation process (Lefevre and Subirade, 2000). Fine-stranded gels are less stiff and more elastic that particulate gels; the critical protein concentration required to form the gel is higher and the gel is weaker (Lefevre and Subirade, 2001).

Aggregation at low pH values (pH 2.0, e.g.) and low ionic strength seems to be a one-step process unlike aggregation at close to pI and neutral pH values. At this pH both light scattering and FTIR spectroscopy analisys show evidence that dimers and monomers coexist. After the dimers dissociation and partial denaturation occur long rigid fibrils (amyloid-type) are formed at low ionic strength by connecting monomers and dimers. These fibrils have a periodic structure of ca. 25 nm and a thickness of one or two protein monomers (Arnaudov and others, 2003). The length of the fibrils decreases as ionic strength increases (Foegeding, 2006). This process is dependent on β-Lg concentration (Euston and others, 2007) and at low ionic strength they only form above approximately 2.5 % w/w (Arnaudov and others, 2003). Part of the partially denatured monomers is not incorporated into fibrils and forms low molecular weight complexes. At low concentrations these "dead-end" species are predominant and no fibrils are formed (Arnaudov and others, 2003). These gels fracture at very low strain (unlike the fine-stranded gels at pH 7) which can be explained by the absence of intermolecular disulfide bonds. β-sheets in this kind of gels are more strongly hydrogen-bonded than in fine-stranded gels formed at pH 7 (Ikeda and Li-Chan, 2004). The distance between crosslinks is shorter and the strains are straighter and stiffer than in the gels at pH 7 (Langton and Hermansson, 1992).

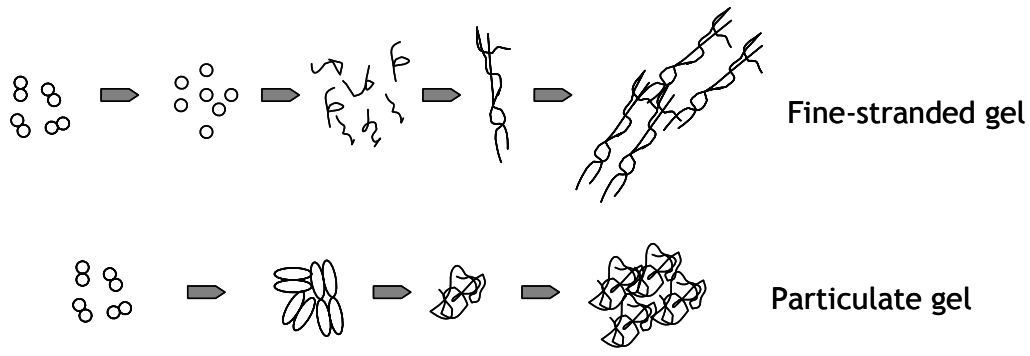


Figure 2-7 Representation of the gel network formation of β-Lg (adapted from Lefevre and Subirade, 2000)

Gelation is an important functional property of WP that has been extensively studied by different authors (see, for example, Langton and Hermansson, 1992; Hines and Foegeding, 1993; Le Bon and others,

1999, Boye and others, 2000; Kavanagh and others, 2000). The gelling ability of whey proteins provides important textural and water holding properties in many foods.

The two major proteins in the whey fraction of bovine milk are β-Lg and α-La (70-80 % of total protein). β-Lg can form gels by itself when heated, depending on the environmental conditions used, while α-La has poor gelation ability. It is generally accepted that the characteristics of β-Lg dominate the behaviour of WPC and WPI. However, it is likely that the gelation behaviour of β-Lg is altered by the presence of the other whey proteins (Havea and others, 2001). Although BSA corresponds to only ca. 5 % of the total whey proteins, it has also good gelling properties (Boye and others, 2000). The inclusion of BSA in β-Lg solutions accelerates the formation of heat-set gels resulting in a gel with increased elastic modulus and strength (Hines and Foegeding, 1993; Kehoe and others, 2007). This synergistic effect was also reported by Gezimati and others, 1996. Gels formed with mixtures of α-La in β-Lg are similar to gels formed with β-Lg alone and do not reflect the poor gelling ability of α-La (Hines and Foegeding, 1993).

Havea and others, 2001 presented a model for the aggregation and gelling of mixtures of the three proteins at neutral pH values (Figure 2-8). Due to differences in thermal stability of these proteins, BSA will begin to unfold and aggregate before β-Lg. The exposed thiol groups of BSA can also react with one of the disulphide bonds of α-La resulting in mixed aggregates BSA/α-La.

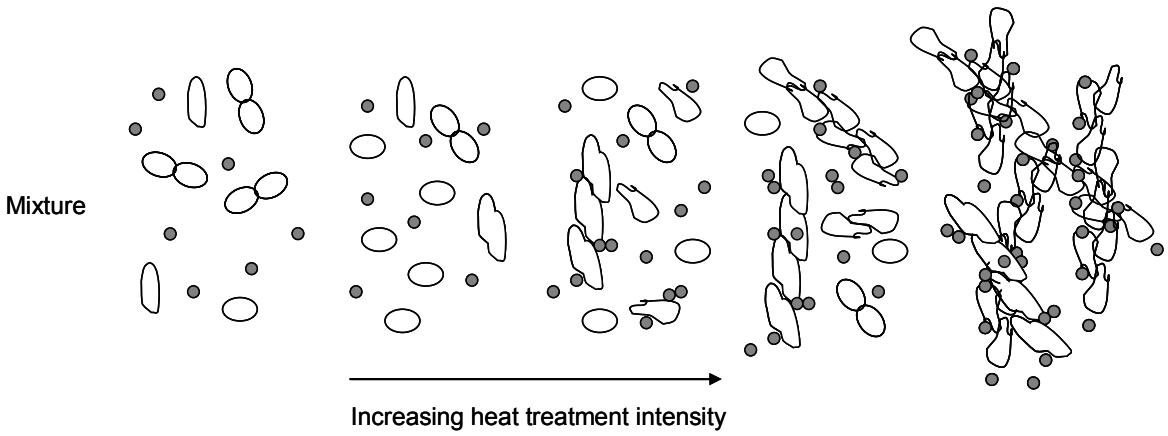


Figure 2-8 Representation of the gel network formation in a heated solution of a mixture of BSA, β-Lg and α-La in the proportion 2:1:1 (adapted from Havea and others, 2001)

● - α-La monomer; ⬭ - β-Lg native monomer; ⬭ - BSA monomer; ⬭ - β-Lg non-native monomer; ⬭ - BSA aggregate

As the temperature rises, β-Lg will partially unfold and the thiol group becomes exposed, initializing the sulfhydryl/disulphide (SH/S-S) interchange reactions with other β-Lg molecules or with α-La molecules,

resulting in dimers, trimers or bigger polymers of β-Lg, α-La or mixed aggregates of the two proteins. Almost all BSA molecules are aggregated already and no free BSA is available to react with β-Lg or α-La at this stage. The resulting gel network consists of gel strands of disulfide-linked co-polymers of β-Lg and α-La with BSA aggregates (that were formed before the other aggregates) embedded in the main gel strands. These BSA aggregates can potentially interact with the β-Lg and α-La aggregates and strands if there are free thiol groups available.

In the heat-gellation of WPC the same type of aggregates would form, but in different relative amounts (Havea and others, 2001). As the ratio of the major whey proteins (β-Lg: α-La: BSA) in a commercial WPC is about 10:4:1, it is expected that gel strands will be formed essentially by β-Lg, with some α-La and very little BSA.

Another group of authors analysed WPI gelation at neutral pH but at NaCl concentrations higher than 0.1 M; under these conditions a particulate gel forms at a temperature of 68.5 °C and with WPI concentrations of 35 to 89 g/L (Verheul and Roefs, 1998). SEM pictures indicated that nested structures were formed: native protein molecules form building particles of ca. 0.1 μm, which in turn form larger flocs that will space-fill the gel structure as described for β-Lg aggregates (Verheul and Roefs, 1998). It is suggested that the gel forms in two steps: there is the formation of a primary spacial structure at the gelling point with part of the protein; the second gelling phase will not change this primary structure but will "thicken" and "strengthen" the strands that build the structure with the rest of the protein (Verheul and others, 1998b).

2.6 Influence of enzymes on the gelling ability of WP

Gels confer structure, texture and stability to food products; they also allow the retention of large quantities of water and other small molecules inside the food matrix. These aspects are appreciated by processed food manufacturers (Ferreira and others, 2007). However, these interesting gelling properties of whey proteins limit their application in some types of food products including beverages, baby-formulae, and salad dressings because a weak or non-gelling character is desirable for these products. Therefore, the capability of designing the gelation characteristics of whey proteins potentially could expand their utilization (Huang and others, 1999). Physical, chemical, and enzymatic methods can modify gelation and/or other functional properties of whey proteins.

Food proteins can be modified by proteases, peptidoglutaminase, transglutaminase (TG) or protein kinase (Dickinson, 1997). Transglutaminase is one type of enzyme that can be used to form protein gels which can be used in protein gel foods. It catalyzes the formation of peptides between lysine and glutamine allowing intermolecular cross-linking of proteins (Nio and others, 1985; Kang and others, 1994). In some cases proteolysis alone was found to reduce gel strength to below that of the control, but limited proteolysis was shown to be a suitable pretreatment to cross-linking with TG, a frequently used cross-linking enzyme (Pinterits and Arntfield, 2007). Thus opening the protein structure through proteolysis prior to TG treatment can increase the availability of lysine and glutamine residues and enhance its effectiveness. Gels of canola protein treated with both a protease and TG were signifficantly stronger than those treated with TG alone (Pinterits and Arntfield, 2007). Improved gelling behaviour was also reported when an immobilized recombinant fusion protein trypsin-streptavidin (Trypsa) followed by an immobilized recombinant fusion protein streptavidin-transglutaminase were used (Wilcox and others, 2002). A disadvantage of transglutaminase when compared to proteases is the higher cost and the availability (Burke and others, 2002). A microbial “broad-range” transaminase was also used to alter the rheology of heat-set WPI gels at pH 4 (Burke and others, 2002).

Limited proteolysis can also improve the functional properties of proteins by changing the molecular size, conformation, and strength of the inter- and intramolecular bonds of the protein molecules (Guan and others, 2007).

A mixture of peptides and proteins is present in protein hydrolysates. The types of chemical forces that are involved in mixed protein gel networks are the same as those involved in holding together individual protein molecules (Dickinson, 1997). Four main types of intermolecular interactions can be present in whey protein gel formation: hydrophobic interactions (5 - 10 kJ/mol), hydrogen bonds (10 - 40 kJ/mol), electrostatic interactions (25 - 80 kJ/mol) and covalent bonds (200 - 400 kJ/mol). When hidden non-polar amino acid residues are exposed through unfolding or rearrangement of the molecular structure with the heat treatment, intermolecular hydrophobic interactions take place. Hydrogen bonding results from the interaction of polar amino acid side-chains and their presence is characterized by a decrease of the gel strength at high temperatures. Repulsive electrostatic interactions are important at low ionic strength and pH away from the isoelectric point. They contribute to the formation of fine-stranded network structures with good water-holding characteristics. The most common intermolecular covalent bond in protein geling results from the permanent S – S crosslinking formed by sulphydryl-disulphide interchanges (Dickinson, 1997).

Four possible mechanisms depending on the type of mixed protein gel achieved have been described (Chen and others, 1994):

(1) Filled gels are obtained when additional components are spread throughout the primary gel network; the filler remains soluble for a single-phase gel;

(2) A "nongelling" component may associate with the primary network in a random fashion via nonspecific interactions;

(3) Two or more proteins may copolymerize to form a single heterogeneous network;

(4) An interpenetrating polymer network can be formed, in which networks of two gelling components are continuous throughout the sample.

Intrinsic (e.g. hydrophobicity, amino acid composition, molecular weight) or extrinsic factors (protein concentration, pH, temperature, ionic strength, type of salts present, pressure, etc.) can affect the gel forming ability of a protein and the type of gel that will form, by affecting the balance between all these attractive and repulsive reversible "physical" and/or covalent interactions (Dickinson, 1997; Totosaus and others, 2002). Hydrolysis of proteins may alter some intrinsic factors influencing thus the gelling properties of the "native" protein. For instance, hydrophobic interactions can play a major role in the gelation of several proteins and limited treatment with proteases leads to some protein hydrolysis and consequently partial unfolding of the protein structure (Pinterits and Arntfield, 2007). Unfolding of the native protein exposes buried hydrophobic groups and other interactive groups, which are then free to interact with neighboring polypeptides (Kang and others, 1994).

The effect of hydrolysis on the whey protein gelation ability is dependent on environmental conditions and on the degree of hydrolysis (DH). In fact, extensive hydrolysis of WPI can impair its gelation properties (Huang and others, 1999). Therefore, the proteolysis reaction must be carefully monitored and controlled in order to manufacture products with desired functionality (Guan and others, 2007).

The type of enzyme chosen is also important and the specificity is different from one enzyme to another. Some enzymes can induce gelation following whey protein hydrolysis; others impair gelling properties (Doucet and others, 2001; Foegeding and others, 2002; Otte and others, 2000).

For instance, ficin and bromelain revealed to be more effective at improving the properties of soy protein gels than trypsin (Pourel and Swenson, 1976). At pH 4, a very limited hydrolysis of hen egg white

ovalbumin by porcine pepsin is achieved. Only a single peptide bond in the original ovalbumin (MW 45000) is cleft, and a peptide with a molecular weight of about 3000 is released. This hydrolysate gives a heat-induced transparent gel whereas the ovalbumin gives a turbid gel with the same heating condition. This transparent gel is not bitter and its hardness is similar to that of the turbid gel propered from the nonproteolyzed ovalbumin (Kitabatake and others, 1988). This means that a slight hydrolysis of protein can result in a drastic change of the functional properties of food proteins (Kitabatake and others, 1988).

Ju and others (1995) achieved a 10 times stronger heat set gel at neutral pH and 12 % protein content after whey protein hydrolysis by the *B. lichenifomis* protease when compared with the correspondent whey protein gel without hydrolysis. No gelling was achieved at pH 7.0 and pH 3.0 with trypsin hydrolysates and there was a pronounced weakening of the gels formed at pH 5.2. Hydrolysis by Neutrase® did not change the ability to form a gel, but the gels formed at pH 5.2 and 7.0 were weaker and, at pH 3, were slightly stronger than the control gels. Later, Otte and others (1996) studied the effect of the proteolysis by the three enzymes on the gel microstructure. They came to the conclusion that partial hydrolysis of whey proteins can dramatically change the microstructure of the final gels. The most marked effect of partial whey protein hydrolysis was seen on the gels from *B. lichenifomis* protease hydrolysates set at pH 5.2 and on the gels from Neutrase® and from *B. lichenifomis* protease hydrolysates set at pH 7. The former had a particulate structure but much looser than the gels from WPI at the same pH and consisting of small aggregates and large pores. At pH 7 both hydrolysates caused formation of an aggregate type of gel. Although the structure of the gel from the WPI treated with Neutrase® was fluffy and heterogeneous, the gels from *B. lichenifomis* protease hydrolysates were as strong as the gels from WPI at pH 5.2. However they were less opaque and with protein aggregates 10 times smaller arranged as a regular network, indicating an intermediate microstructure between the particulate and fine-stranded structures characteristic for whey protein gels. Otte and others (2000) reported that limited hydrolysis of β-Lg with a *B. lichenifomis* protease prior to thermal gelation resulted in coarser gels with thicker protein strands and larger pores. The gel achieved with a low degree hydrolysis was stiffer than the gel made from unhydrolysed β-Lg but this increase was smaller than the one achieved by Ju and others, 1995) with WPH. The gel strength increased with low degrees of hydrolysis, but decreased after prolonged hydrolysis (Otte and others, 2000). Therefore it seems that there is an optimal degree of hydrolysis (*DH*) for gelling properties.

Unlike Ju and co-workers, Huang and others (1999) obtained gels at pH 7 from tryptic whey protein hydrolysates. These gels were particulate and weaker than the ones achieved with non-hydrolysed whey

protein isolate, but their gelling point was lower. Chen and others (1994) achieved a gelling time of 7 min after reaching 80 °C with β-Lg hydrolysates from immobilized trypsin while the value was 38 min at 80 °C for native β-Lg. However, the value of *G'* for 7 % β-Lg was about twice that obtained with gels from hydrolyzed β-Lg. Furthermore β-Lg hydrolysates were able to form strong gels at 60 °C at 15 % and a weak gel at 7 % while β-Lg only forms very weak gels (*G'* of β-Lg hydrolysates gel was 61 times higher than *G'* of β-Lg gel at 15 % and 12 times higher at 7 %). The authors suggest that the existence of structured domains with lower thermal stability can explain the altered gelation characteristics. Although only about 15 % of the protein is hydrolyzed to yield core β-barrel domains, the thermal stability of the mixture is reduced and gelation occurs at lower temperatures than with the intact protein. Since hydrolyzed β-Lg probably contains multiple protein fragments the mechanism for the gellation may be a combination of several of the four mechanisms described above. However, the lower gel point of the hydrolyzed protein suggests that the gel structure is a copolymerized matrix (Chen and others, 1994).

Although extensive enzymatic hydrolysis of whey proteins usually improve solubility and decrease gelling properties, non heat-set gelation occurs during extensive hydrolysis of whey protein isolate at high solids content (20 % w/v) with Alcalase 2.4L®, a protease from *B. lichenifomis* (Doucet and others, 2001). This gel forms slower and is weaker than the gel obtained with heat-set gelation of β-Lg and both gel mechanisms require the formation of aggregates before gelation. The fact that only a small amount of β-Lg and α-La remains in the hydrolysate when the solution becomes viscous suggests that native proteins do not play a major role in the gelation process (Doucet and others, 2001). The gel is formed by small molecular weight peptides (< 2000 Da) that seem to be held together by non-covalent interactions. It has been suggested that the gelation of WPI is by physical aggregation, mainly via hydrophobic interactions with hydrogen bonding and electrostatic interactions playing a minor role and that disulfide bonds are not involved in the gel network (Doucet and others, 2003a; Doucet and others, 2003b). Actually, it has been referred that, when highly concentrated solutions of proteins are incubated with proteases, water-insoluble and gel-forming products may appear (Doucet and others, 2003a). The mechanism of this is not well established and there are authors that consider it to be a reaction of peptide synthesis inverse to the hydrolysis due to the high protein concentration (Lorenzen and others, 1997) and authors that think it is mainly a physical aggregation mechanism (Andrews and Alichanidis, 1990).

Gels from hydrolysates of α-La made with a *B. lichenifomis* protease specific for Glu-X and Asp-X bonds have a totally different character and a microstructure most exceptional for food protein gels (Otte and others, 2004). Ipsen and others (2001) obtained gels that were almost transparent and more than 20

times stiffer than equivalent gels made from β-Lg at the same concentration. The microstructure of the gels consisted of non-branching, apparently hollow strands with a uniform diameter close to 20 nm, similar in overall structure to microtubules. These gels form from building blocks that self-assemble into nanometer-sized tubular highly organized structures. One possible application of these hollow nanotubes is that they can serve as vehicles for delivery or controlled release of drugs and other encapsulated molecules, such as vitamins and enzymes, or to protect or mask encapsulated compounds (Graveland-Bikker and de Kruif, 2006). Gelation properties of whey proteins, thus, can be manipulated by limited proteolysis.

2.7 Interaction between polyssacharides and whey proteins

The use of mixed protein/polysaccharide systems can improve or modify the functional behaviour of proteins and/or polysaccharides (Turgeon and Beaulieu, 2001). In particular, when one of the macromolecules (or both of them) is a gelling agent, competition between gelation and phase separation can lead to a number of complex gel microstructures, resulting in a wide range of properties (Tolstoguzov, 1991; Doublier and others, 2000).

2.7.1 Protein/polysaccharide mixed solutions

Mixing a protein with a polysaccharide into an aqueous solution may drive to one of several situations (Tolstoguzov, 1991; Syrbe and others, 1995; Syrbe and others, 1998; Doublier and others, 2000; de Kruif and Tuinier, 2001) depending on the polymer-polymer and solvent-polymer attraction or repulsive interactions present and on the polymer molecular masses (**Figure 2-9**):

1. Co-solubility, though this is the least typical situation;

2. Incompatibility; in this case the system can have: a) only one phase when the biopolymers concentrations are lower than the phase-separation threshold; or b) two phases (segregation), when the concentrations are above the threshold; in this case, each phase contains predominantly one biopolymer as the biopolymers are mutually segregating from each other; in fact, interactions between the biopolymers are repulsive and/or the biopolymers have different affinity towards the solvent; in this last case, considering two biopolymers (biopolymers 1 and 2), both solvent – biopolymer 1 or solvent – biopolymer 2 interactions are favoured against biopolymer 1 – biopolymer 2 and solvent – solvent interactions;

3. Complexing: in this case also one or two phases can occur and the soluble complexes are stabilized through electrostatic interaction and hydrogen bonding; two phases arise when polysaccharides are adsorbed onto the protein or bridge between several protein molecules, therefore concentrating both polymers in one phase leading to the exclusion of the solvent from their vicinity; one phase is, thus, enriched in both polymers while the other phase is depleted in both polymers and solvent enriched; this phenomenon has been called complex coacervation (or complexation, or aggregative phase separation) and is typical of oppositely charged anionic poysaccharides and proteins, however it can also be observed between polymers with similar charges.

Thus, the addition of a polysaccharide (linear polysaccharides are normally non-ideal even at low concentrations) to a protein solution may result in the thermodynamical incompatibility of biopolymers and system demixing, or in the formation of interbiopolymers complexes with a new set of properties (Tolstoguzov, 1992). Under specific compositions and conditions (protein-to-polysaccharide ratio, pH, ionic strength, temperature, mixing, processing steps) proteins and polysaccharides form water-soluble hybrids (complexes or conjugates), and do not coacervate, often resulting in enhanced functional properties in comparison to the proteins and polysaccharides alone (Benichou and others, 2007). Nevertheless, as a first approach, biopolymers tend to segregate even if this phenomenon is not observable at the macroscopic level due to the stability of the system in the state of water-in-water emulsion (Doublier and others, 2000). In fact, for polymers with different shape and structure, segregation leads to a reduction of the polymer (polysaccharide) concentration near the other (protein) particle, due to a reduction of conformational entropy at the interface (a polymer molecule looses conformational entropy when confined between neighboring colloidal particle surfaces); a reduction of polymer concentration near an interface is called depletion; exceeding a certain polymer concentration leads to a phase separation into a protein-enriched and a polysaccharide-enriched phase; in the case of segregative interaction of very large polymers and relatively small colloidal spheres this reduction of conformational entropy is small and the concentration needed for the phase separation is so high that it is not attainable in practice (Tuinier and others, 2000; de Kruif and Tuinier, 2001).

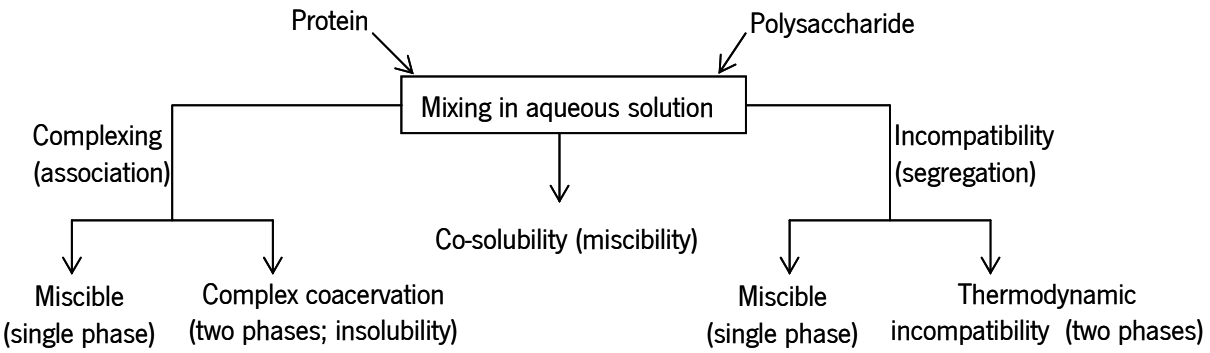


Figure 2-9 Representation of the behaviour of aqueous mixed proteins and polysaccharides solutions (adapted from Tolstoguzov, 1991; Syrbe and others, 1998; and de Kruif and Tuinier, 2001)

Although food systems phase separation may be thermodynamically favoured, they can be kinetically hampered by high viscosity (Tolstoguzov, 1992). Thus both kinetics and thermodynamics should be considered when predicting the behaviour of a mixed biopolymer solution.

Globular proteins can behave very differently in their native and denatured state and incompatibity can come up in a miscible protein – polysaccharide system if the protein is, e.g., heated. Syrbe and others, 1995) showed that, from a large range of polysaccharides used, native whey proteins only showed incompatibility with neutral polysaccharides (arabinogalactan, maltodextrin, dextran, methylcellulose, guar gum) at pH values near the isoelectric point while heat-denatured whey proteins were incompatible also with some anionic polysaccharides (e.g. κ-carrageenan) at pH 7. Thus, although mixtures of anionic polysaccharides and globular proteins are usually compatible over a wide range of concentrations, phase separation can be observed if globular proteins are associated into large clusters (which can be e.g. heat-induced). On the other side, if a mixture of a neutral polysaccharide and a protein close to the isoelectric point is considered, incompatilility is likely to prevail as the protein self-assembly is high (Olsson and others, 2002b).

Many different authors studied mixtures of whey proteins and neutral or anionic polysaccharides and reported positive effects on their functional properties. Methyl cellulose, carboxymethyl cellulose, dextran, galactomannans (guar gum, locust bean gum, tara gum, *Cassia javanica* galactomannans), pectin, xanthan gum, exo-polysaccharides from lactic acid bacteria, maltodextrin, ι-carrageenan, κ-carrageenan, gum Arabica, chitosan are a few examples of the polysaccharides used in those studies (Syrbe and others, 1995; de Kruif and Tuinier, 1999; Tuinier and others, 2000; Michel and others, 2001; Girard and others, 2002; Ibanoglu, 2002; Tavares and da Silva, 2003; Girard and others, 2004; Gonçalves and others, 2004b; Gonçalves and others, 2004a; Baeza and others, 2005; Beaulieu and others, 2005; Ibanoglu,

2005; Guzey and McClements, 2006; Kim and others, 2006; Akhtar and Dickinson, 2007; Benichou and others, 2007; Ercelebi and Ibanoglu, 2007; Kika and others, 2007; Neirynck and others, 2007; Sun and others, 2007).

2.7.2 Whey protein/polysaccharide mixed gels

The gels resulting from heat-set gelation of WPI/polysaccharide mixed solutions can have homogeneous or phase separated microstructures, depending on the nature of the polysaccharide and on the pH, the ionic strength, the temperature and the concentrations used (Turgeon and Beaulieu, 2001; van den Berg and others, 2007). The homogeneous structure can be interpenetrating, when the two components gel separately and form independent networks, or coupled, when favourable intermolecular interactions between WPI and the polysaccharide are present (complex coacervate network).

A division can be made between active polysaccharides (that form part of the molecular network) and non-active polysaccharides (that are merely contained within the network structure). The network of the resulting gel could (Totosaus and others, 2005):

1. Be made of a protein network containing a nonactive polysaccharide; the presence of the polysaccharide will reduce the solubility of the protein and promote intra- or intermolecular protein-protein interactions, thus the polysaccharide can modify the order–disorder transition of the protein without the need of direct protein-polysaccharide interactions; the polysaccharide function may also be to act as a filler; it will be entrapped within the protein gel matrix and influence the formation of the continuous gel structure during heat-induced gelation, modify the viscosity of the aqueous phase and/or influence the texture and appearance of the gel due, e.g., to its particle size, distribution and rheological properties.

2. Be made of both protein and active polysaccharide, when the polysaccharide also has gelling ability.

Thus, on phase separation the system develops two-phase interspersed microstructures. It may be that a bi-continuous two-phase system is formed or that one phase is dispersed in the other continuous phase (de Kruif and Tuinier, 1999). WPI/polysaccharide mixed cold-set gels with several different polysaccharides (gellan gum, locust bean gum, κ-carrageenan, high methyl pectin), for instance, showed protein continuous, bicontinuous or coarse stranded microstructures (van den Berg and others, 2007). β-

Lg/κ-carrageenan mixed gels showed also a bicontinuous profile while gels only from β-Lg showed a mono-continuous profile indicating the formation of phase-separated gels (Eleya and others, 2006).

The network structure will depend on the degree of phase separation prior to gelation, and hence will be sensitive to the processing conditions (Totosaus and others, 2005).

Phase separated structures are the most likely outcome of the gelation because the interaction of two polymers is usually not favoured. This phase separation usually leads to the presence of protein-rich microdomains. In such situations the morphology of the protein gels is determined by the competition between phase separation, on the one hand, and aggregation and gelation, on the other (Durand and others, 2002). In this case, there is a particular ratio (A/B) called the phase inversion point where the system changes from a matrix of gel A containing inclusions of gel B to a matrix of gel B containing inclusions of gel A (Boye and others, 2000).

In fact, phase separation in whey protein/polysaccharide mixed gels was reported by many authors. Gonçalves and others (2004b) reported phase separation in the systemt whey protein isolate (WPI) gel/ *Cassia javanica* gum galactomannan at a neutral pH. For low polysaccharide concentrations (< 0.68 %) there was an increase in the elastic modulus of the mixed gel but for higher concentrations (> 0.68 %) the effected was the reverse. With tara gum this inversion was neither reported at neutral pH nor at 4.6, but the concentration of galactomannan was only tested until 0.63 %. The higher the concentration of tara gum the coarser the formed mixed gel (Sittikijyothin and others, 2007). Tavares and da Silva (2003) showed that whey protein formed a phase separated gel on a micrometre scale in the presence of locust bean gum during heating (pH 7). The presence of the galactomannan increased the elastic modulus of the mixed gel suggesting that the protein network formed a continuous phase which accommodated the polysaccharide chains, acting as filler. Li and others (2006) also reported weaker WPI gels in the presence of more than 0.5 % (w/w) of xanthan.

Microscopy and rheology measurements on WPI/κ-carrageenan, α-La and β-Lg/carboxymethylcellulose, WP/pectin, β-Lg/sulphate dextran, WPI/xanthan heat set gels also showed phase separation (Turgeon and Beaulieu, 2001; Zhang and Foegeding, 2003 Roesch and others, 2004; Bertrand and Turgeon, 2007; Capitani and others, 2007).

Another study describes the gelation on β-Lg in the presence of amylopectin and concludes that the higher the concentration and the higher the viscosity of the amylopectin, the lower was the gelling temperature,

the faster formed and the stronger the gel was. However high viscosity amylopectin used in high concentration was found to restrict the particle aggregation and the clusters formation and thus to counteract the aggregation to a connected protein network (Olsson and others, 2002b). An additional study from the same group of authors analised the microstructure of these gels. They concluded that an increase in the cluster size, pore size and connectivity results in increased gel strength. Nevertheless a decrease in gel strength could occur despite of, for example, a strand dimension increase, due to decreased connectivity (Olsson and others, 2002a). When only low methoxyl pectins were used, increasing the amount of pectin (up to 1.5 %) and the calcium concentration made mixed gels firmer (Beaulieu and others, 2001).

Synergistic effects were once more observed in the gelation of κ-carrageenan in the presence of β-Lg, native and denatured soy protein (Baeza and others, 2002). Although many synergistic effects are reported in the literature, also many antagonistic effects can be found. For instance, dynamic rheological data indicated an antagonistic trend in 5 % total solids heated WPI/cross-linked waxy maize starch dispersions due to phase-separated networks and structural incompatibility (Ravindra and others, 2004).

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Chapter 3 Hydrolysis of whey protein concentrate with free proteases

3.1 Introduction

Whey proteins can be used for a variety of technological functional applications, once they can act as gelling agents, emulsifiers or foaming agents (Foegeding and others, 2002). β-lactoglobulin (β-Lg) is considered to be responsible for most of the functional properties of whey protein products (Madsen and others, 1997) and these properties can be modified by limited enzymatic hydrolysis. By this method, it is also possible to improve thermal stability of the whey proteins (Doucet and others, 2001). Thus it is possible to manipulate the food process in order to achieve a product with pre-defined (desired) characteristics.

From a dietary point of view, protein hydrolysates can also be used to e.g. reduce allergenicity or improve digestability (Silvestre, 1997) or to produce bioactive peptides.

In any case, it has been shown that, for dietary purposes, protein hydrolysates should be rich in low molecular weight peptides and that large molecular weight peptides are presumed to be associated to the functional properties of whey protein hydrolysates (Silvestre, 1997). Therefore hydrolysis must be carried out under strictly controlled conditions to a specified degree of hydrolysis (*DH*) (Adler-Nissen, 1979) and a general method of determining *DH* is needed, particularly for quality control.

Several different methods of determining the *DH* of the peptidic bonds are available. They are based in three main fundamental principles: determining the amount of nitrogen released by the hydrolysis, which is soluble in the presence of a precipitation agent such as trichloroacetic acid; determining the amount of free α-amine groups, for instance with ninhydrin, trinitrobenzenesulphonic acid (TNBS) or ο-phthaldialdehyde (OPA); or by titration of the released protons (Silvestre, 1997; Nielsen and others, 2001; Spellman and others, 2003).

a) pH-stat method:

The principles of the pH-stat method were developed in the Carlsberg Laboratory in Denmark by Jacobsen and co-workers in 1950's (Adler-Nissen, 1986; Dzwolak and Ziajka, 1999; Nielsen and others, 2001). This method has the big advantage of allowing on-line control of the hydrolysis degree. It is also simple and non-denaturing.

During hydrolysis, a new carboxyl and a new amino group are released for each cleaved amide bond. Therefore, the number of hydrolysed peptide bonds can be deduced from the determination of the number of newly formed C- and/or N-terminal groups in hydrolysates. As explained before, the amino

and carboxyl groups are more or less (de)protonated after hydrolysis, depending on the pH of the solution. If the hydrolysis is performed in a pH-stat set-up, the amount of added acid or base can be used to calculate the *DH* directly, since the addition of acid or base is related to the amount of liberated amino and carboxyl groups (Adler-Nissen, 1986; Diermayr and Dehne, 1990). However, the pH-stat method is only applicable for hydrolysis at neutral/alkaline (pH > 7) or acidic pH (pH < 4). At pH values between 5 and 6 there is no net release or uptake of protons, as the protonation and deprotonation of the acid/base groups are in equilibrium. However, the ionisation of amino acid side chains and the increase in buffer capacity influence the pH-stat efficiency. Thus, if absolute values are needed, the pH-stat method should be calibrated with another method, such as TNBS or OPA (Turgeon and others, 1991). Moreover, at extreme pH values the pH-stat method is inoperable due to the high buffer capacity (Adler-Nissen, 1986). Moreover, pK values, used to calculate the degree of dissociation (α) of the acid/base groups, are not constant during hydrolysis since they depend on the peptide chain length and on the side chain of the terminal amino acid (Adler-Nissen, 1986; Diermayr and Dehne, 1990; Camacho and others, 2001). At low values of α the determination of the *DH* is subjected to much more uncertainty. As a result the pH-stat method is not recommended to measure *DH* at acid pH values and the osmometer technique, that involves measuring the freezing point depression with an osmometer (cryoscope) to calculate changes in the osmolality of the sample (the *DH* is proportional to the increase in osmolality), is more suitable in this case (Adler-Nissen, 1986; Dzwolak and Ziajka, 1999).

b) TNBS method

The amount of released α-amino groups can be measured using reagents that react specifically with amino groups, yielding derivatives that can be detected spectrophotometrically. TNBS reacts with primary amino groups at slightly alkaline conditions to form a chromophore with a maximum absorvance at 340 nm (Adler-Nissen, 1979). The reaction is stopped by lowering the pH. This method is of wider application than OPA (that gives underestimated values of *DH* when the protein is rich in cystein, as is the case of α-La) or pH-stat methods, but cannot be used for real-time monitoring of *DH* due to time-consuming incubation and cooling steps (Spellman and others, 2003).

Hydrolysates' peptide composition, and consequently their properties, depends on the protein and on the enzyme used, as well as on hydrolysis conditions (temperature, pH, enzyme to substrate ratio and reaction time). Hydrolysates can be characterized according to several molecular characterization methods, which reflect their molecular properties (Mota and others, 2006). Usually chromatographic or

electrophoretic methods are used to characterize hydrolysates from proteins. Several authors used high performance liquid chromatography (HPLC) at the reverse phase mode (RP-HPLC) to separate and characterize peptides from whey protein hydrolysates according to their hydrophobicity (including Silvestre, 1997; Bordenave and others, 2000; Groleau and others, 2003; Mercier and others, 2004; Creamer and others, 2004). The ion-exchange chromatography may also be used (e.g. Lieske and Konrad, 1996; Silvestre, 1997). The size-exclusion chromatography (HPLC or Fast Protein Liquid Chromatography) is also interesting because it allows to separate peptides due to differences in the peptide molecular volume (Kinekawa and Kitabatake, 1996; Madsen and others, 1997; Doucet and others, 2001; Barros and Malcata, 2002; among others). Electrophoretic methods as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have also been used by many authors (for instance, Guo and others, 1995; Kinekawa and Kitabatake, 1996; Madsen and others, 1997; Sannier and others, 2000; Barros and Malcata, 2002; Creamer and others, 2004; Kim and others, 2007). More than one technique may be used when there is the need to isolate the peptides or for a better characterization (Madsen and others, 1997; Creamer and others, 2004; El-Zahar and others, 2005). The outlet of the HPLC analyser can also be connected to a mass spectrometer and the molecular mass of peptides can thus be determined. Peptides can also be analized and separated through 2D electrophoresis (see, fo example, Lindmark-Mansson and others, 2005).

After fractioning, the identification of the formed peptides in protein hydrolysates can be made through the determination of the amino acid composition (for instance, through Edman degradation), with the N-terminal analysis using a protein sequencer or through mass spectrometry accopled to a theoretical analysis of the expected peptide molecular weights (e.g. Madsen and others, 1997; Silvestre, 1997; Caessens and others, 1999; Hernandez-Ledesma and others, 2002; Groleau and others, 2003; Creamer and others, 2004; Hernandez-Ledesma and others, 2005; Roufik and others, 2006).

Until recently, fractionation was often performed rather arbitrarily. With new analytical and statistical techniques it has become possible to analyse a multitude of data simultaneously, which allows the establishment of correlations between several hydrolysate characteristics (Mota and others, 2006).

The objectives of the present study were:

a) To preliminarly choose the working enzymes by monitoring the hydrolysis of whey protein concentrate (WPC) at different temperatures and pH values using three enzymes – pepsin,

trypsin and Alcalase®; general RP-HPLC hydrolysate profile and gelling ability were also considered;

b) To choose and establish the applicability range and operating conditions of the detection methods to be used (degree of hydrolysis, peptide profile);

c) To study the hydrolysis of whey protein isolate with the choosen enzyme and determine the best operational conditions. The monitoring was carried out during 4 hours by measuring the degree of hydrolysis; further analyses were made by RP-HPLC/UV; peptides were separated according to their polarity and degradation of α-lactalbumin and β-lactoglobulin was evaluated.

3.2 Materials and methods

3.2.1 Reagents and enzymes

All reagents used were of analytical grade and supplied by Sigma, Co. Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg (one BAEE unit will produce a ΔA_{253nm} of 0.001 per min at pH 7.6 at 25 °C using BAEE as substrate; in a reaction volume of 3.2 mL and 1 cm light path), trypsin from bovine pancreas with an activity of 11000 BAEE units/mg (chymotrypsin ≤ 0.2 %), pepsin from hog stomach with an activity of 975 units/$mg_{protein}$ (one unit will produce a ΔA_{280} of 0.001 per min at pH 2.0 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate in a reaction final volume of 16 mL and 1 cm light path), pepsin from hog stomach with an activity of 2540 units/$mg_{protein}$ (89 % w/w protein) and a protease from *Bacillus licheniformis*, Alcalase® 2.4 L (BLP) with an activity of 2.77 Anson units/g were also obtained from Sigma Chemical, Co. A comercial spray dried whey protein concentrate (WPC) from Armor Protéines, France with the reference HG80 with 80 % wt of protein (dry basis) and 6 % humidity was used as substrate. Whey protein isolate (WPI) powder (Lacprodan DI-9212, batch R320215) was kindly supplied by Arla Foods Ingredients (Viby, Denmark) and was also used as subtrate for the hydrolysis. According to the suppliers, the WPI protein content was 91 % dry basis, moisture was 5.5 % (maximum), the ash content was 3 % and the ion content was: sodium, < 0.1 %, phosphorus, 0.2 %, chloride, 2.2 %, potassium, < 0.1 % and calcium, < 0.1 %.

3.2.2 WPC hydrolysis

Solutions of WPC 80 were prepared by suspending 25 g of WPC in 500 mL of distilled water. The resulting solutions were then stirred for one hour, then heated to the hydrolysis temperature and

adjusted to the desired pH with HCl or NaOH (2.0 M). Enzymatic degradations were performed with pepsin with an activity of 975 U/mg (concentrations ranging from 0.5 to 2 g/L), trypsin with an activity of 1800 BAEE units/mg (0.05 to 0.2 g/L) or with BLP (2 mL/L) in a 0.5 L stirred, tank-type, batch reactor equipped with pH and temperature control as represented in Figure 3-1. The pH was kept constant at the desired value with 0.5 M HCl or 0.5 M NaOH and the temperature was kept at constant values ranging from 30 to 70 °C, depending on the experiment.

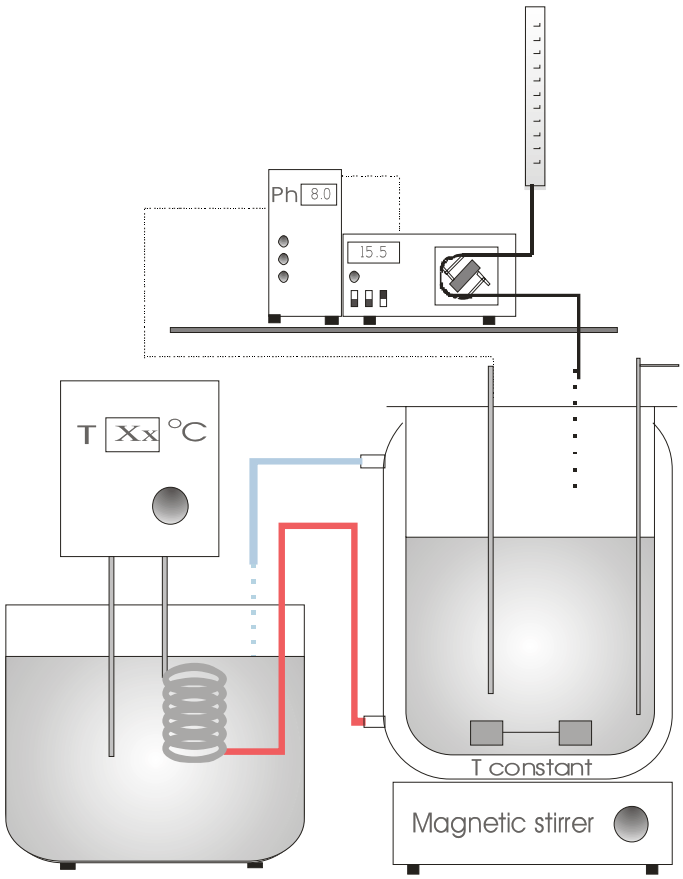

Figure 3-1: Schematic representation of the hydrolysis apparatus

The degree of hydrolysis achieved was measured by the TNBS method in the case of experiments with pepsin (although it was roughly followed on-line through the acid consumption), and by the pH-stat method in the case of experiments with trypsin or alcalase. For each experiment, samples were collected, before (time zero) and during the hydrolysis of bovine milk whey protein concentrate, at variable intervals of time, during 3 hours. The reaction of the samples was stopped by immersion of the samples in a water bath at 80 °C for 30 min, in the case of trypsin and BLP or by increasing the pH to 7.0 in the case of pepsin and the samples were stored at -20 °C until performing RP-HPLC. After hydrolysis, the enzyme was inactivated by increasing the pH to 7.0 in the case of pepsin or by rapidly

increasing the temperature to 80 °C and holding it for 15 min in the case of trypsin and for 30 min in the case of BLP. The resulting suspensions were immediately frozen and lyophilised for further analysis.

3.2.3 WPI hydrolysis

WPI solutions of 2.5 g in 50 mL of distilled water were prepared as described in section 3.2.2. Enzymatic degradations were performed with pepsin (0.4 mg/mL) or trypsin (0.43 mg/mL) in a 0.05 L reactor similar to the one described in 3.2.2. The pH was kept constant at the desired value with 0.25 M HCl in the case of pepsin or 0.25 M or 0.1 M NaOH in the case of tryptic hydrolysis. The temperature was kept at constant values ranging from 37 to 60 °C for tryptic hydrolysis, depending on the experiment, and at 37 °C for peptic hydrolysis. The degree of hydrolysis achieved was measured by the TNBS method in the case of experiments with pepsin and by the pH-stat method in the case of experiments with trypsin. For each experiment, samples were collected, before (time zero) and during the hydrolysis of WPI, at variable intervals of time, during 3 hours. The enzyme in the samples was inactivated by increasing the pH to 7.0 or decreasing it to 3.0, in the case of pepsin and trypsin respectively. In the case of trypsin, this inactivation was partially reversible (Adler-Nissen, 1986). The samples were then stored at -20 °C until the RP-HPLC analysis (3.2.5).

3.2.4 Quantification of the protein degree of hydrolysis

a) pH-stat method

Quantification of *DH* by the pH stat method was carried out by measuring the amount of NaOH used to keep the pH constant. The *DH* was calculated by (Adler-Nissen, 1986):

$$DH(\%) = 100 \times B \times N_b \times \frac{1}{\alpha} \times \frac{1}{m_P} \times \frac{1}{h_{tot}},$$

where B is the base consumption in mL, N_b the normality of the base, α the average degree of dissociation of the α-NH_2 groups, m_P the mass of protein being hydrolysed (g), and h_{tot} the total number of peptide bonds in the protein substrate (meqv peptide bonds per gram of protein). The h_{tot} for whey protein concentrate is 8.8 meqv per g protein (Adler-Nissen, 1986).

The degree of dissociation (α) for the α-amino groups was calculated as follows:

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)'}}$$

where *pK* is the average dissociation value for the α-amino groups liberated during hydrolysis. *pK* is dependant on temperature, peptide chain length and the nature of the terminal amino acid (Dzwolak and Ziajka, 1999; Spellman and others, 2003). The average *pK* values for the α-amino groups of peptides and proteins used were the ones presented by Adler-Nissen (1986).

b) TNBS method

Samples were diluted 30 times except samples from time zero and the 50 g/L whey protein solution that were diluted 20 times. All samples and standard solutions were diluted in 1 % (w/v) SDS. Standard solutions of L-leucine in 1 % (w/v) SDS were prepared ranging from 0 to 2.0 mM. The TNBS method related by Adler-Nissen, 1979 was used as described by Spellman and others, 2003. A solution of 0.1% (w/v) of TNBS was prepared with water as solvent. Duplicate aliquots (0.25 mL) of test or standard solutions were added to test tubes containing 2.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2). TNBS reagent (2.0 mL) was then added to each tube. The tubes were homogeneized and incubated in the dark at 50 °C for 60 min. After incubation, the reaction was stopped by the addition of 4.0 mL of 0.1 M HCl to each tube. Samples were then allowed to cool at room temperature for 30 min, before absorbance values were measured at 340 nm in a UV-Vis spectrophotometer V-560, Jasco (Japan). *DH* values were calculated using the following formula:

$$DH\% = 100\left(\frac{AN_2 - AN_1}{Npb}\right)$$

where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg $g_{protein}^{-1}$), AN_2 the amino nitrogen content of the protein substrate after hydrolysis (mg $g_{protein}^{-1}$), and *Npb* the nitrogen content of the peptide bonds in the protein substrate (mg $g_{protein}^{-1}$). A value of 123.3 was used for whey protein (Adler-Nissen, 1979; Spellman and others, 2003). The values of AN_1 and AN_2 were obtained by reference to the standard curve of Abs at 340 nm versus mg L^{-1} amino nitrogen generated with L-leucine.

3.2.5 Peptide profile of hydrolysates

To evaluate the native proteins degradation (α-La and β-Lg) and peptide formation, samples were analysed by RP-HPLC. Prior to analysis, samples were diluted 20 times with ultra purified water. They were then injected in a reverse phase column C18 Symmetry 300, Waters, USA (5 mm, 300 Å, 250 ×

4.6 mm² i.d.) installed on a liquid chromatograph (formed by an intelligent HPLC pump PU-2080 Plus, a ternary gradient unit LG-2080-02 and a 3-Line Degasser DG-2080-53, all from Jasco, Japan) to promote peptide separation according to their polarity. A Symmetry guard column (Waters, USA) was used as pre-column. The elution flow rate was 0.75 mL min^{-1} with the following gradient of eluents (A: 0.1 % TFA in water; B: 0.1 % TFA in acetonitrile): 0 to 30 min, 100 to 50 % A; 30 to 35 min, 50 to 20 % A; 35 to 40 min, 20 % A. Under these conditions the retention time for α-La was 30.3 min and for β-Lg was 32.2 min. Monitoring was made at 215 nm and 35 °C by a diode array detector LabChrom L-7455, Merck Hitachi, Japan.

3.3 Results and discussion

3.3.1 Preliminary studies on the hydrolysis of WPC with several enzymes

Pepsin

The main results achieved with pepsin are presented in Figure 3-2 and Figure 3-3.

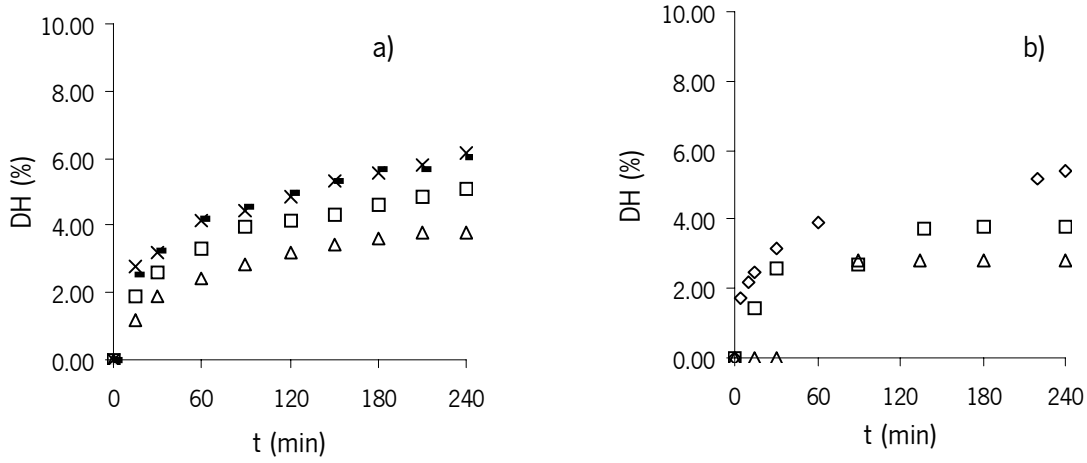

Figure 3-2: Degree of hydrolysis of WPC by pepsin: a) 40 °C and pH 2 – Δ E/S = 0.5/40; □ E/S = 1/40; × E/S = 1.5/40; - E/S = 2/40; b) 37 °C and E/S = 1.5/40 – Δ pH 4; □ pH 3; ◊ pH 2

From Figure 3-2a it is possible to conclude that for experiments at 40 g/L of protein (50 g/L of WPC), an increase in the enzyme concentration above 1.5 g/L does not lead to a significant increase in the reaction rate. For the pH values tested, optimal pH seems to be around 2 (Figure 3-2b), which is in accordance with the 1.8 to 2 pH optimal values found in literature (Godfrey, 1996). Results at pH 1 are

not presented because pH-stat method at this pH did not allow any conclusions (as expected). In fact, at this pH value results are very sensitive to slight pH oscillations (which correspond to high volumes of pH reagent) and buffer effects can be quite relevant. Adler-Nissen (1986) suggest the value of 3 as the lower limit to the applicability of the method and, even so, those authors do not recommend its use at acid pH values because at values above 3 the method is very sensitive to slight pH oscillations due to big oscillations in the value of α of the carboxyl groups. However some authors refer the possibility of using it at acid pH values. For instance, Zhao and others (1994) have used pH-stat method at pH 2 and Diermayr and Dehne (1990) at pH < 3. In both cases a calibration was made with the TNBS method.

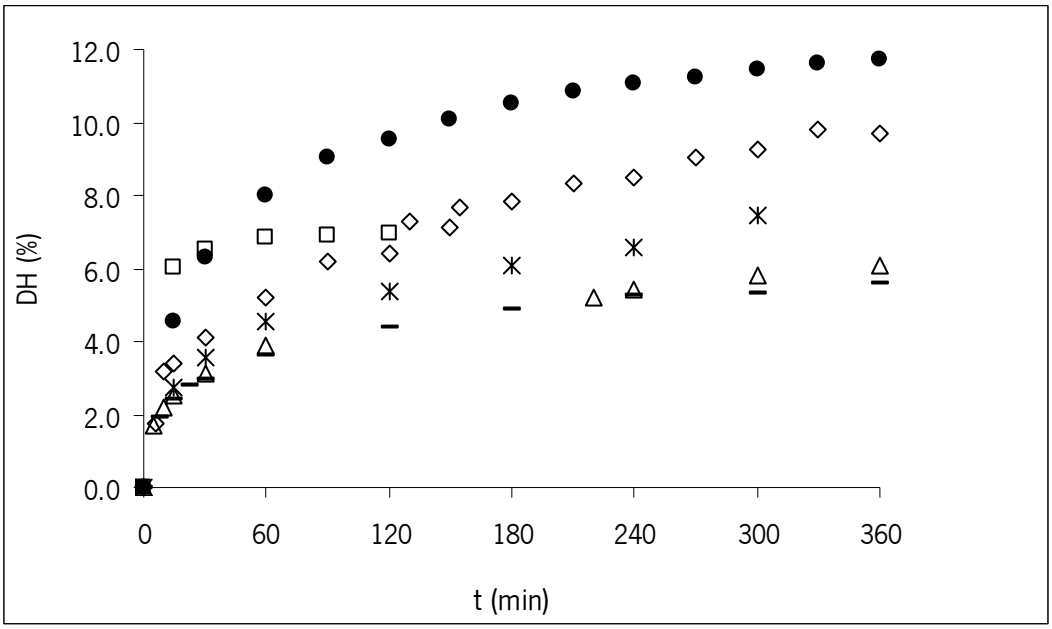

Figure 3-3: Degree of hydrolysis of WPC by pepsin at pH 2 and E/S=1.5/40

– 30 °C; Δ 37 °C; ∗ 40 °C; ◊ 50 °C; • 60 °C; □ 70 °C

Optimal temperature for the hydrolysis of WPC with pepsin at pH 2 was found to be 60 °C (Figure 3-3), in agreement with the value found in literature (Godfrey, 1996). In fact, at higher temperatures the enzyme inactivates quickly and the hydrolysis stops in a few minutes. Anyway, at 50 °C there is already a relatively high rate of hydrolysis and the final *DH* achieved is close to the value achieved at optimal temperature. Thus, this temperature should be considered when the denaturation or destruction of some heat sensitive relevant compounds is important. The final *DH* achieved (close to 12 %) is lower than the values of DH referred in the literature. For instance, Perea and others (1993) and Camacho and others (1998) refer values of 20 to 30 % with other enzymes at alcaline pH values. This result was expected. α-La and β-Lg (the two main whey proteins) react differently towards enzymatic hydrolysis. In fact, β-Lg is resistant to the hydrolysis at pH 2 while α-La is completly digested (Bordenave and others, 1999). As the

two proteins are present in whey protein concentrate in an approximate proportion of 2/3 of β-Lg to 1/3 of α-La, it is expected that final degree of hydrolysis is lower in comparison with hydrolysis at higher pH values, where both proteins are hydrolysed, though in different extensions, depending on the specificity of the enzymes used.

No correction to the degree of dissociation of the carboxyl groups was made in the experiments at different temperature. Although this value changes much more with the pH than with temperature, these results should be regarded as being only indicative.

This study is, at this stage, only comparative and absolut hydrolysis degrees are not very important. Even if the *DH* has a high associated error conclusions are still possible through comparison with other experiments with an associated error of the same order of magnitude.

Trypsin

The influence of the pH and the temperature on the degree of hydrolysis of WPC with trypsin is presented in Figure 3-4.

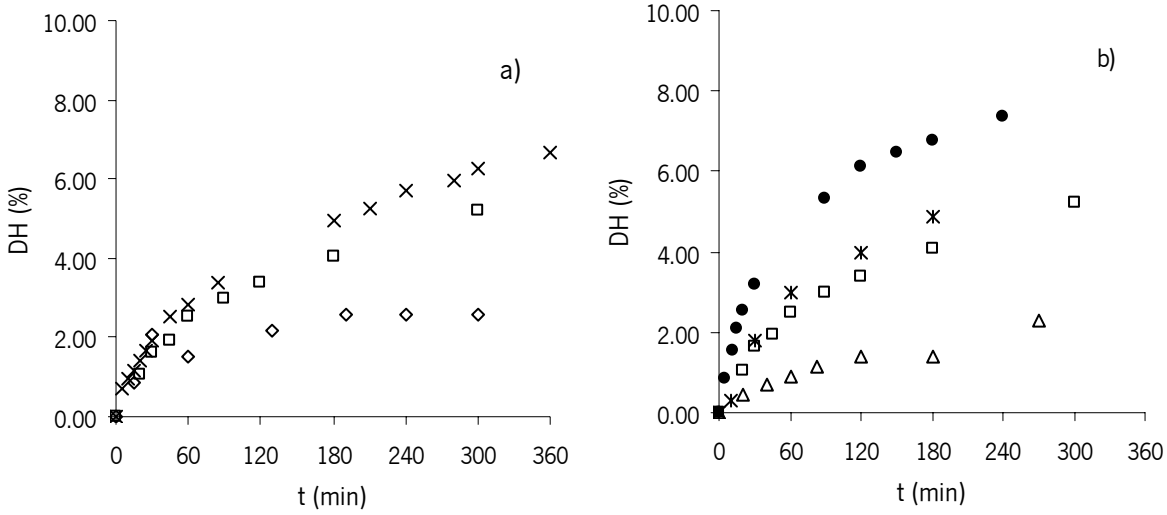

Figure 3-4: Degree of hydrolysis of WPC by trypsin (E/S = 0.2:40, except otherwise satated):

a) pH 8; b) 37 °C

◊ 37 °C, pH 7; □ 37 °C, pH 8; × 37 °C, pH 9; ∗ 40 °C, pH 8; ● 60 °C, pH 8; Δ 50 °C, pH 8, E/S = 0.05:40

The temperature of 50 °C leads to the higher hydrolysis degree among the tested temperatures at a pH value of 8. The best pH value was 9, although values with pH 8 are quite close. These values are close to the optimal values reported in literature for this enzyme (50 °C and pH 8.5; Godfrey, 1996).

BLP

Results for the *Bacillus licheniformis* protease (Alcalase) are presented in Figure 3-5.

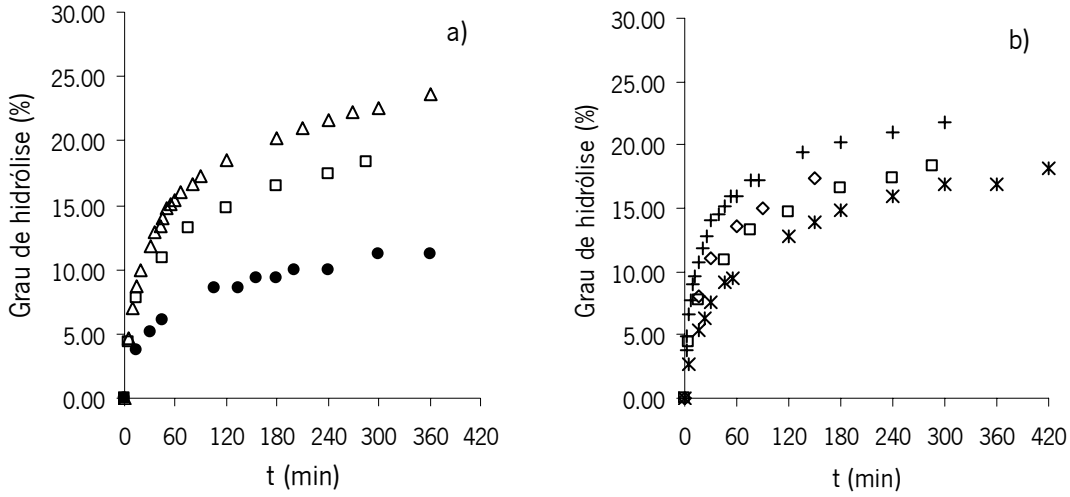

Figure 3-5: Degree of hydrolysis of WPC by BLP (E/S = 2 mL : 40 $g_{protein}$): a) pH 8; b) 37 °C • pH 7; □ pH 8, 37 °C; Δ pH 9; ∗ 30 °C; ◊ 40 °C; + 50 °C

The optimal temperature within the tested values was 50 °C, close to the value indicated by the supplier (between 55 and 60 °C). However, for high operational times (to get high degrees of hydrolysis) at 55 °C a slight inactivation of the enzyme occurs, and after 90 min the *DH* achieved at 50 °C was higher than the *DH* achieved at 55 °C (Adler-Nissen, 1986). This effect is more evident and happens sooner when higher temperatures are employed. The best tested pH value was 9 while the suggested by the supplier was 8 - 8.5. However, Godfrey (1996) states that optimum values for this kind of enzymes can go up to 10-11 and Adler-Nissen, 1986 reports a value of 9 as being the best for the hydrolysis of casein or soya isolate (among pH values of 7, 8 or 9) at 50 °C, stating that the influence of pH differs with the substrate in use.

As expected, the final degree of hydrolysis was higher than 20 %, in accordance with the values reported by Perea and others, 1993 and Camacho and others, 1998 with similar enzymes. In fact, this BLP is a subtilisin with a broad specificity with general preferences for hydrophobic amino acids except for proline

(Adler-Nissen, 1986). Thus, as whey proteins have approximatly 30.7 % (molar basis) of hydrophobic amino acids (excluding proline), it is expected that the final hydrolysis degree is high.

HPLC profile and gelling properties

After 15 min of hydrolysis, the three enzymes gave quite different hydrolysates at 37 °C (pH 8 for BLP and trypsin and pH 2 for pepsin). Hydrolysates from pepsin and trypsin had almost no intact α-La while β-Lg was only slighly degradated (Figure 3-6 a and b). The hydrolysate from BLP, after the same period of time, had only residual amounts of both intact proteins (Figure 3-6 c). Beyond these differences, the peptide profiles of the resulting hydrolysates are also different, because of the different specificity of the enzymes and different catalytic activity.

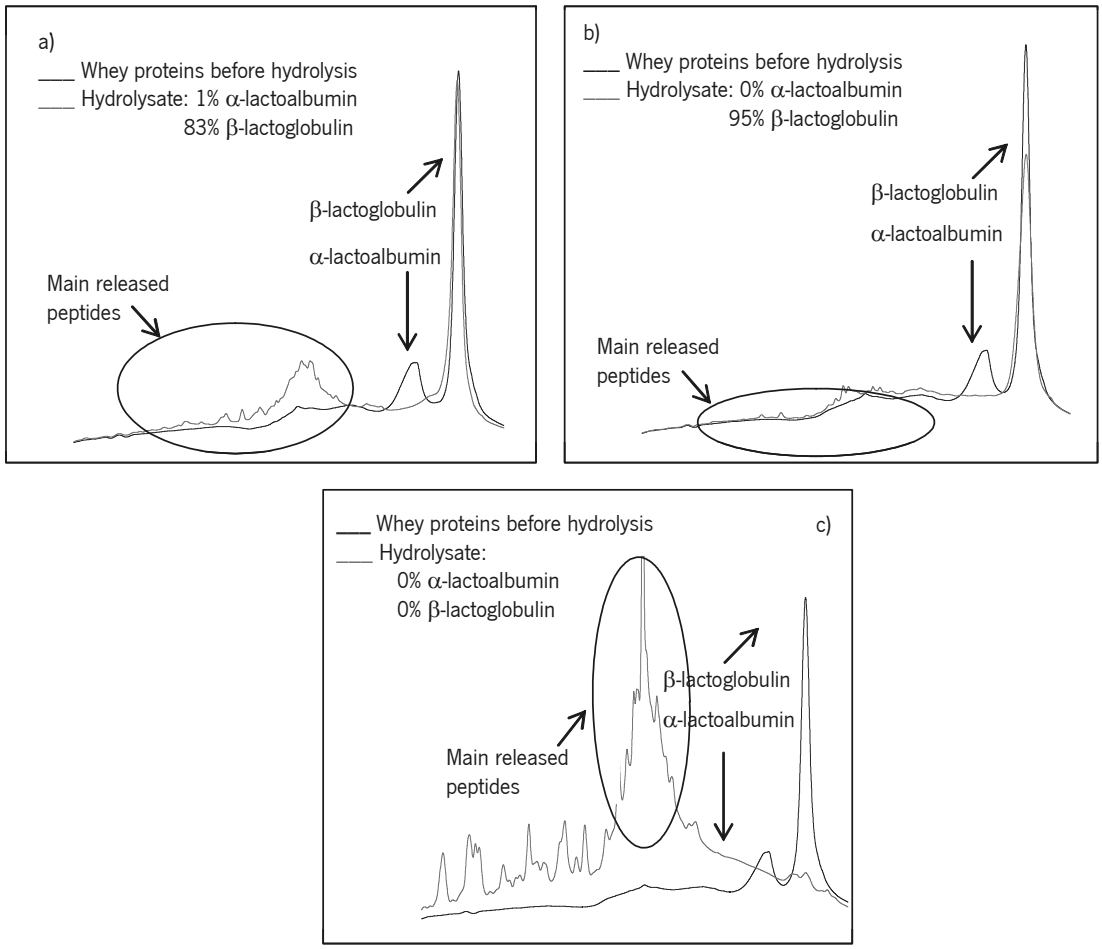

Figure 3-6: HPLC profile of hydrolysates of whey protein after 15 min of hydrolysis: a) Pepsin, pH 2.0, 37 °C; b) Trypsin, pH 8.0, 37 °C; c) BLP, pH 8.0, 37 °C (adapted from Torres and others, 2003)

The heat-set gel formed after hydrolysis with all three enzymes was weaker than the heat-set gel formed with the intact WPC and this decrease was higher with the increase of *DH* (experiences made with small amplitude oscillatory tests with 10 % w/w hydrolysate aqueous solutions; results not shown). Pepsin led to the strongest (although weak, even so) gel when only hydrolysates were considered, although the amount of intact β-Lg was slightly less (83 %) than for trypsin (95 %). Hydrolysates from BLP did not form a gel at all, probably due to the high level of protein degradation detected by chromatography (Torres and others, 2003). Even so, hydrolysates from BLP have been reffered to as improving the gelling ability (Ju and others, 1995; Otte and others, 2000). This contradictory result is probably due to the heat inactivation treatment.

Although β-Lg is resistant to peptic and chymotryptic hydrolysis (Reddy and others, 1988), it is not resistant to tryptic hydrolysis. As a consequence, β-Lg should have been more degradated with trypsin than with pepsin. Pepsin was probably not pure enough and some contaminant may have partially degradated β-Lg. In the case of trypsin, the activity of the chosen enzyme was too low (as well as the used amount of trypsin) and almost no degradation occurred after 15 min. α-La might have been degradated not in those 15 min but during the heat inactivation time, as it denaturates at lower temperatures at which the enzyme is still active and the reaction rates are higher. β-Lg is more heat resistant and was not denatured while the enzyme was still active. On the other hand, β-Lg was eventually also denatured as the heat treatment was long to assure that the enzyme was inactive and its gelling ability was partially damaged. This may be the reason for the stronger gel achieved with the peptic hydrolysate. On the other hand, as the peptic hydrolysate was made at pH 2 and inactivated at pH 7, a higher amount of pH reagents (NaOH and HCl) was added leading to a higher final salt concentration, which can also be responsible for a stronger gel as explained in Chapter 2.

3.3.2 Hydrolysis with trypsin

With the results from the previous subsection and considering that the literature mentions that whey protein hydrolysates from trypsin with a low degree of hydrolysis can form gels, it was decided to remove the heat inactivation step. Instead, the pH of the samples for HPLC analysis was decreased to 3 because at this pH the enzyme is inactive (although this inactivity can be reversible). The samples were kept frozen and were unfrozen only immediately before use (the time at room temperature was minimized). WPI was used instead of WPC. The trypsin used this time was different, with a very low chymotryptic activity (< 0.2 %).

The optimum pH for tryptic hydrolysis of 5 % (w/w) WPI at 37 °C during 3 hours is 8.5 (Figure 3-7).

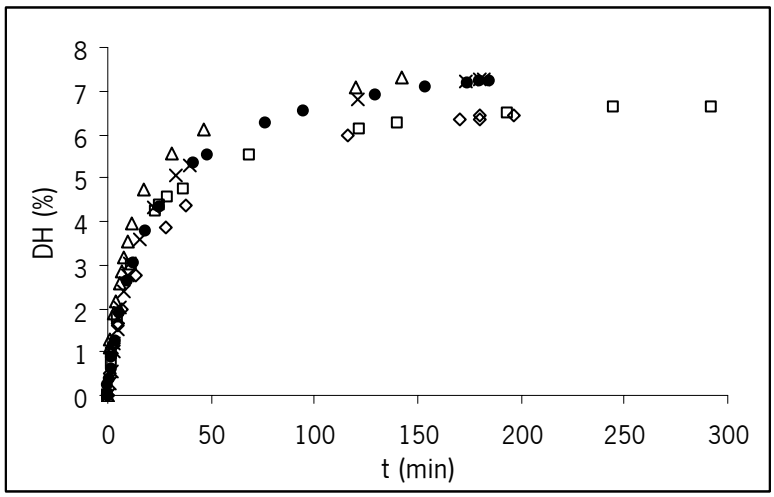

Figure 3-7: Degree of hydrolysis of whey protein isolate with trypsin at 37 °C: ◊ pH 7.5; □ pH 8.0; Δ pH 8.5; ● pH 9.0; × pH 9.5

The optimum temperature for tryptic hydrolysis of 5 % (w/w) WPI at pH 8 during 3 hours seems to be 45 °C (Figure 3-8), although when lower degrees of hydrolysis are intented, shorter times can be used and the optimum can in this case be different. In fact, for processes lasting for instance 20 min the optimum temperature is 50 °C. Above 50 °C the enzyme inactivation starts to be relevant. At 60 °C almost no active enzyme is present after only 10 min of hydrolysis.

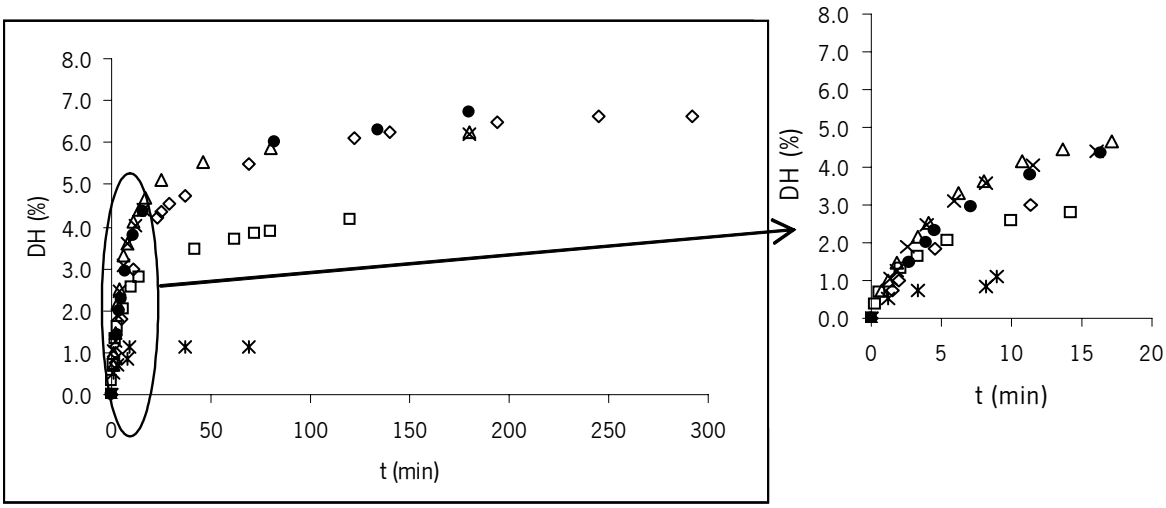

Figure 3-8: Degree of hydrolysis of whey protein isolate with trypsin at pH 8.0: ◊ 37 °C; ● 45 °C; Δ 50 °C; × 50 °C (2nd test); □ 55 °C; ∗ 60 °C

The hydrolysis curves (Figure 3-7and Figure 3-8) show that initial reaction rate is high but after a certain period (ca. 10-20 min) the reaction rate becomes slow. On one side, the cleavable peptide bonds of

whey proteins with trypsin correspond to only ca. 12.3 % of all peptide bonds from whey proteins (Arg and Lys data used to calculated this value from Adler-Nissen, 1986). Consideration of only α-La and β-Lg, which constitute more than 70 % of the total WP, shows that the maximum theoretical *DH* is around 11 % with trypsin (Ju and others, 1995). As the reaction proceeds, fewer and fewer peptide bonds are available per unit of enzyme and this may cause the decrease in the reaction rate. This explanation has also been proposed by Adler-Nissen (1986). However kinetic studies indicate that substrate saturation usually prevails over the hydrolysis reaction. If the enzyme is saturated with substrate, this cannot be the reason for the hydrolysis degree curve (Adler-Nissen, 1986). On the other side, inhibition of serine proteases by the formed peptides has been suggested by several authors (Northrop, 1921; Adler-Nissen, 1986; Margot and others, 1997), as well as inactivation of the enzyme by autolysis or thermal unfolding (Margot and others, 1997; Marquez and Vazquez, 1999). The presence of a protease inhibitor in the whey fraction that acts on trypsin has also been mentioned (Weber and Nielsen, 1991; Camacho and others, 1998). Thus the maximum value of *DH* achieved was around 7.4 %, lower than the theoretically expected, although a slightly closer *DH* might be achieved if more time had been given to the hydrolysis. Perea and others (1993) mentioned a *DH* of 9.9 % for β-Lg and 7.4 % for α-La with free pancreatin trypsin Novo 3.0S at 50 °C and pH 8.0 and 12.4 % for β-Lg and 15.2 % for α-La with a mixture of free trypsin and chymotrypsin. Mullally and others (1994) achieved a *DH* of 6.0 % for the hydrolysis of whey proteins with trypsin at 50 °C and pH 8.0. Mercier, Gauthier and Fliss (2004) reported a *DH* of 12 to 17 % with a mixture of free trypsin and chymotrypsin. Thus, the achieved values are in good agreement with those found in literature.

The non-hydrolysed whey protein isolate has two major peaks corresponding to α-La and β-Lg, as expected, with average retention times of 30.3 and 32.2 min, respectively (Figure 3-9). After a period of incubation the peaks associated with the major whey proteins gave rise to a new group of peaks due to the formation of several peptides in a pattern that indicates the presence of a wide range of peptides with higher polarity. Most of those peaks appear right from the beginning, although some hydrophobic peptides (with higher retention times) were further degraded and the concentration of hydrophilic peptides (with low retention times) increased with the time of hydrolysis. This agrees with the fact that trypsin is a selective enzyme that breaks only specific bonds as also related by Madsen and others (1997).

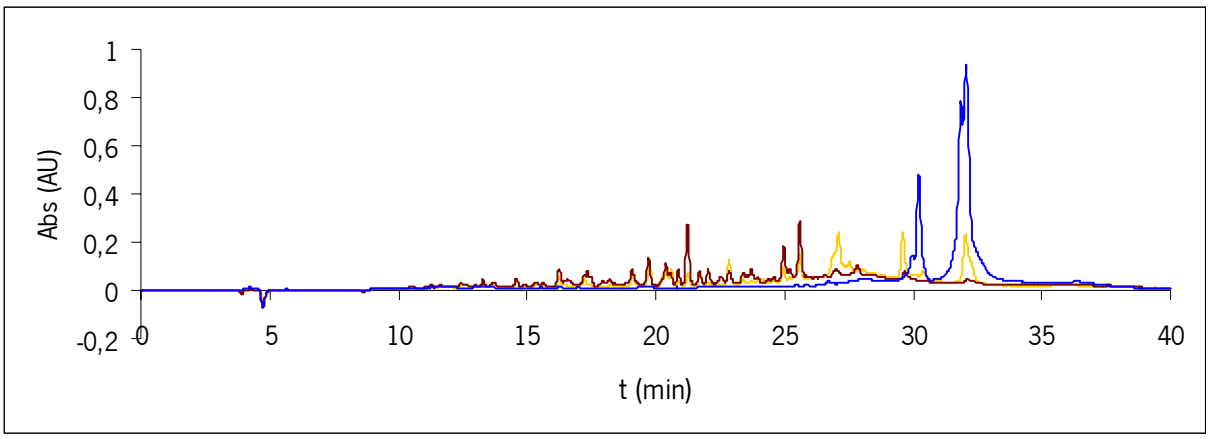

Figure 3-9: RP-HPLC profile of whey protein hydrolysates from trypsin at pH 8.0 and 37 °C: – *DH* 0 % (*t* = 0 min); – *DH* 4.3 % (*t* = 25 min); – *DH* 6.3 % (*t* = 140 min)

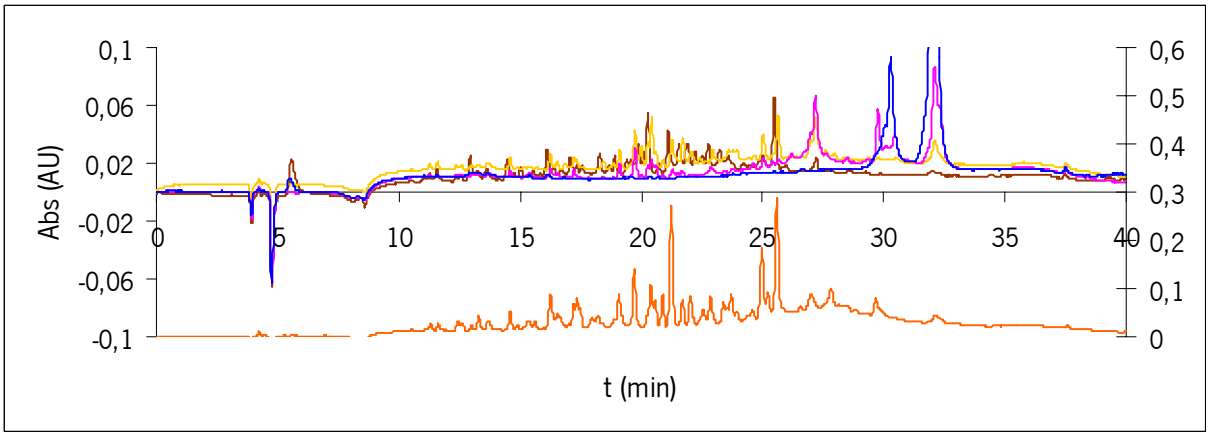

Figure 3-10: RP-HPLC profile of whey protein hydrolysates from trypsin at pH 8.0, 37 °C and 10 g/L of WPI: – *DH* 0 % (*t* = 0 min); – *DH* 1.5 % (*t* = 3 min); – *DH* 2.7 % (*t* = 25 min); – *DH* 3.7 % (*t* = 180 min); – *DH* 4.3 % with 50 g/L of WPI (*t* = 25 min)

As already observed by several authours (including Adler-Nissen, 1986), the RP-HPLC profile from hydrolysates produced at 50 g/L of WPI and from hydrolysates from 10 g/L of WPI are slightly different (Figure 3-10), especially in the more hydrophobic region (higher retention times), where more peaks can be observed in the case of 50 g/L (for degrees of hydrolysis around 4 %). At lower and medium retention times, the peptides formed seem to be similar, although the relative amounts are different for some peaks (or some peptides). It should also be noticed that hydrolysis is faster for higher substrate concentration.

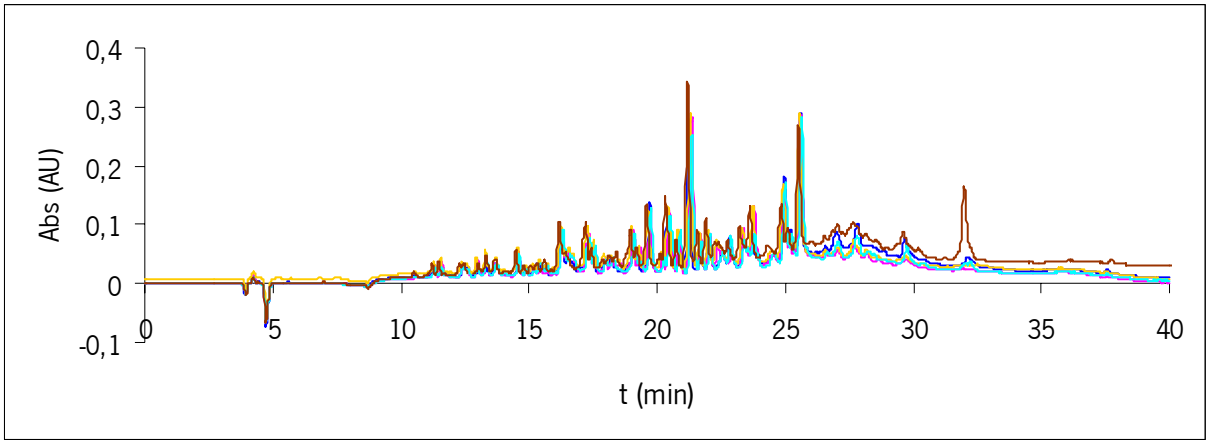

Figure 3-11: RP-HPLC profile of whey protein hydrolysates from trypsin at 37 °C and 50 g/L of WPI: – pH 7.5 (t = 180 min; *DH* = 6.4 %); – pH 8.0 (t = 140 min; *DH* = 6.3 %); – pH 8.5 (t = 142 min; *DH* = 7.3 %); – pH 9.0 (t = 180 min; *DH* = 7.2 %); – pH 9.5 (t = 180 min; *DH* = 7.2 %)

Although between pH 8 and pH 9.5 the peptide profiles seem to be the same, this is not the case at pH 7.5, although at this pH and at pH 8 the *DH* used for the comparison is slightly lower (Figure 3-11)). However, the profile at pH 8 is comparable to the profile at pH 7.5 and the differences remain. At pH 7.5, β-Lg seems to be more resistant to the hydrolysis and more intermediate peptides in the range of 26-30 min of retention time remain after 180 min (*DH* of 6.4 %). This may be ascribed to the quaternary structure of β-Lg. At pH 7.5 some β-Lg molecules may be associated in dimers, making the hydrolysis more difficult, although at 37 °C the monomer form prevails (see Chapter 2).

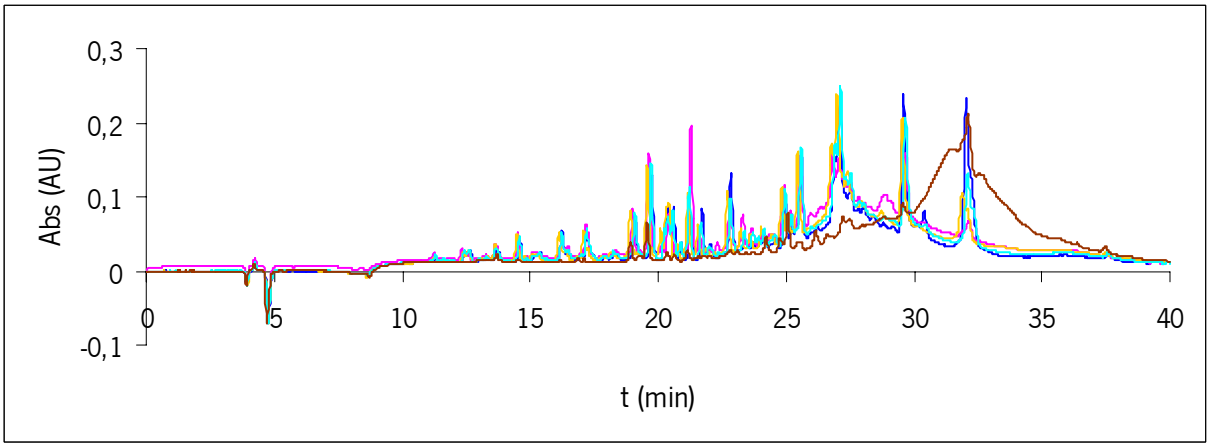

Figure 3-12: RP-HPLC profile of whey protein hydrolysates from trypsin at pH 8.0 and 50 g/L of WPI: – 37 °C (t = 25 min; *DH* = 4.3 %); – 45 °C (t = 16 min; *DH* = 4.3 %); – 50 °C (t = 11.6 min; *DH* = 4.0 %); – 55 °C (t = 120 min; *DH* = 4.2 %); – 60 °C (t = 69 min; *DH* = 1.1 %)

The peptide profiles from tryptic hydrosysates produced at different temperature are also very similar (Figure 3-12). Exception can be made for the hydrolysate at 60 °C, although small differences already appear at 55 °C. At 60 °C a wide peak is observed in the region where the peaks of α-Lg and β-Lg appear. This does not happen for the lower temperatures at the same *DH* (chromatograms not shown) and can be caused by thermal denaturation of β-Lg and α-Lg.

β-Lg is slightly more resistant to tryptic attack than α-La (Table 3-1). These differences are small and decrease with the increase of temperature. This quicker degradation of α-La corresponds to the appearance of a small peak with a slightly lower retention time that may correspond to an "intermediate" of α-La.

Table 3-1 Degradation of α-La and β-Lg with trypsin

pH	*T* (°C)	*C* (g/L)	Hydrolysis time (min)	Degree of hydrolysis (%)	α-La concentration (% of initial α-La concentration)	β-Lg concentration (% of initial β-Lg concentration)
8	37	50	25	4.3	2.9	14.3
8	37	50	140	6.3	0	0.9
8	37	10	3	1.5	9.2	36.3
8	37	10	25	2.7	0	6.3
8	37	10	180	3.7	0	0.3
7.5	37	50	180	6.4	0	6.2
8.5	37	50	142	7.3	0.6	1.4
9.0	37	50	180	7.2	0	0
9.5	37	50	180	7.2	0	0
8	45	50	16	4.3	0.7	7.6
8	50	50	11.6	4.0	0.5	5.0
8	55	50	120	4.2	0.2	1.8
8	60	50	69	1.1	n.d.	n.d.

Only one profile is presented for each sample, although the injections were all made in duplicate. The RP-HPLC analysis was found to be qualitatively highly reproductible and examples of three samples with different dilutions are presented in Figure 3-13.

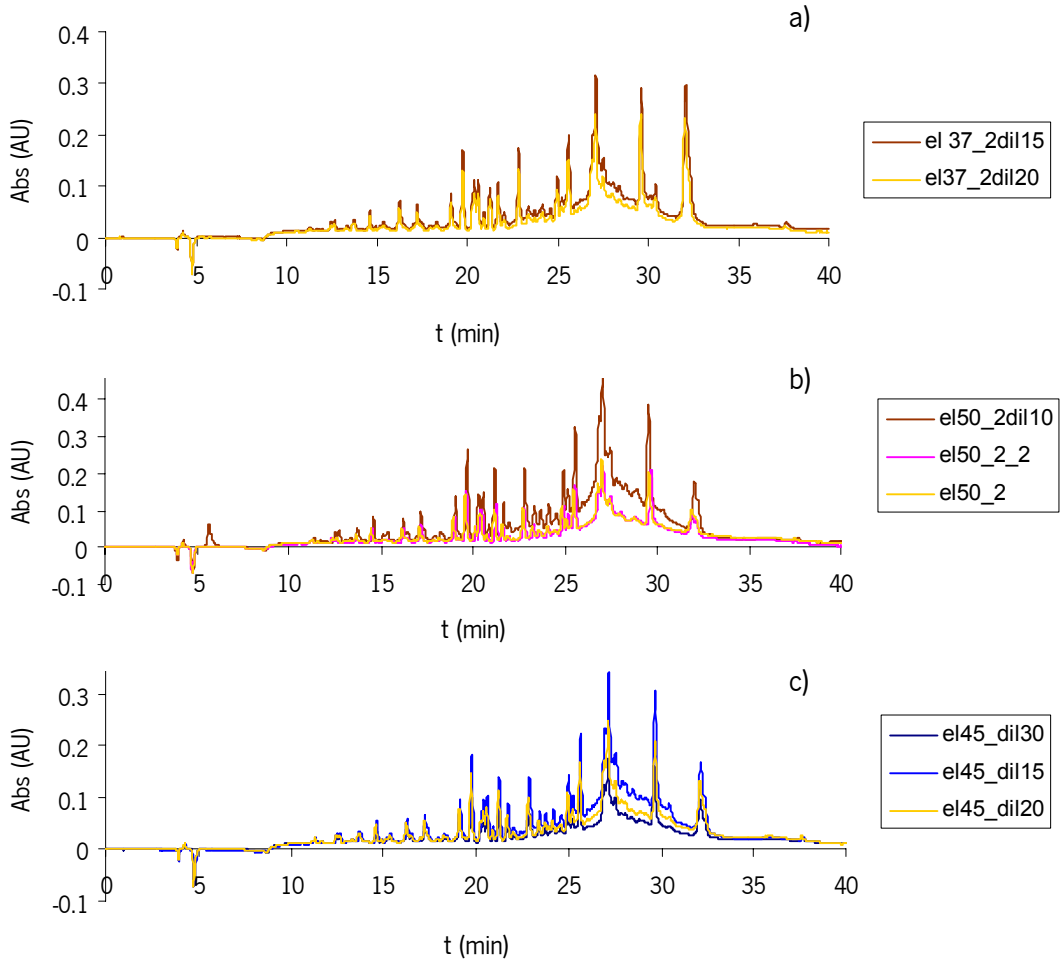

Figure 3-13: RP-HPLC profile of: a) hydrolysate (*DH* = 4.3 %) with free enzyme at 37 °C and pH 8 diluted 15 and 20 times; b) hydrolysate (*DH* = 4.0 %) with free enzyme at 50 °C and pH 8 diluted 10 and 20 times (the last one in duplicate); c) hydrolysate (*DH* = 4.3 %) with free enzyme at 45 °C and pH 8 diluted 15, 20 and 30 times

3.3.3 Hydrolysis with pepsin

Due to the results of section 3.3.1, the type of pepsin was changed to a more purified one and the resulting samples and hydrolysates were dialysed with a 100 Da membrane against distilled water for 24 hours at 4 °C to remove the excess of salt (water was changed four times).

Although initial hydrolysis rate with trypsin is much higher than with pepsin, which is understandable because β-Lg has particularly stable conformation at pH 2 (unless pre-heated) and is not easily hydrolysed, at the end of the hydrolysis the reaction rate with trypsin is lower than with pepsin, and probably if enough time had been given the final *DH* might eventually had been higher with pepsin than with trypsin (Figure 3-14).

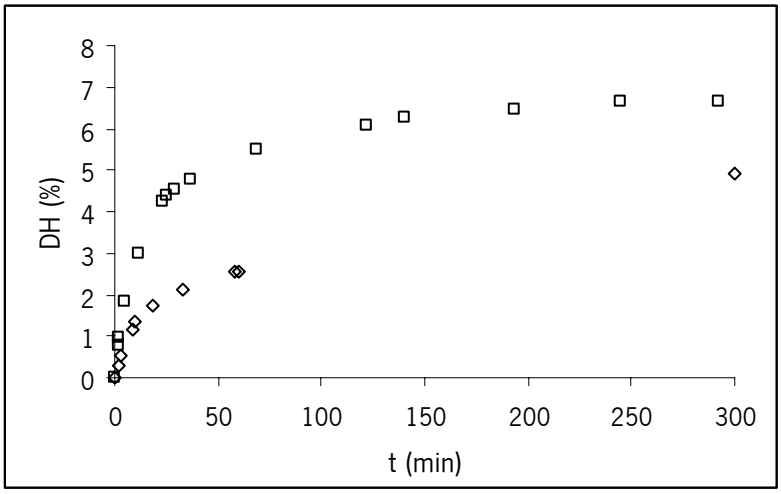

Figure 3-14: Degree of hydrolysis of WPI with trypsin (□) or pepsin (◊) at pH 8.0 and 37 °C

In fact, pepsin has a fairly broad specificity with a preference for cleaving after hydrophobic residues (Davis and others, 2005), while trypsin is very specific for arginine and lysine amino acids (present in lower amount than hydrophobic amino acids). After 300 min, the hydrolysis with pepsin was thus less extensive than with trypsin (final *DH* with pepsin was 4.9 %; final *DH* with trypsin was 6.6 %).

As expected, after 60 min of hydrolysis with pepsin all α-La was degradated and almost all the β-Lg remained intact (Figure 3-15; Table 3-2). In fact, at neutral pH, denaturation temperatures of β-Lg and α-La are reported to be around 78 °C and 64 °C, respectively. Lowering pH significantly increases the denaturation temperature of β-Lg (85 °C at pH 3), but decreases the denaturation temperature of α-La to 58.6 °C at pH 3.5 (Ju and others, 1999). Due to differences in the enzymes' specificity the peptide profiles obtained are quite different. Besides cleaving peptide bonds at different amino acid residues, the peptides from peptic digestion are mainly originary from α-La while the peptides from tryptic digestion are mainly originary from β-Lg (present in a higher amount in whey protein isolate). Peptic peptides seem to be more hydrophylic (in average) as bigger peaks appear at lower retention times (this would probably be more notorious for higher peptic hydrolysis degree). This is also expected because pepsin is able to cleave more peptide bonds resulting in smaller peptides, usually more hydrophilic. On the other

hand, comparing Figure 3-9 and Figure 3-15 at initial hydrolysis stages, it seems that with trypsin some highly hydrophobic intermediates are formed that are further degradated into smaller peptides while with pepsin most of the early formed peptides have already a retention time lower than 27 min. This might indicate that the hydrolysis mechanism with trypsin is, in this case, similar to a "one-by-one" type reaction and the hydrolysis mechanism with pepsin is more similar to a "zipper" type reaction (described in Chapter 2), with almost no intermediate species present. A similar behaviour was described by Adler-Nissen (1986) for trypsin and pepsin at the same pH values using serum albumin as substrate.

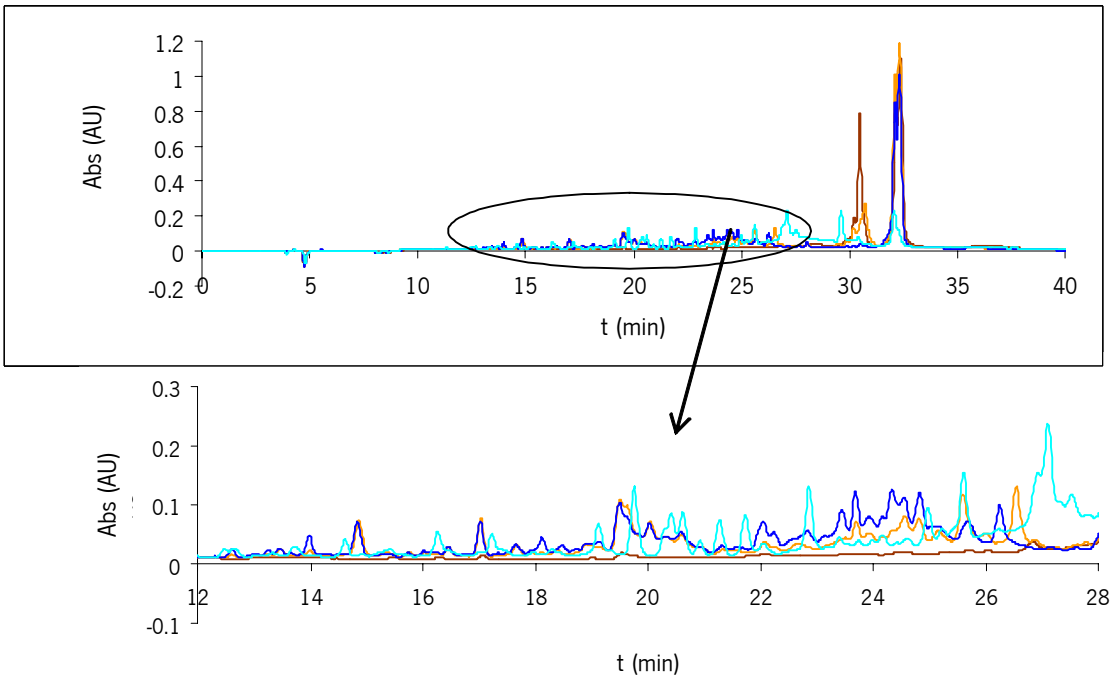

Figure 3-15: RP-HPLC profile of whey protein hydrolysates from pepsin at pH 2.0, 37 °C and 50 g/L: – DH 0% (t=0 min); – DH 1.8% (t=18 min); – DH 2.6 (t=60 min); – DH 4.4 Trypsin (t=25 min)

Table 3-2 Degradation of α-La and β-Lg with pepsin

Hydrolysis time (min)	Degree of hydrolysis (%)	α-La concentration (% of initial α-La concentration)	β-Lg concentration (% of initial β-Lg concentration)
18	1.8	59.1	100
60	2.6	0.3	96.1

3.4 Conclusion

The choice of the hydrolysis enzyme is particularly important in determining the properties of the resulting hydrolysates. Besides choosing the type of enzyme it is also important to select an adequate form of the choosen enzyme with the adequate purity and treatment (for instance a treated trypsin with low chymotryptic activity) for the desired application, as different hydrolysates are achieved with different forms of the enzyme.

The selection of the adequate operational conditions (time, pH and temperature) also determines the composition of the resulting hydrolysate. Higher reaction times lead obviously to higher degrees of hydrolysis and smaller peptides (usually more hydrophobic) and pH and temperature determine the resistance of whey proteins to the hydrolysis as well as the activity of the enzyme.

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Chapter 4 Trypsin immobilization

4.1 Introduction

Trypsin is a widely used enzyme for protein hydrolysis and can be used to improve functional and nutritional properties of foods. Its immobilization on solid carriers can offer several advantages over the free enzyme including easy handling, recovery from the reaction medium, reuse and operation in continuous reactors.

Enzyme immobilization has been widely studied since the early 1960's. Trypsin, as well as pepsin, urease and invertase were often used as "model" enzymes and were immobilized in many different kinds of supports (Bareli and Katchalski, 1960; Epstein and Anfinsen, 1962; Bareli and Katchalski, 1963; Levin and others, 1964; Glazer, 1967; Habeeb, 1967; Gabel and others, 1970; Goldstei and others, 1970; Goldstei, 1973; Royer and Uy, 1973; Stoner and others, 1975). Axen and Porath (1966) immobilized trypsin in Sephadex conjugates involving isothiourea linkages and achieved relative activities of 17-24 % of the free enzyme activity observed with N- α-benzoyl-L-arginine ethyl ester (BAEE) as substrate and 2.5-4 % relative activity with casein as substrate. Habeeb (1967) achieved relative activity yields of 70-86 % with trypsin immobilized on aminoethylcellulose derivatives. Royer and Uy (1973) immobilized trypsin on a diazotized arylamine derivative of porous glass in the presence and absence of a specific substrate, BAEE. Later, trypsin was immobilized on chitosan by glutaraldehyde coupling with a specific activity of 38 % of its initial specific activity. The pH-activity profile of trypsin was slightly shifted toward alkaline values, and its thermal stability was increased. Immobilized trypsin was found to be less sensitive to its natural inhibitors than the soluble enzyme (Leuba and Widmer, 1979).

Some more recent works with trypsin immobilization are summarized in Table 4-1.

Traditional carriers include porous silica, porous glass and cellulose derivatives.

In the pursuit of better carriers, zeolites have attracted much attention in more recent years (Mukherjea and others, 1977; Diaz and Balkus, 1996; Gonçalves and others, 1996; Liu and others, 1997; Knezevic and others, 1998a; Serralha and others, 1998; Xing and others, 2000; Yiu and others, 2001; Seetharam and Saville, 2002; Lei and others, 2004; Chang and others, 2006a; Carvalho and others, 2007; among others) since they: (i) have unique structural characteristics (high mechanical strength and resistance to sterilization, e.g.) and are resistant to biodegradation; (ii) possess novel properties such as high surface areas, hydrophobic or hydrophilic behavior and electrostatic interactions; (iii) can be readily prepared with pore sizes ranging from micropore (< 20 Å) to mesopore (20 to 500 Å) and in a wide range of particle sizes (Xing and others, 2000).

Table 4-1: Some results on trypsin immobilization

Support	Immobilization methods / binding agents	Immobilized protein*	Retained activity	Reference
Agarose	Covalent, glycidol followed by periodate to achieve glyoxyl-agarose	-	75 %	Blanco and Guisan, 1989
Aminopropyl porous glass (200-400 mesh, 175 $\overset{o}{A}$)	Covalent, 10 % glutaraldehyde, without and with inhibitor (soybean trypsin inhibitor)	92/100 %	10 %	Sears and Clark, 1993
Poly(ethylene terephthalate) fibres through grafted chains	Covalent, 1-ethyl 3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride.	-	-	Kulik and others, 1993
Oxirane-acrylic beads (Eupergit C)	Covalent, epoxide	85 %	80 % BAEE; 75 % casein	Lorenzen and Schlimme, 1995
MCM-41 silica molecular sieve (40 Å)	Adsorption/ entrapment through silanation	4.7 mgg^{-1} / -	- / 13 %	Diaz and Balkus, 1996
Porous zirconia and porous silica	Covalent, activated with 3-isothiocyanatopropyltriethoxysilane, imidazole	11-20 %/16–36 %	21-46 %/22–36 % BAPNA	Huckel and others, 1996
Diatomaceous earth (Celite™)	Covalent, activated to aminopropyl-Celite or derivatized to succinamidopropyl-Celite, 1.2 % glutaraldehyde	34.7 %/44.5 %	18 %/22 % TAME	Huang and others, 1997
Acrylic copolymers with acrylonitrile, butyl acrylate, ethyl acrylate and hydroxypropyl methacrylate as co-monomers; cross-linked with divinylbenzene, ethylene glycol dimethacrylate or trimethylolpropane triacrylate	Covalent, 2 % glutaraldehyde	1.7-13.9 $mg.cm^{-2}$	4.9-115 U_{casein} $/cm_2$	Bryjak and Kolarz, 1998
Methacrylic-methacrylate polymer (Eudragit S-100)	Covalent, carbodiimide; physical adsorption	75-86 %/30 %	56-64 %/3 %	Kumar and Gupta, 1998
Eudragit S-100 (methacrylic acid and methyl methacrylate)	Entrapment, covalent with EDC, covalent with EDC and benzamidine, covalent with EDC removing noncovalent binding protein with Triton X-100	76 %/95 %/86 %/82 %	55 %/22 %/76 %/35 % BAPNA	Arasaratnam and others, 2000
Polyester fleece	Covalent, N-hydroxysuccinimide activated, N, N'-dicyclohexylcarbodiimide, via different spacers (PEG-diamine, aldehyde dextran, amino dextran and bovine serum albumin)	11.3 $\mu g/cm^2$ –38.2 μgcm^{-2}	2.9–15.5 $\mu mol_{Bapna} min^{-1} cm^{-2}$	Nouaimi and others, 2001
Crosslinked mercerized cellulose	Covalent, epoxy with 1,4-butanediol diglycidyl ether, glutaraldehyde and epoxy followed by diazotization with 1,4-phenylenediamine and $NaNO_2$	-/-/67 %	3 %/13 %/28 % BAPNA	Ruckenstein and Guo, 2001
Copolymers of vinylene carbonate and β-hydroxyethylene Acrylate	Covalent	14.4-37.9 %	18.7-72.8 %	Ding and others, 2002
Chito-Xan (hydrogel with chitosan and xanthan)	Entrapment	62–92 %	low	Magnin and others, 2003

* Except stated otherwise, the units are % of the immobilized protein over the total protein used in the immobilization procedure

Table 4.1 (cont) Some results on trypsin immobilization

Support	Immobilization methods / binding agents	Immobilized protein*	Retained activity	Reference
Aminopropyl controlled pore glass (80-120 mesh, 700 Å average pore size)	Covalent, Glutaraldehyde 1 %; with sodium cyanoborohydride	68 %	13 % BAEE	Migneault and others, 2004
Silica gel supported macroporous chitosan bead	Adsorption and covalent, epoxy with 1,2-ethylene diglycidyl ether, epoxy with 1,4-phenylenediamine to form an aminoaryl derivative and diazotization with $NaNO_2$, and aminoaryl derivative activated with 2.5 % glutaraldehyde.	12 %/72 %/76 %/80 %	-/50 %/63 %/45 %	Xi and others, 2005
Ferromagnetic polyethylene terephthalate	Covalent, activated with hydrazine, glutaraldehyde.	37 %	29 %	Amaral and others, 2006
Extracellular cellulosic polysaccharide produced by *Zoogloea* sp. from sugarcane	Covalent, sodium metaperiodate (to form carbonyl groups) without and with BSA as a spacer	17.2 %/26.9 %	37.2 %/9.2 %	Cavalcante and others, 2006
Poly(methyl methacrylate-ethyl acrylate-acrylic acid) latex	Covalent with 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride (EDC), preadsorption and covalent with EDC, covalent with EDC and spacer arm (6-Aminocaproic acid (6-ACA)	30.3 %/46.0 %/ 50.1 %	203/218/ 186 U_{casein}/g; -/11.7 %/-	Kang and others, 2006
N-isopropylacrylamide, 2-hydroxyethyl methacrylate and glycidyl methacrylate modified by amination	Adsorption followed by crosslinking with glutaraldehyde;	23.4-49.1 %	0.10-18.7 % BAPNA; 0.0-1.5 % casein	Hamerska-Dudra and others, 2007
Siliceous mesostructured cellular foams (MCF) and silica gels	Covalent, derivatized with 3-aminopropyltriethoxysilane, 2-aminoethyl-3-aminopropylmethyldimethoxysilane, 2-aminoethyl-3-aminopropyltrimethoxysilane and 3-glicydoxypropyl-triethoxysilane and activated with glutaraldehyde	24.1–71.1 % for MCF; 33.8–70.7 % for silica	73-100 % BAPNA; 1.4–14.7 % casein; 0-1 % silica	Jarzebski and others, 2007

* Except stated otherwise, the units are % of the immobilized protein over the total protein used in the immobilization procedure

They are built from a three dimensional framework of silica and alumina tetrahedra with water and cations occupying the pores (with a defined diameter, due to the structure of the zeolite). By changing the Al/Si ratio, carriers with different hydrophobic/hydrophilic characters can be generated (Gonçalves and others, 1996). Microporous zeolites usually have high superficial areas with low size pores allowing the deposition of high quantities of biocatalyst only on carrier surface, leading to reduced internal resistance to diffusion of products and substrates. Due to its ionic structure, the enzyme-carrier interactions are essencially ionic and, thus, stronger than hydrophobic interactions that usually exist between the adsorbed enzyme and the immobilization support (Veloso, 1999).

The well-known zeolites A, X, and Y are commonly used as supports for the immobilization of enzymes. They have negatively charged (due to the presence of alumina) aluminosilicate crystalline structures and

can be used as cationic exchangers. Their chemical formula can be described as $\left[Na_x\left(AlO_2\right)_x\left(SiO_2\right)_y\right]zH_2O$ (where x is 12 for NaA, 86 for NaX and 56 for NaY; y is 12 for NaA, 106 for NaX and 136 for NaY; z is 27 for NaA, 264 for NaX and 250 for NaY) and consists of a three-dimensional arrangement of SiO_4 and AlO_4 tetrahedral linked to each other by a shared oxygen atom. The surface area and effective pore diameter of zeolites X and Y are approximately 800 m^2/g and 7–8 Å, respectively, which are twice the values of zeolite A (4 Å). The Si/Al ratios in zeolites A, X and Y are 1.0, 1.23, and 2.43, respectively (Rolison, 1990; Chang and others, 2006b). These zeolites present a hydrophilic character with a remarkable water affinity.

One of the most referred application of microporous zeolites to enzyme immobilization is when the enzyme has to act in a non-aquous solvent (Gonçalves and others, 1996; Gonçalves and others, 1997; Knezevic and others, 1998a; Knezevic and others, 1998b; Serralha and others, 1998; Xing and others, 2000; Serralha and others, 2001a; Serralha and others, 2001b; Serralha and others, 2004). In this case the activity and quantity of the water retained by the immobilization support are very important factors for the hydrolytic enzyme activity. In fact, zeolites are known to be capable of storing a large amount of water in their intracrystalline void space, and one could easily envisage the use of the external surface of the zeolitic material as the interface between the aqueous phase, contained within the zeolite's framework, and the organic medium where the substrate lies, in much the same way as one uses a reversed micellar medium, only with the distinct advantage of the use of a solid support, thus facilitating enzyme recovery and reuse (Gonçalves and others, 1996; Knezevic and others, 1998a).

Spent grains are a brewing by-product with a high content in cellulose and can also be interesting as carriers for enzyme immobilization because, besides having the necessary conditions (as stability, rigidity, low mass transfer limitations), they are cheap and food grade (Branyik and others, 2001).

Most proteins are immobilized through its amine groups. There are two main types of amine groups exposed to the medium: ε—amine groups of lysine residues (the most abundant), usually with a pK around 10.5-10.7, and terminal amine-groups with a pK around 7-8. Most of the reactive agents used for immobilizing proteins (glutaraldehyde, cyanogen bromide, etc.) are able to yield very stable enzyme-support bonds under mild immobilization conditions (e.g., neutral pH values). The high reactivity of these agents makes them very unstable at alkaline pH values, where the reactivity of lysine residues may be more suitable for the reaction (Mateo and others, 2005). Therefore proteins should be mainly immobilized via the amino terminal group at neutral pH values when using these carriers.

Glyoxyl or glutaraldehyde activated supports have proven to be quite efficient in increasing the tertiary enzyme stability via multipoint covalent attachment (Lopez-Gallego and others, 2007). Immobilization on glyoxyl-carriers occurs at alkaline pH, via the richest area in lysines on the proteins surface (Rocchietti and others, 2004). Although immobilization of proteins at neutral pH values is possible when performed in the presence of a Schiff's base reducing or stabilizing agent (e.g., cyanoborohydride), the low reactivity of the lysine at neutral pH hardly permits very intense multipoint covalent attachment under these conditions. To increase the multipoint covalent attachment after the first immobilization, and thus probably increase immobilized enzyme stability, it is necessary to increase the pH value (Mateo and others, 2006).

To enhance the activity retention of enzyme molecules attached to the support, the glyoxyl-carrier may be aminated with ethylenediamine what results in a reversible Schiff base that can be reduced with sodium borohydride to stable amino groups (after oxidation with sodium periodate). The activation of the support is then done with glutaraldehyde, allowing immobilization to occur at milder values of pH.

The use of glutaraldehyde for covalent immobilization can be done in several ways. Immobilization of enzymes on supports previously activated with glutaraldehyde or pre-adsoption of proteins onto supports with primary amino groups followed by treatment with glutaraldehyde are two possible alternatives (Alonso and others, 2005).

Non-porous support particles must be small in order to hold sufficient catalytic activity per unit volume of support. Magnetic particles suspended in liquids can be effectively handled at much smaller diameters than non-magnetic particles because their separation from the reaction medium can be greatly accelerated in the presence of a magnetic field (Munro and others, 1975). Thus a magnetic particle as carrier offers the convenience of easy washing procedures and easy separation of the enzyme from the reactional medium. Several authors have been immobilizing enzymes on magnetic carriers (Munro and others, 1975; An and Su, 2001; Akgol and others, 2001; Shaw and others, 2006; Hong and others, 2007, among others). Bruno and others (2005) successefully used magnetic polysiloxane-polyvinyl alcohol (POS-PVA) composite to immobilize a lipase. In this case tetraethoxysilane (TEOS) and polyvinyl alcohol (PVA) were used for the formation of a matrix combining the PVA property to covalently retain proteins, via glutaraldehyde, with excellent optical, thermal and chemical stability of the host silicon oxide matrix. PVA is a synthetic non-toxic soluble polymer that can be activated with glutaraldeyde to render a more biocompatible surface for covalent immobilization. The resulting composite was then conjugated to

magnetite (Fe_3O_4), allowing the separation and recovery of the enzyme and carrier by magnetic force without loss of enzymatic activity.

In this chapter trypsin is immobilized onto spent grain or modified spent grain, POS-PVA, zeolites NaA, NaX and NaY and silica through different methods. Adsorption, ionic binding, covalent attachment (with glutaraldehyde and with glycydol, for spent grain) and a combination of adsorption and covalent binding (physical adsorption of the protein onto the carrier and intermolecular crosslinking with glutaraldehyde as a bi-functional reagent) was tested. The efficiency of immobilization and activity, operation and storage stability of free and immobilized enzyme on the supports were studied.

4.2 Materials and methods

All reagents used were of analytical grade and supplied by Sigma Chemical Co. Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg was also obtained from Sigma Chemical Co. (one BAEE unit will produce a ΔA_{253} of 0.001 per min at pH 7.6 at 25 °C using BAEE as substrate, in a reaction volume of 3.2 ml and 1 cm light path). Several carriers were tested: porous silica with 30-45 mesh and 375 Å of pore diameter (ref. 27706, Fluka, Switzerland), commercial zeolites NaY, NaA and NaX from Sigma (EUA), spent grains (kindly supplied by UNICER S. A., Porto, Portugal) and a magnetic hybrid inorganic-organic composite based on polysiloxane and polyvinyl alcohol (POS-PVA), produced as described below. The pH 6 and pH 7 buffers were prepared with phosphate, pH 8 buffer was prepared with tris(hydroxymethyl)aminomethane (TRIS) and HCl in the presence of 0.02 M $CaCl_2$ (to reduce enzyme auto-digestion) and pH 10 buffer with carbonate.

4.2.1 Supports

Silica and zeolites

Silica and zeolites were used without prior treatment for adsorption tests. For other tests they were firstly derivatized with 3-aminopropyltriethoxysilane: 3 g of carrier were added to 60 ml of 10 % (p/v) 3-aminopropyltriethoxysilane in acidified distilled water; pH was adjusted to 3-4 with HCl 6 N. The mixture was activated at 75 °C during 2 hours; carriers where then recovered by vacuum filtration or centrifugation, washed several times with distilled water and dried overnight at 105 °C. Carriers were

then activated with 1 % glutaraldehyde (w/v) for 2 hours in 0.05 M pH 7 phosphate buffer, except stated otherwise.

Spentgrains

Dry spent grains were prepared as described by Branyik and others (2001). Dry spent grains (100 g) were mixed in 1500 mL of 3 % (v/v) HCl solution at 60 °C for 2.5 hours in order to hydrolyse the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was cooled and washed with water. The remaining solids were partially delignified by shaking (120 rpm) in 500 mL of 2 % (w/v) NaOH solution at 30 °C for 24 hours. After being several times washed with water until neutral pH and dried, the carrier (ca. 10 g) was ready to be used.

Diethylaminoethyl-modified spent grains (DEAE-cellulose) were also prepared according to the method described by Branyik and others (2001), and ionic attachment to the carrier was tested.

Spent grains were activated using glutaraldehyde 1 % (w/v) for 2 hours in 0,05 M pH 7 phosphate buffer at room temperature or glycidol (2,3-epoxypropanol) with subsequent oxidation with periodate as described by Guisan (1988). Shortly, 5 g of spent grain were suspended in destilled water until the final volume reached 50 mL; 13.9 mL of NaOH 1.7 M with 0.40 g $NaBH_4$ were added to the suspended carrier; glycidol was gently poured into the suspension refrigerated on ice until a final concentration of 2 M, and allowed to react overnight with gentle mixing at 4 °C. The carrier was then washed with abundant distilled water and $NaIO_4$ was added to a final concentration of 0.1 M. The suspension was diluted 10 times and left to oxidise during 2 more hours. The carrier was then washed with distilled water and stored at 4 °C. Glyoxyl-spent grains were also further activated with 1 M ethylenediamine at pH 10.05 for 2 hours to amine-spent grains. Sodium borohydride was added and the carrier was gently stirred for 2 hours more. The carrier was then washed consecutively with pH 4 sodium acetate buffer (to destroy $NaBH_4$), with pH 9 sodium borate (to reduce electrostic interactions) and with water (Lopez-Gallego and others, 2007).

POS-PVA synthesis and magnetization

POS-PVA hybrid composite beads were synthesized by the hydrolysis and polycondensation of tetraethylorthosilicate (TEOS) as described by Barros and others (2002). Briefly: 6 ml of 2 % w/v polyvinyl alcohol (PVA), 5 ml of ethanol and 5 ml of TEOS were carefully mixed and stirred for 5 min at

60 °C, followed by the addition of 2–3 drops of concentrated HCl, in order to catalyze the reaction. After an incubation period of 40 min, the material was transferred to microwells of tissue culture plates and allowed to solidify for about 48 h at room temperature until complete formation of the interpenetrated network of POS-PVA.

POS-PVA was then magnetized according to Coelho and others (2002). The beads were powdered by using a mortar and pestle and 10 g of the powder were suspended in deionized water (500 mL) and coprecipitated with 50 mL of 0.6 M $FeCl_2.4H_2O$ and 1.1 M $FeCl_3.6H_2O$ (1:1) added drop-wise under stirring. The pH was adjusted to 11 with 33 % (w/v) NH_4OH. After 30 min incubation at 100 °C, under stirring, the magnetized particles were washed with deionized water until reach pH 7. To collect the particles a magnetic field was always used from this stage onwards. These particles were dried at 50 °C overnight and sieved (< 100 μm). For some of the tests, they were further activated with 1 % glutaraldehyde (w/v) for 2 hours in 0.05 M pH 7 phosphate buffer, except stated otherwise.

4.2.2 Trypsin Immobilization

Adhesion to the carriers was tested without chemical modification of the carrier surface (by physical adsorption) and with activation using glutaraldehyde (silica and zeolites were previously derivatized with 3-aminopropyltriethoxysilane) or glycydol (only for spent grain) alone or with further modification to amine-spent-grain. All immobilizing tests were performed at least in duplicate.

For each carrier, trypsin was incubated with the carrier and apropriate buffer overnight at 4 °C. Benzamidine (3 mM as final concentration) was used in some cases as a reversible trypsin inhibitor to prevent auto-proteolysis that could promote enzyme inactivation. When testing crosslinking, glutareldehyde (the bi-funtional reagent) was added in the next morning to a final concentration of 1 % for 1 h more at the immobilization pH.

Schiff bases are expected to be formed from the nucleophilic attack on ε-amino groups of the lysine residues in the protein (Blanco and Guisan, 1989) and from the reaction between glutaraldehyde and the terminal amine groups of the enzyme. Thus, these reversible Schiff bases have to be reduced into stable amines. Sodium borohydride hydrolysis is acid catalised (Davis and Swain, 1960), therefore it is far more stable at alkaline pH values. The hydrolysis rate is also reduced with the increase of ionic strength. When borohydryde was used to reduce Schiff base bonds, it was added at the end of the test in the proportion of 1 mg /mL and the mixture was allowed to stand for more 30 minutes, at the immobilization pH, in the

case of silica and spent grains with glutaraldehyde, and at pH 10 for POS-PVA and glyoxyl or amine spent grain.

Except otherwise stated, the standard conditions used were as follows:

- Silica: 100 mg of trypsin and 200 mg of silica in 5 mL of pH 8 TRIS/HCl 0.05 M buffer with 0.02 M of $CaCl_2$; in the cases of trypsin covalently bond to silanized silica, the carrier was previously activated with a 1 % glutaraldehyde solution in 0.05 M pH 7 phosphate buffer, except otherwise stated; or 25 mg of trypsin on 50 mg of activated silanized silica in 2 mL of buffer; except stated otherwise, activation was done for 2 h with 1 % glutaraldehyde solution in 0.05 M pH 7 phosphate buffer;

- Spent grain: 100 mg of trypsin and 200 mg of carrier in 5 mL of buffer; immobilization with 0.05 M phosphate buffer, pH 7.0 overnight at 4 °C, except for glyoxyl-spent grain which was incubated in 0.05 M carbonate buffer, pH 10; urea was used when covalent binding was involved;

- POS-PVA: 15 mg of trypsin and 40 mg of carrier in 1.5 mL buffer;

- Zeolites: 100 mg enzyme and 200 mg carrier in 5 mL buffer; immobilization with 0.05 M Tris/HCl buffer, pH 8.0 with 0.02M $CaCl_2$ overnight at 4 °C;

Samples were taken and the Bradford method was used for protein determination in the supernatant. The supernatant was separated from the particles by centrifugation, in the case of zeolite and silica, by vacuum filtration in the assays with spent grains and applying a magnetic field in the case of POS-PVA. The carrier was then washed several times, first with the immobilization buffer and then with TRIS/HCl buffer without $CaCl_2$ and filtered. The washing procedure with TRIS/HCl buffer was repeated four times. Urea 6 M was used to denaturate and remove unadsorbed/bond enzyme.

4.2.3 Measurement of Trypsin Activity

Trypsin activity of immobilized and native enzyme preparations was monitored hydrolyzing N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) in 0.05 M TRIS buffer with 0.02M $CaCl_2$ at pH 8.0 (Erlanger and others, 1961).

Hydrolyses of 1 mM BAPNA in TRIS buffer (a dilution from a 25 mg/mL BAPNA solution in DMSO was freshly prepared) with immobilized enzyme were carried out at 25 °C in a 0.05 L stirred, tank-type, batch reactor equipped with temperature control. Samples of 1 mL were collected and the reaction was stopped with 0.25 mL of acetic acid 30 % (v/v). The supernatant was once again centrifuged, in the case of zeolite and silica, and vacuum filtered in the case of spent grains. The rate of p-nitroaniline formation was determined by measuring absorvance of supernatant at 410 nm in an ELISA (Synergy HT, Bio-Tek, USA). The extinction coefficient used was 8.8 mL/μmol.cm^{-1} (Huckel and others, 1996) and the activity was calculated by:

$$Activity(U/mg_{protein}) = \frac{slope \times dilution\_factor \times V_{reaction}(mL)}{8.8 \times m_{protein}(mg)} \quad \text{Eq. 4-1}$$

or

$$Activity(U/g_{carrier}) = \frac{slope \times dilution\_factor \times V_{reaction}(mL)}{8.8 \times m_{carrier}(g)} \quad \text{Eq. 4-2}$$

Activity retention for the immobilized enzyme was determined by the ratio between the activity of the immobilized enzyme and the activity of a similar amount of the free enzyme.

4.2.4 Storage Stability and Reusability

Storage stability was determined incubating the immobilized enzyme in TRIS-buffer with 0.020 % (w/v) sodium azide at 4 °C for 60 days. The remaining enzyme activity was determined with BAPNA as above and compared with the initial activity.

The reusability (or operational stability) of immobilized trypsin was studied by measuring the residual activity after four operational cycles and comparing it with the initial activity. Each time, immobilized trypsin was washed and centrifuged/filtered four times with TRIS buffer.

4.3 Results and discussion

4.3.1 Silica

Immobilization efficiency (expressed as the percentage of immobilized mass protein referred to the initial mass protein in solution) in carriers with chemical modification of the surface was better than with simple adsorption, with efficiencies above 40 % (Table 4-2) within the range of those referred in literature

(Table 4-1). As chemical bonds are stronger these results were obviously expected. Operational and storage activity loss of the immobilized trypsin through simple adsorption were also very high (73 and 63 %, respectively) for the same reason. The use of borohydride to reduce and stabilize the bonds between the protein and the carrier led to a 50 % decrease of activity retention (or recovered activity) in comparison with the same procedure with no reduction with borohydride (also visible on Table 4-3), though better behaviour through storage is expected (not tested). This deleterious effect could probably be reduced if the borohydryde was used at pH 10 (Blanco and Guisan, 1989).

The use of a higher amount of glutaraldehyde (2.5 % instead of 1 %) did not significantly influence the immobilization results. The use of a smaller ionic strength gave better immobilized protein values (76 % with a 20 mM buffer against 48 % with a 50 mM buffer), but the activity retention of the carrier was slightly lower. Even so, the resulting carrier had a slightly higher overall activity. However, the operational activity loss was high (45 %) maybe indicating a higher non-specific weak adsorption, being the original carrier (with 50mM buffer) still better. Adsorption of the protein into the carrier followed by intermolecular crosslinking with the bi-functional reagent glutaraldehyde, creating an enzyme "envelope" around each particle (Haynes and Walsh, 1969), was the best alternative with an activity retention of 23 %, corresponding to a carrier with an activity of 13.3 % U/g.

The use of a higher concentration (the same amount in a smaller volume) of enzyme and carrier, with the same ratio between the two (1:2) led to an increase in the amount of immobilized protein (48.2 to 60.3 %, within the experimental error) but with a much lower activity retention, probably due to hydrophobic or electrostatic repulsions possibly caused by the "proximity" of the particles (Table 4-2 and Table 4-3).

Immobilization at pH 7 significantly improved both the amount of immobilized protein and the retention of activity (Table 4-3) even though immobilization at pH 8 was done in the presence of $CaCl_2$, which should have improved the specific activity by reducing autodigestion of trypsin (e.g. Huckel and others, 1996) and without $CaCl_2$ at pH 7 (because there was precipitation of the calcium ion in pH 7 phosphate buffer). Probably, as the immobilization was done at low temperatures, the enzyme was poorly active and almost no autolysis occurred. On the other hand, a decrease of 50 % in the recovered activity was observed when immobilization was performed at pH 6, although immobilized protein was slightly higher. The lower immobilization rates at higher pH values could be associated with the deactivation of the

reactive glutaralgehyde groups of the support under these conditions, although some authors describe that this only occurs at pH 9 and higher (Mateo and others, 2005).

Activation during 2 hours instead of 15 min resulted in similar protein immobilization and activity retention (within experimental error), but storage stability was greatly improved (Table 4-3).

The amount of immobilized protein achieved with activation with glutaraldehyde performed at pH 8 was lower, as expected, because glutaraldehyde works better at pH near neutrality, and although activity retention was similar to that obtained at pH 7, storage stability was worse (24 % of activity loss against 2 % when activation is done at pH 7), maybe indicating that some protein was physically adsorbed and not covalently bond.

Table 4-2: Immobilization results of 100 mg of trypsin on 200 mg of silica in 5 mL of pH8 TRIS/HCl 0.05 M buffer with 0.02 M of $CaCl_2$; in the cases of trypsin covalently bond to silanized silica, the carrier was previously activated with a 1 % glutaraldehyde solution in 0.05 M pH 7 phosphate buffer, except otherwise stated

Immobilization strategy	Immobilized protein (%)	Immobilized protein (mg/g carrier)	Activity (U/g carrier)	Activity retention (%)	Operational activity loss * (%)
Adsorption at pH 8	22.4±22.8	0.111	0.654	10.6	73.3±2.7
Adsorption and crosslinking with glutaraldehyde pH8	55.6±5.6	0.277	13.3±4.1	23.0	7.5±2.4
Covalently attached w/ glutaraldehyde	48.2±6.5	0.242	4.39±1.69	11.2	28.8±6.7
Covalently attached with glutaraldehyde and reduction with bh	46.3±13.7	0.218	2.78±0.15	5.03	28.4±4.9
Covalently attached w/ 2.5 % glutaraldehyde	40.5±21.3	0.200	4.26±1.39	10.6	18.3±13.6
Covalently attached w/ glutaraldehyde in a 20mM TRIS/HCl buffer	76.2±15.6	0.386	6.45±0.33	7.72	44.6±12.8

*Ratio between the enzyme activity after four cycles and the initial enzyme activity

In short, good operating conditions for the immobilization of trypsin on silanized silica activated with glutaraldehyde seem to be: activation at pH 7 with 1 % glutaraldehyde, immobilization at pH 7 in a 50 mM buffer. Stabilization of protein carrier binding with borohydride does not appear to be necessary if the enzyme is going to be used only during 60 days, since storage activity loss after 60 days was only 2-5.5 % (when the activation was made at pH 7). Longer stability tests or more reactive storage media would probably result in a worse performance of the unreduced carriers.

Table 4-3: Immobilization results of 25 mg of trypsin on 50 mg of activated silanized silica in 2 mL of buffer; except stated otherwise, activation was done for 2 h with 1 % glutaraldehyde solution in 0.05 M pH 7 phosphate buffer

Immobilization strategy	Immobilized protein (%)	Immobilized protein (mg/g carrier)	Activity (U/g carrier)	Activity retention (%)	Storage activity loss (%)
Activation during 15 min; immobilization at pH 8	60.3±8.7	0.267	1.31±0.02	2.04	27.3±6.2
Immobilization at pH 8	64.4±3.2	0.315	1.22±0.08	1.60	2.0±1.4
Activation at pH 8; immobilization at pH 8	57.4±2.9	0.275	1.19±0.03	1.72	24.2±15.3
Immobilization at pH 7	73.8±2.3	0.371	2.58±0.57	2.47	5.5±2.0
Immobilization at pH 7 and reduction with bh	74.0±4.7	0.381	0.931±0.186	1.03	-
Immobilization in pH 6 phosphate buffer; reduction with bh	83.1±6.4	0.401	0.488±0.10	0.51	-

4.3.2 POS-PVA

In order to determine the optimum amount of protein to be immobilized, several experiments with the same amount of carrier and buffer were performed with initial protein concentrations ranging from zero to 60 mg/mL. The results are presented in Figure 4-1.

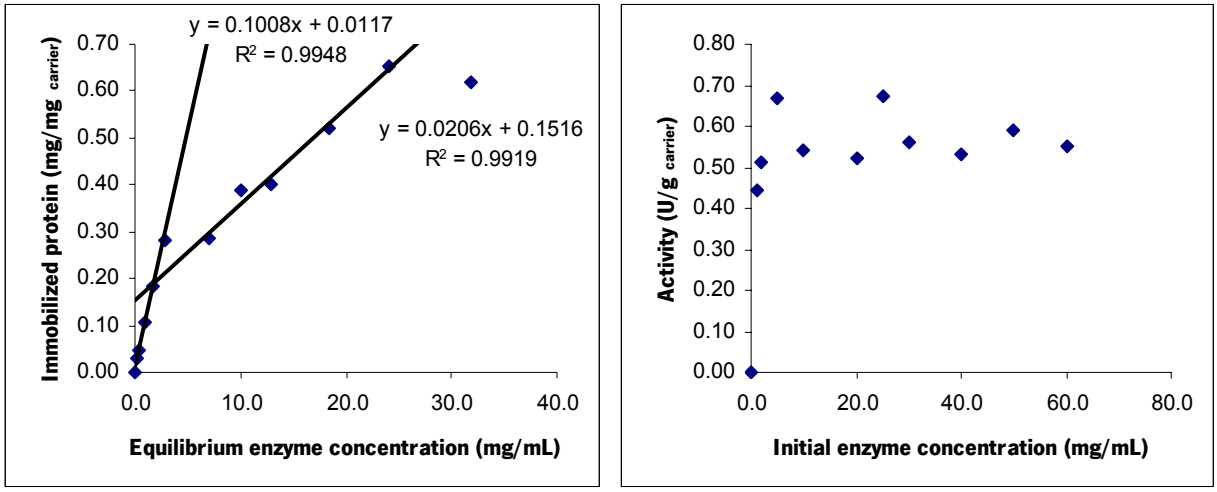

Figure 4-1 Influence of the enzyme concentration on the amount of immobilized protein (experiments with 40 mg of POS-PVA, glutaraldehyde 1 %, pH 7 and borohydride at the end)

The equilibrium binding curve is steeper for low enzyme equilibrium concentration (Figure 4-1a) and a big change in the slope occurs around 2.8 mg/mL (corresponding to an initial enzyme concentration of 15 mg/mL). The first slope possibly corresponds to the formation of a monolayer of enzyme, while the second slope corresponds to the binding of enzymes on top of the monolayer. In the first part of the curve, reaction rate of the enzyme with the functional groups of the support might be determinant, and in the second part, the adsorption rate becomes lower and mass transfer rate becomes important. The corresponding carrier activity shows that after a certain concentration (5-10 mg/mL), that roughly corresponds to the equilibrium enzyme concentration at which the slope changes, no increase in activity is observed, so there is no point on using higher initial enzyme concentrations (for 40 mg of POS-PVA and 1.5 mL of buffer). The activity of covalently immobilized trypsin was almost constant after "saturation" which may also indicate that the enzyme activity is dependent on substrate diffusion.

More protein was immobilized on carriers with chemical modification of the surface or when the enzyme was cross-linked with glutaraldehyde, as expected (Figure 4-2).

Activity retention when the enzyme was adsorbed to the carrier was higher (19 %) than when enzyme was covalently bond with glutaraldehyde (14 %; Table 4-4). Even so, no significant loss was observed when urea was used to wash the carrier after immobilization, indicating that the enzyme was "well" bond (stabilized) to the carrier (maybe through stronger binding than simple adsorption). The use of urea also did not reduce the activity on the other tested POS-PVA carriers, although it led to a major decrease on

the operational activity loss, indicating that enzyme not strongly adsorbed to the carrier had been removed.

A more active and stable immobilized trypsin (Table 4-4 and Figure 4-3) was accomplished with the use of benzamidine during the immobilization procedure at pH 7, although the activity loss was slightly higher (9.3 against 11.6 %).

Glutaraldehyde reactivity at pH 10 is low and therefore the amount of immobilized protein was low when the enzyme was immobilized at pH 10 (Figure 4-2). In spite of that, activity retention was higher (36 %) as the immobilization occurred close to the isoelectric point of trypsin, where electrostatic repulsions are minima, and the resulting immobilized enzyme was more active (4.4 U/g carrier).

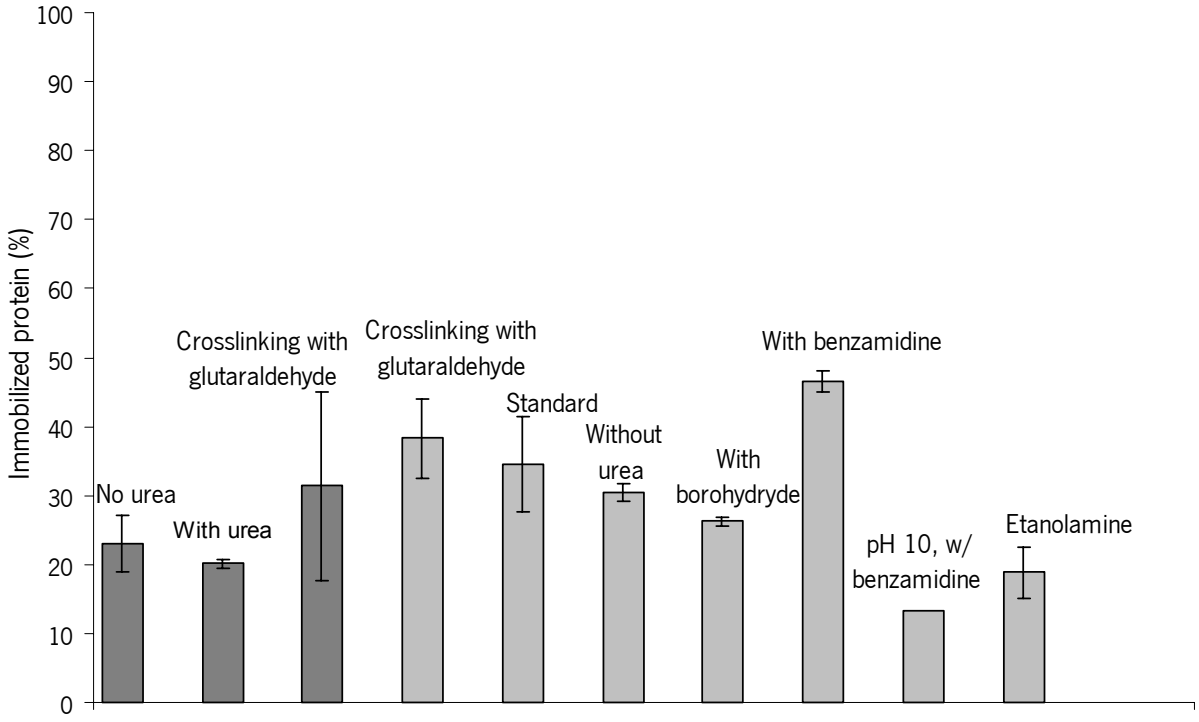

Figure 4-2 Immobilization efficiency: ■ - assays with covalent attachment with glutaraldehyde; ■ – adsorption at pH 7 (assays with covalent attachment with glutaraldehyde or/and crosslinking were made with urea and without borohydride unless otherwise stated)

The use of etanolamine to block the unreacted carrier points did not lead to a better performance of POS-PVA-trypsin.

Once again, immobilized enzyme activity highly decreased when borohydride was used after the immobilization to reduce unstable Schiff base bonds (Table 4-4) without a considerable improvement on enzyme stability (Figure 4-3), even though the pH was rised to 10 before the adition of borohydryde. Thus, no significant difference between borohydride at pH 8 and borohydride at pH 10 seems to be achieved (although the comparison is made on different carriers), probably because borohydride solutions were used immediately after preparation and there was enough time for enzyme binding reduction before significant hydrolysis.

Once more, the better performance was achieved with adsorbing the enzyme or covalently binding it to the carrier and subsequently crosslinking it with glutaraldehyde. Although the amount of immobilized protein was not the finest, retained activity (29-33 %) and stability were very good, leading to a highly active carrier (7.5 – 9.4 U/g).

Table 4-4: Activity retention

Immobilization method	Activity (U/g carrier)	Specific activity (U/mg protein)	Activity retention (%)
Adsorption at pH 7	3.11±0.02	0.0433	18.8
Adsorption at pH 7 with urea	3.76±0.01	0.0480	20.8
Adsortion at pH 7 and crosslinking w/ glutaraldehyde w/ urea without bh	7.46±0.62	0.0754	32.8
Covalently attached w/ glutaraldehyde and crosslinking w/ glutaraldehyde w/ urea without bh	9.38±1.63	0.0674	29.3
Covalently attached w/ glutaraldehyde; w/ urea without bh	2.99±0.45	0.0267	11.6
Covalently attached w/ glutaraldehyde without urea without bh	3.27±0.27	0.0315	13.7
Covalently attached w/ glutaraldehyde with urea with bh	0.90±0.02	0.0099	4.3
Covalently attached w/ glutaraldehyde w/ benzamidine with urea without bh	3.77±0.27	0.0213	9.3
Covalently attached w/ glutaraldehyde w/ benzamidine at pH 10 with urea without bh	4.41±	0.0821	35.7
Covalently attached w/ glutaraldehyde w/ etanolamine without bh	0.946±0.08	0.0147	6.4

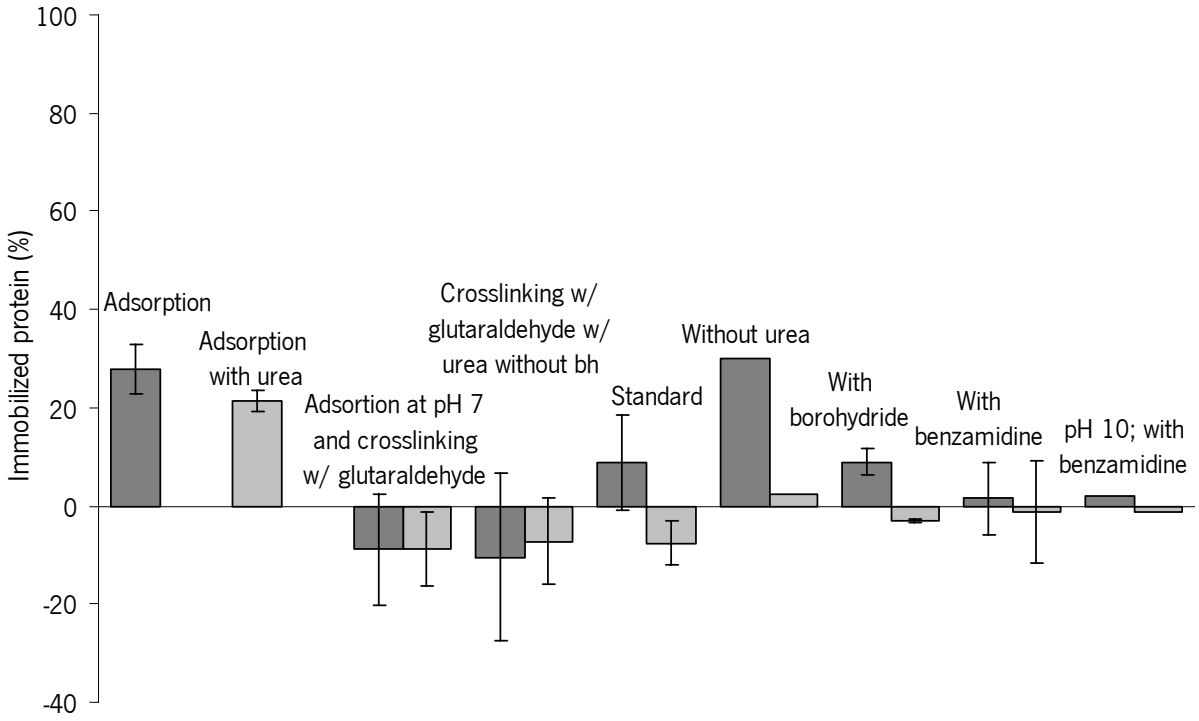

Figure 4-3 Operational stability (■) after four cycles and storage stability (■) after 60 days in TRIS/HCL buffer at 4 °C (assays with covalent attatchment with glutaraldehyde with urea and without borohydride - standard - unless otherwise stated)

4.3.3 Spent grain

Immobilization efficiency in carriers with chemical modification of the surface was better than with simple adsorption, with efficiencies around 60 % (Figure 4-4).

The best results concerning protein retention were obtained with glyoxyl and glyoxylEDA (amine) spent grain (almost 70 % in the former case and around 60% in the latter). The driving force for the immobilization with glyoxyl-carriers is the density of lysines in the enzyme (the reactive group). As the pKa of lysine is around 10, immobilization always takes place at alkaline pH values. Trypsin may have rich lysine areas which could explain why this support has been successfully used. As the isoelectric point of trypsin is 10.5 (Diaz and Balkus, 1996), electrostatic repulsions are probably lower making binding more efficient.

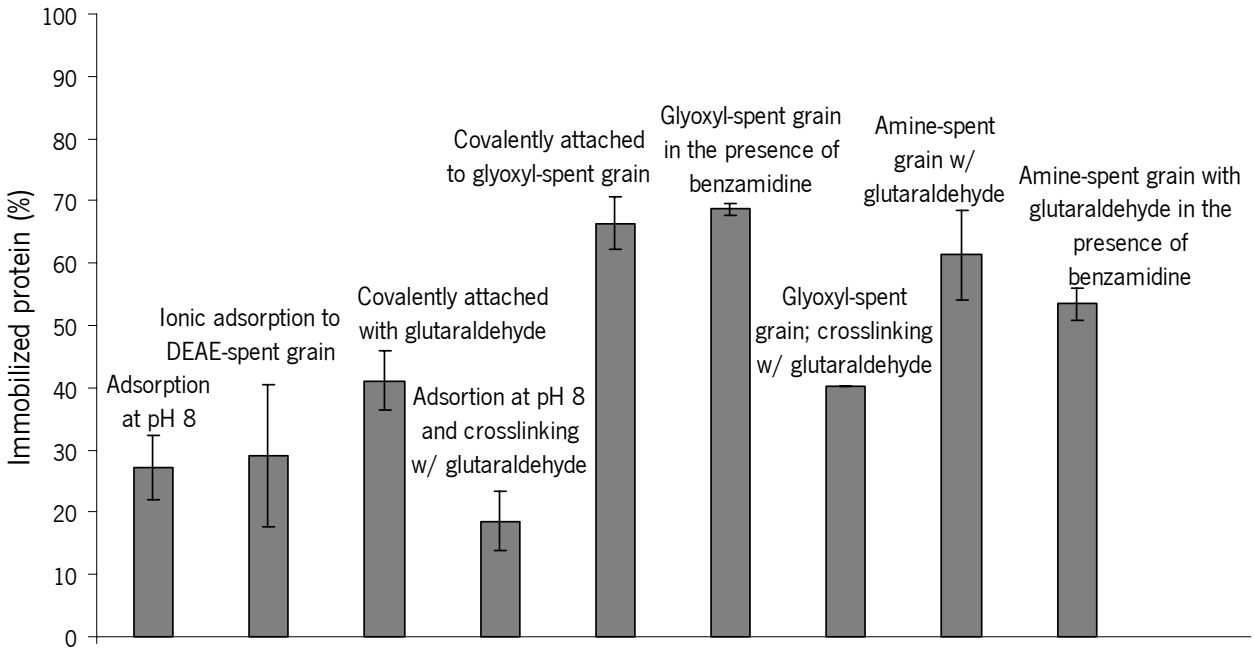

Figure 4-4 Immobilization efficiency

In the case of DEAE-spent grains no improvements from simple adsorption were observed. Higher pH should be tested, as the isoelectric point of the enzyme is 10.5 and DEAE-cellulose is an anionic exchanger (positively charged), though reversible bonds are involved and the optimum working pH of the free enzyme is 8. Transforming spent grains in a cationic exchanger (as a carboxymethyl-derivative) is probably a better alternative.

Although the amount of immobilized protein was higher when chemical bonds were involved, the specific activity was lower, indicating enzyme inactivation (Table 4-5). The necessary conditions to the covalent attachment of an enzyme to a carrier are such that some loss of activity is inevitable. Besides, the active sites may not be as accessible to the substrate by partial obstruction or their conformation may be altered.

Even so, spent grain with glutaraldehyde showed a much higher enzyme inactivation during immobilization than the others (only 3 % of the initial activity was retained). Probably there were not many points of attachment to the support and the enzyme was not stabilized. On the other hand, the enzyme might be too close to the carrier and could have been denatured by hydrophobic interactions. The use of a spacer arm might be beneficial. It should be noted that protein retentions above 95 % were achieved with glyoxyl- and glyoxylEDA-carriers (presumably by multipoint attachment) using a slightly

lower ratio quantity of trypsin/quantity of carrier (1:3) suggesting that the carrier's surface might be saturated and the enzyme might be adsorbing in multilayers (Figure 4-5). It has been reported immobilized trypsin on glyoxyl supports with seven lysine residues per trypsin molecule that have reacted with the activated support (Blanco and others, 1989).

Table 4-5: Activity retention

Immobilization method	Activity (U/g carrier)	Specific activity (U/mg protein)	Activity retention (%)
Adsorption at pH 8	5.01±0.56	0.0428	15,2
Ionic adsorption to DEAE-spent grain	-	-	-
Covalently attached w/ glutaraldehyde	1.07±0.36	0.0064	3.06
Adsorption at pH 8 and crosslinking with glutaraldehyde	1.14±0.004	0.013	6.08
Covalently attached to glyoxyl-spent grain	9.07±4.14	0.026	11.5
Covalently attached to glyoxyl-spent grain in the presence of benzamidine	8.25±0.96	0.024	10.5
Covalently attached to glyoxyl-spent grain and crosslinking with glutaraldehyde	2.15±0.19	0.0152	6.59
Covalently attached to glyoxylEDA-spent grain with glutaraldehyde	5.60±0.05	0.0196	8.51
Covalently attached to glyoxylEDA-spent grain with glutaraldehyde in the presence of benzamidine	5.06±0.45	0.0202	8.80

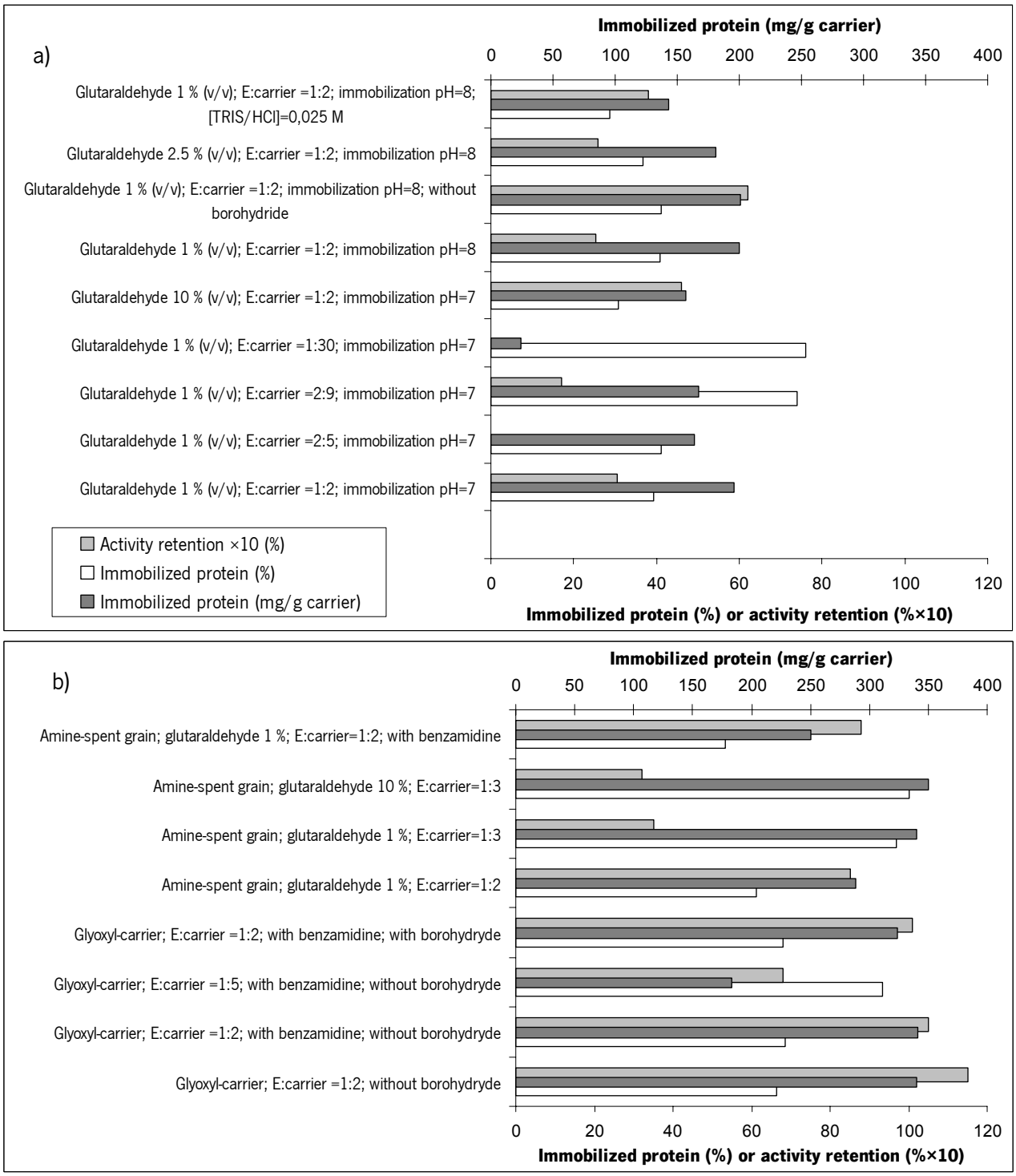

Figure 4-5 Influence of operational conditions on the immobilized protein and retained activity (a) Spent grain with glutaraldehyde; b) Glyoxyl and amine spent grain; ■ – activity retention; □ immobilized potein (%); ■ immobilized protein (mg/g carrier)

In order to be economically interesting, this kind of systems must be re-usable. Thus, operational stability was tested (Figure 4-6). The operational activity loss is high (above 50%) when only (weak) physical bonds are involved, probably due to enzyme leaching during washings. Operational stability of

immobilized enzyme with glutaraldehyde is much higher and with glyoxyl and EDA-glyoxyl it is close to 100 %.

Carriers with glyoxyl are also able stable during storage and they retained all activity during 60 days at 4 °C (Figure 4-6).

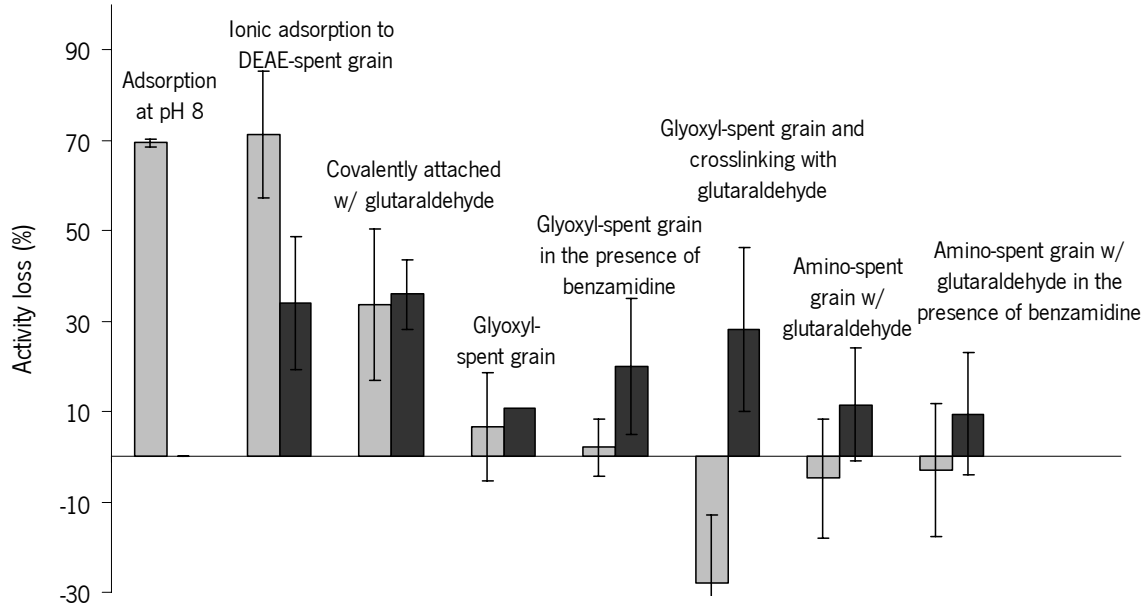

Figure 4-6 Operational stability after four cycles and storage stability after 60 days in TRIS/HCL buffer at 4 °C; ■ operational stability; ■ storage stability

It has been referred that the presence of an enzyme inhibitor during the immobilization process can reduce enzyme inactivation. Benzamidine, a competitive inhibitor of trypsin, has been used during trypsin immobilization and it strongly adsorbs on the active site of the enzyme, greatly reducing the inactivation of trypsin (Arasaratnam and others, 2000, e.g.). On the other hand, derivatives prepared in the presence of benzamidine are more active but less stable than derivatives prepared in the absence of the inhibitor (Blanco and Guisan, 1988). However, in this work, no significant differences were perceptible with spent grain carriers.

In the case of glyoxyl spent grains the reduction of activity due to the use of borohydryde was almost null (Figure 4-5b). The use of pH 10 may be responsible for this. Another possible reason can be that, as the enzyme is attached to spent grain through several points, it is more strongly stabilized against denaturating agents such as borohydryde. Even so, the reduction of Schiff bases when multipoint

attachment is involved is not so important as in monopoint attachment, as if one bond is reversibly destroyed the others can still hold the enzyme. When glutaraldehyde is used, instead of glycydol, the use of borohydryde is again very harmful to the immobilized enzyme activity (Figure 4-5a), as with silica and POS-PVA.

4.3.4 Zeolite

Immobilization efficiencies achieved using zeolites NaA, NaX and NaY are presented in Figure 4-7.

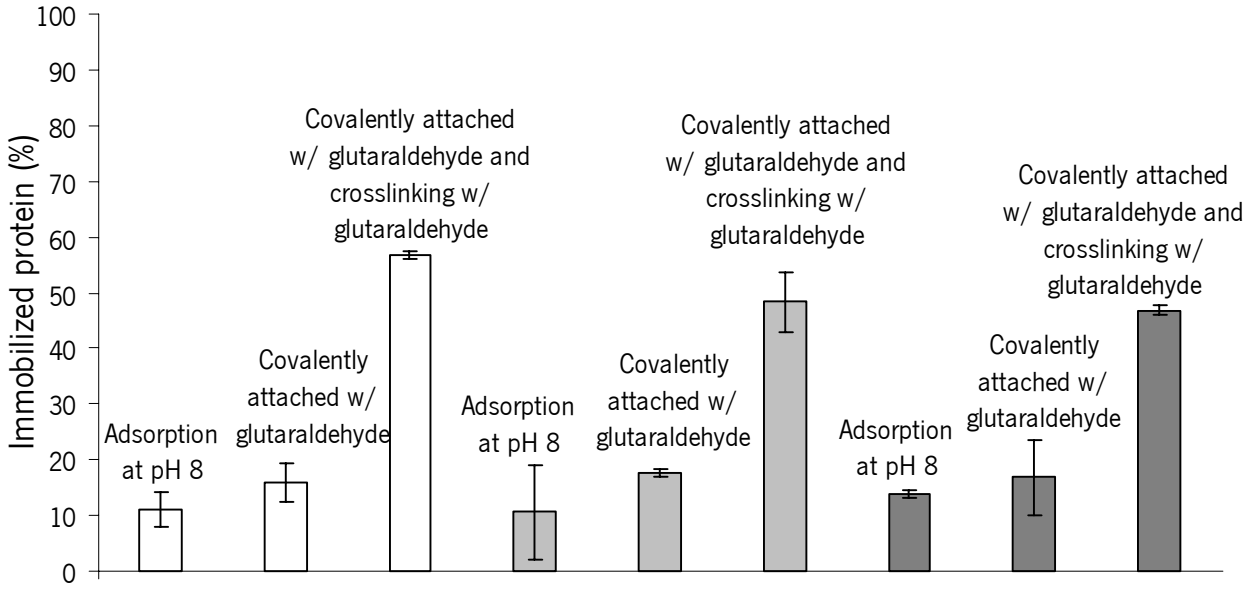

Figure 4-7 Immobilization efficiency (white – zeolite NaA; light grey – zeolite NaX; dark grey – zeolite NaY)

The interaction between enzyme molecules and zeolite is complicated. Apart from the electrostatic action between the charged groups on the surface of the enzyme and the cations from the framework of molecular sieves as well as the negatively charged oxygen atoms in the zeolite framework, there are some hydrogen bonds between the H-bond acceptors on the enzyme and the hydroxyl groups on the zeolite. The cations and -OH groups in the zeolite decrease with increasing the Si/Al ratio (Xing and others, 2000). Thus, there are slightly more cations and hydroxyl groups that can form stronger hydrogen bonds with trypsin molecules in NaY than in NaX or NaA zeolites. Therefore, immobilized enzyme in NaY zeolite should have a slightly higher activity and stability than the other two (which can be seen on Figure 4.8, although the difference is within the experimental error). Zeolites with a high aluminium content are more acidic than zeolites with a lower aluminium content (Tavolaro and others,

2007). All the zeolites used are very acidic, but zeolite Y is the least acidic. It has also been reported that usually, the amount of enzyme adsorbed is larger on hydrophobic than on less hydrophobic supports. The adsorption on hydrophobic supports also leads to larger conformational changes on the enzyme molecule; however, higher enzymatic activities are usually obtained under these conditions. As Si/Al ratio is slightly higher for zeolite NaY, it presents a lower framework charge and higher hydrophobic character than the other two zeolites.

On the other hand, at pH values near neutrality, trypsin is positively charged. Although electrostatic repulsions between carrier and protein should be low as they are oppositely charged, electrostatic repulsions between trypsin molecules are very high, as they are very protonated. In fact, several authors have reported that adsorbed protein is maximum when working near the isoelectric point (Gonçalves and others, 1997; Yiu and Wright, 2005, Tavolaro and others, 2007), where electric repulsions between protein molecules are minima. Working at pH values near 10.5 (to neutral protein) to minimize repulsions or slightly below 10.5 to reduce electrostatic repulsions between protein molecules but allow ionic interaction between carrier and protein would probably allow better immobilization efficiencies.

Increasing immobilization temperature would probably lead to an increase in the amount of immobilized protein by increasing the adsorption rate (Tavolaro and others, 2007) but probably the loss in activity would be higher as the autolysis of the enzyme would be enhanced.

The amount of immobilized protein achieved by covalently immobilizing trypsin to silanized Na zeolites, was slightly higher than for adsorption, indicating stronger bonds, as expected. Even so, only about 20 % of the total enzyme was immobilized, which may be due to a relatively low density in hydroxyl groups (Tsai and others, 2006) besides the already referred highly charged trypsin. There are more hydroxyl groups available for silazination and further covalent binding with glutaraldehyde in NaY than in NaX or NaA zeolites, as its Si/Al ratio is almost twice as that of the other two zeolites. Therefore, immobilized enzyme in NaY zeolite showed higher activity and stability than the other two.

Covalent binding with glutaraldehyde, followed by crosslinking also with glutaraldehyde led to very good enzyme activity retention (48 % for NaA, and 67 % for NaX and NaY) and very stable immobilized enzymes (both operational and storage activity losses were close to zero, in all zeolite carriers). The relative amount of immobilized protein was around 50 %. Although the adequate amount of enzyme per gram of support was tested, it was not fully optimized and a smaller quantity of enzyme might lead to

even better results. The use of a lower ionic strength or higher immobilization pH could also help, but its effect on the enzyme activity would have to be considered.

It is interesting to notice that almost no activity was observed when carriers were dehydrated (overnight, at 105 °C; results not shown) prior to use, although trypsin is negatively charged and the cationic exchanger character is higher when the carrier is dried. Complete loss of enzyme activity was also observed by Carvalho and others, 2007 when immobilizing horseradish peroxidase on NaY zeolite (by adsorption, covalent binding and crosslinking), but the pre-treatments of the zeolite were not referred. Some residual activity was found but it was attributed to enzyme leaching from the support. It seems clear, in any case, that the contact with zeolite induced a change in the functional groups and/or conformation of the enzyme. On the other hand, highly stable tyrosinase was obtained when glutaraldehyde was adsorbed to NaY zeolite and then used for crosslinking the enzyme (Seetharam and Saville, 2002). Hydrophilic supports can bind water and so compete with the enzyme for the available water; however, when the enzyme and the support are fully hydrated, the hydrophilic supports also lead to a higher water concentration in the microenvironment of the enzyme (Gonçalves and others, 1997) and thus allowing proteolytic activity.

Table 4-6: Activity retention

Carrier	Adsorption		Covalent binding with glutaraldehyde		Covalent binding with glutaraldehyde and crosslinking	
	Activity (U/g carrier)	Activity retention (%)	Activity (U/g carrier)	Activity retention (%)	Activity (U/g carrier)	Activity retention (%)
Zeolite A	-	-	0.297±0.037	1.8	29.9±1.2	47.6
Zeolite X	0.231±0.053	1.9	0.314±0.041	1.9	35.9±1.3	67.2
Zeolite Y	0.282±0.036	1.8	0.679±0.216	5.3	35.6±1.5	66.8

It might be important to test if the substrates or products of reaction are not being adsorbed to the carrier. Apparently in the case of covalent binding with glutaraldehyde and crosslinking this may not happen in a significant extent once trypsin is highly adsorbed to the carrier thus leaving negligible available surface for substrate or product adsorption or reaction. This hypothesis is reinforced by the fact that, being the activity detected spectrophotometrically by measuring the rate of formation of a product, such product is effectively detected (proving that the reaction took place and that the products are effectively formed and released).

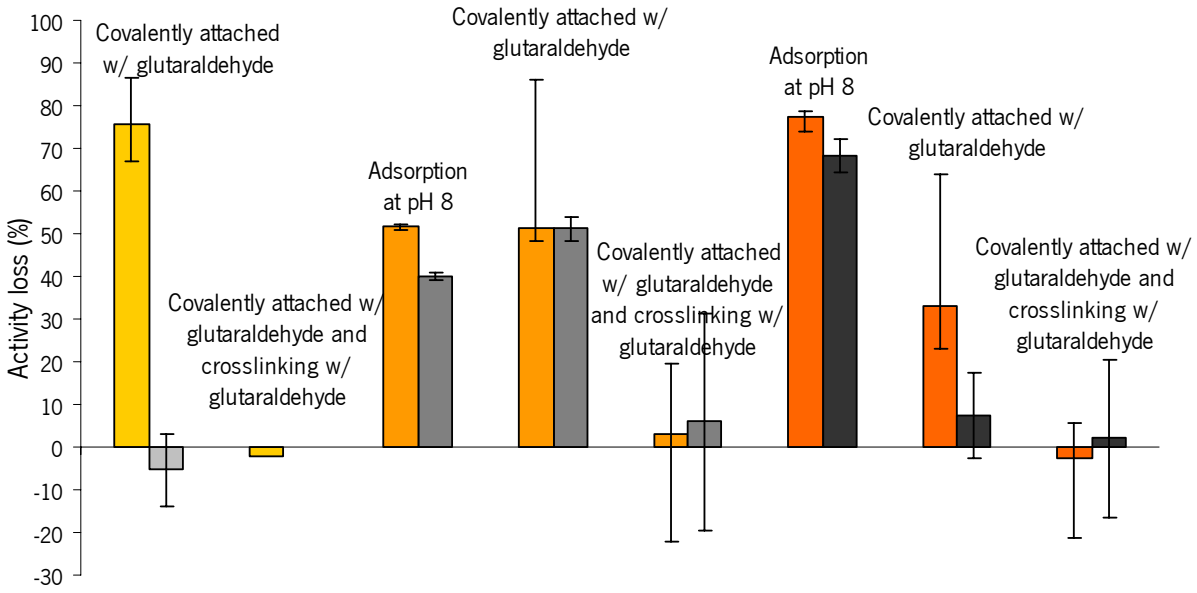

Figure 4-8 Operational stability after four cycles and storage stability after 60 days in TRIS/HCL buffer at 4 °C; orange scale – operational activity loss and grey scale storage activity loss; light colour – zeolite A; medium colour – zeolite X; dark colour – zeolite Y

4.3.5 General discussion

The results achieved are in accordance with those found in literature, although the factors influencing the immobilization process are so many that the referred results have a huge range of values depending on the used conditions. For instance, Sears and Clark (1993) achieved 38 mg of immobilized protein per gram of carrier (porous size controlled glass), corresponding to 95 % of protein. However, only 4 mg of enzyme per gram of carrier were active. Huckel and others (1996) obtained 16 mg g^{-1} carrier in porous silica, corresponding to the immobilization of 29 % of total protein. Kumar and Gupta (1998) were able to immobilize 75 to 86 % of protein by chemical bond to Eudragit S-100 and 30 % by physical adsorption to the same polymer. Some more results are presented in Table 4-1. With respect to stability, oxirane-acrylic beads (Eupergit C) loss in proteolytic activity was of approximately 25 % after nine cycles of repeated use (Lorenzen and Schlimme, 1995). Bryjak and Kolarz (1998) found that acrylic polymers retained activity after 30 days in borate buffer with 20 mM $CaCl_2$ (pH 7.0, 4°C) between 12.8 and 76.6 %. Nouaimi and others (2001) immobilized trypsin on N-hydroxysuccinimide activated polyester fleece via different spacers (PEG-diamine, aldehyde dextran, amino dextran and bovine serum albumine - BSA), to try to minimize the poor long term stability of the enzyme due to direct immobilization of enzyme to a

polyester fleece, which may be ascribed to the relatively low surface polarity of the polymer. In this case, spacer molecules may be used to shield the enzyme from the polymer surface rather than to convey flexibility onto the enzyme molecule. Trypsin activity with BSA spacer was 0.15 mmol/min/cm^2, five times the trypsin activity of the directly immobilized enzyme.

Thus, the use of a spacer arm such as BSA could have improved the results in this work. However, this is not always the case. Sometimes direct immobilization yields a preparation with higher specific activity retention. Probably, steric hindrance impaired the immobilized enzyme via BSA due to protein overloading since this derivative fixed higher amount of trypsin compared to the direct procedure (Cavalcante and others, 2006).

Free enzyme stability tests during forty days lead to a 40 % decrease in the activity when the enzyme was stored in 0.05 M TRIS/HCl buffer at pH 8 and in the presence of $CaCl_2$ (so with reduced autolysis), and to a 90 % decrease when the enzyme was stored in TRIS/HCl buffer at pH 8 or phosphate buffer at pH 7. As the storage stability and operational stability losses are most of the times significantly less than 40 % (Table 4-2, Table 4-3, Figure 4-3, Figure 4-6 and Figure 4-8) and sometimes even close to zero (crosslinked trypsin on zeolite, e.g.), the enzyme was effectively immobilized and stabilized and this losses cannot be attributed only to the presence of $CaCl_2$ that inhibites the autodigestion of trypsin. The retention of activity after urea 6 M was used to wash the immobilized protein also leads to the same conclusion.

The activity loss was high (above 50 %) when the enzyme was immobilized by adsorption. In spite of only (weak) physical bonds being involved, probably considerable enzyme leaching took place during washings and operation resulting in high losses.

Operational stability of immobilized enzyme with glutaraldehyde is high for silica, POS-PVA and spent grains but not for zeolites. However, if we analyze only the loss of activity from the 2nd to the 3rd cycle (results not shown), that loss is technically null (except for zeolite A). This may indicate that there was still some enzyme weakly bonded to the support (physically adsorbed) that leached during washings between the first two cycles.

Sometimes, particularly with spent grain and POS-PVA (Figure 4-3 and Figure 4-6) the retained activity of the carrier increased with repeated reaction cycles or with storage. Although the immobilized enzymes were washed with urea once and with immobilization buffer four times, this may indicate that some

noncovalent coupled proteins were still present and did not allow the binding of the substrate to the inner trypsin molecules. On the other hand, as some outer molecules may be physically adsorbed they are more easily inactivated due to conformational changes and denaturation, and their activity may become lower than the activity of the inner molecules. This fact has already been described, for instance, with Eudragittrypsin and emphasizes the importance of the removal of noncovalently bound proteins after covalent coupling and the use of covalent coupling of a ligand when the conjugate is aimed to be used repeatedly (Arasaratnam and others, 2000).

Although the amount of immobilized protein was high in many cases, particularly when covalent binding or crosslinking were involved the specific activity was generally very low, indicating strong enzyme inactivation. The necessary conditions for covalent attachment of an enzyme to a carrier are such that some loss of activity is inevitable. Besides, the active sites may not be as accessible to the substrate by partial obstruction or their conformation may be altered. Also some autolysis is likely to occur, especially in the cases where immobilization was done at pH 8 (the optimum pH for the enzyme). Even so, trypsin immobilized on spent grain with glutaraldehyde showed an activity (U/g carrier) four times higher than trypsin immobilized on silanized zeolite NaY with glutaraldehyde (Table 4-5 and Table 4-6). Although the structure of the used zeolites are some of the most open of all the zeolites, the pore size is still too small (< 20 Å) and the inclusion of trypsin (38 Å) in the pores of the zeolite (microporous structure) is impossible (Diaz and Balkus, 1996). This means that the area available for immobilization is only the external surface area. Spent grains are plain sheet-like carriers with a high average particle area (ca. 0.5 mm^2). This may be the reason for the better activity results achieved with spent grains. On the other side, subtrate (BAPNA) molecules might be strongly adsorbed inside the pores of the zeolites (as already described by Yiu and others, 2001), and as the trypsin molecules are too big to enter these pores, the BAPNA molecules are effectively separated from the enzyme and a low activity is observed.

Silica with glutaraldehyde presented higher activity than spent grains with glutaraldehyde (4.4 against 1.1 U/g carrier; Table 4-2 and Table 4-5). The used silica pore diameter is higher (375 Å) than for zeolites and ten times bigger than the trypsin molecule allowing its immobilization inside silica pores. Thus, the available area for immobilization is high. This may be the reason for the better activity results achieved with silica. In the case of POS-PVA, the activity of the carrier with glutaraldehyde was around 3 U/g, slightly lower than for silica.

Photographs taken on a classical SEM microscope are shown in Figure 4-9, for details on supports structure.

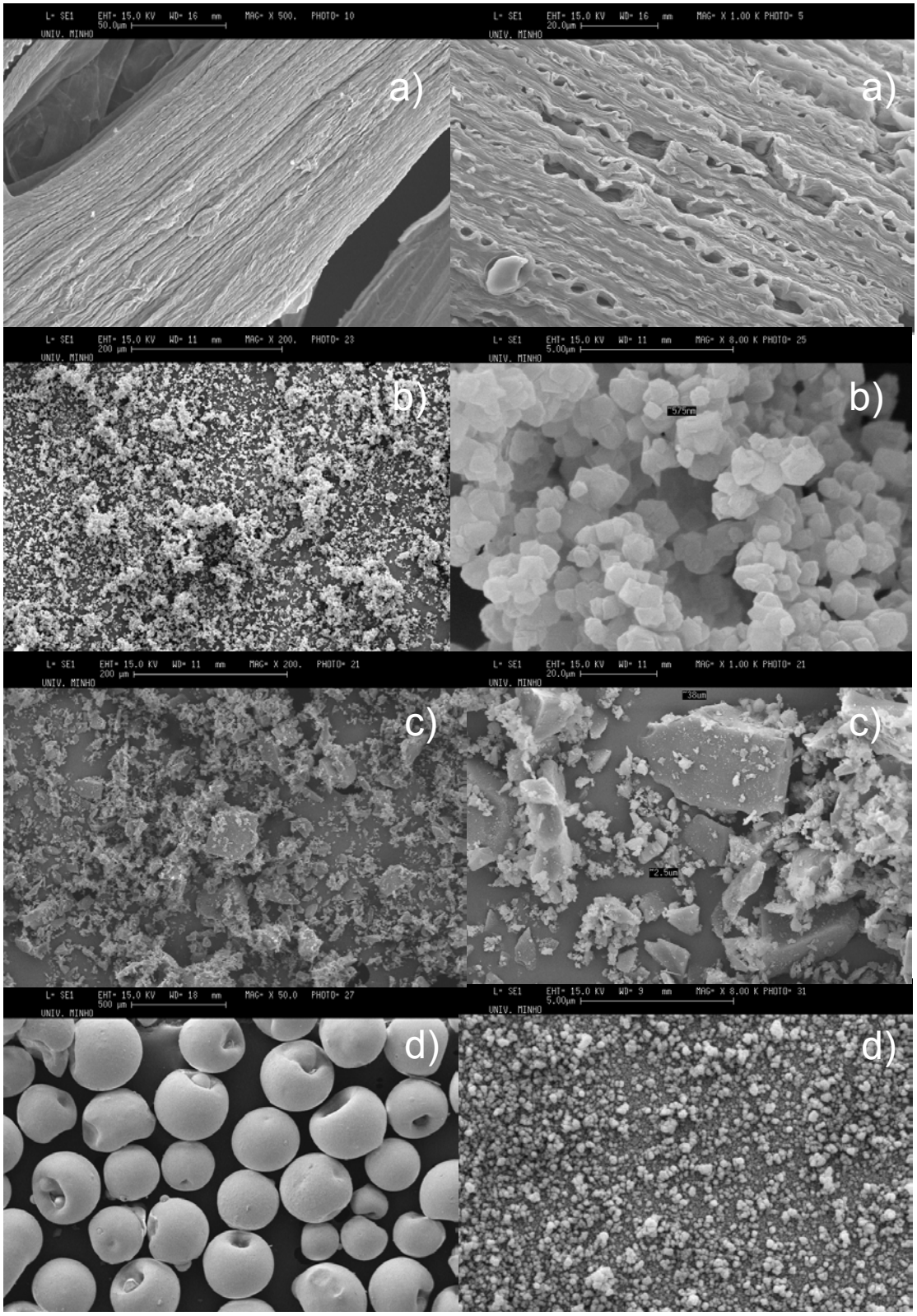

Figure 4-9 SEM photographs of the different supports: a) Spent grain; b) Zeolite Y; c) POS/PVA; d) silica

Immobilized trypsin adsorbed onto the carrier or covalently attached and further crosslinked with glutaraldehyde showed the highest activity in all carriers except spent grain (13.3, 9.4, 1.1 and 35.6 U/g for silica, POS-PVA, spent grain and NaY zeolite, respectively). For zeolite, this difference was much bigger, although the immobilized protein was almost the same (40-50 %). Thus the activity retention was in this case very high (67 %), unlike all the other carriers. Trypsin immobilized on glyoxyl spent grain had an activity of 9.1 U/g, of the same order of magnitude of the crosslinked trypsin on silica and POS-PVA.

Generally the recovered activity after the immobilization procedure was low. This might be owed to the use of an enzyme that was not very purified. In fact it has been mentioned in the literature (Goradia and others, 2005, e.g.) that when a crude enzyme is used the activity retention is much smaller than the activity retention achieved with a highly purified enzyme. Thus, a more purified enzyme (a bovine trypsin with a BAEE activity of 11000 BAEE U/g and chymotryptic activity $\leq$ 0.2 %) was used. Glyoxyl spent grains, POS-PVA activated at pH 7 with glutaraldehyde and zeolite NaY were tested with the new enzyme. Immobilization on glyoxyl spent grain was done at pH 10 in the presence of benzamidine, on POS-PVA it was done at pH 7 with borohydride at the end and on zeolite NaY trypsin was adsorbed at pH 7 and then crosslinked with glutaraldehyde 1 %. Each carrier was prepared 14 times and protein concentration in the "bulk" solution was always determined. Only three of these prepared carriers were tested for trypsin activity towards BAPNA.

Table 4-7: Immobilized protein and activity recovery achieved with the purified enzyme (a ratio of 1 mg of enzyme:6.5 mg of carrier was used for spent grain carrier and 1:7.5 for the other two)

Carrier	Immobilized protein (%)	Activity (U/g carrier)	Retention of activity (%)
Zeolite	70.0±4.1	52.6±1.9	63.5
POS-PVA	41.6±7.7	37.6±5.6	73.7
Glyoxyl spent grain	69.8±3.9	44.3±2.2	46.0

Although experimental conditions still have to be adjusted (namely the ratio mass of enzyme: mass of carrier, the volume of immobilization, among others), the immobilization activity retention was much better in this case (Table 4-7), namely for glyoxyl spent grain and POS-PVA (46 % and 73 % against 11 % and 9 % with the unpurified enzyme). In the case of zeolite, no improvement was observed, but the

activity retention was already high with the unpurified enzyme. This may indicate that trypsin has a higher affinity for zeolites than its contaminants, making them quite handy when an unpurified trypsin is being used. Another possibility is that the enzyme activity is improved not by immobilization but by interaction with the zeolite. Enzyme–zeolite interactions are gaining new potential in catalytic improvements and increased enzyme activity has recently been reported in the presence (without immobilization) of zeolite NaY (Carvalho and others, 2007).

4.4 Conclusion

Activity recovery was low with all carriers except for trypsin crosslinked on zeolites, where it was satisfactory. However, when a more purified enzyme from bovine pancreas was used with glyoxyl-spent grain or POS-PVA with glutaraldehyde, the activity retention was of 46 % and 73 % against 11 % and 9 % with crude enzyme. Thus it can be stated that trypsin was successfully immobilized on spent grains by multipoint covalent attachment using glycidol and on POS-PVA functionalized with glutaraldehyde. Even so, the immobilized trypsin with the highest activity was achieved with covalent binding through glutaraldehyde to silanized zeolite followed by crosslinking with glutaraldehyde, probably due to a positive effect of the zeolite on the enzyme activity.

4.5 References

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Chapter 5 Whey protein hydrolysis with immobilized trypsin

5.1 Introduction

The accurate control of the degree of hydrolysis and, thus, of the hydrolysates' properties is only possible if the hydrolysis can be stoped at the exact moment and with a non-aggressive method. For instance, when a free enzyme is utilized for catalysis, the use of heat to inactivate the enzyme results in the denaturation of protein molecules, causing the loss of tertiary and quaternary structures that are essential for protein functionality (Huang and others, 1999). If the enzyme is immobilized on a carrier which is easily removable from the reaction medium this can be almost instantaneously done without further interferences. However, differences in the hydrolysates may arise from the use of an immobilized enzyme instead of the free enzyme (Duggal and Buchholz, 1982; Lorenzen and Schlimme, 1995). In fact, immobilization can change e.g. the accessibility of the enzyme to the substrate or even the affinity of the enzyme towards a specific substrate.

Several authors have been using immobilized enzymes to hydrolyse proteins. Chen and others (1994) immobilized trypsin on succinamidopropyl controlled-pore glass beads and used it to hydrolyse β-Lg with the aim of improving its gelling properties. Huang and others (1994) used the same enzyme immobilized on the same carrier to hydrolyse β-Lg and Park and others (1998) used it to hydrolyse casein. Trypsin immobilized on succinamidopropyl Celite was used to hydrolyse WPI (Huang and others, 1999). Paramagnetic porous glass beads were used to hydrolyse fetuin with immobilized trypsin (Krogh and others, 1999), while Pedroche and others (2004) used agorose gel activated with glycidol to immobilize trypsin, α-chymotrypsin and carboxypeptidase A and to hydrolyse casein.

Although the immobilization of enzymes on solid carriers can offer several advantages over free enzymes, low mass transfer efficiency of the substrate, operational instability of the immobilized enzymes and, sometimes, high cost of the carrier have limited its application to the hydrolysis of proteins (Bai and others, 1999). Besides, a carrier that works well in the hydrolysis of a protein may have a poor performance in the hydrolysis of a different protein (Bai and others, 1999). For instance, Ge and others (1996) have successfully hydrolysed casein in a column reactor packed with endo- and exo-peptidases immobilized on sliced shrimp chitin hull (to improve mass transfer efficiency), but the hydrolysis of higher molecular weight proteins like soybean or egg white proteins was not possible. Mesoporous materials such as SBA15 can also efficiently immobilize trypsin but, in this case, only small substrates can be hydrolysed

(the enzymes can "select" the low size substrates from the medium, which can be useful in other applications); proteins such as ovalbumin or BSA are not hydrolysed (Yiu and others, 2001).

The aim of the work described in this chapter was to assess the influence of the immobilization process (optimized in Chapter 4) on the whey protein hydrolysis. The enzyme activity, kinetic parameters and the peptide profile of the hydrolysates from free and immobilize enzymes were analysed. Glyoxyl spent grains, zeolite NaY and POS-PVA were used as carriers.

5.2 Materials and methods

All chemicals used were of analytical grade and supplied by Sigma, Co (St. Louis, MO, USA). Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg (one BAEE unit will produce a ΔA253 of 0.001 per min at pH 7.6 at 25 °C using BAEE as substrate; in a reaction volume of 3.2 mL and 1 cm light path) and trypsin from bovine pancreas with an activity of 11000 BAEE units/mg (chymotrypsin ≤ 0.2%) were also obtained from Sigma Chemical, Co. Three carriers were tested: POS-PVA, a commercial zeolite (NaY) from Sigma (EUA) and spent grains (kindly supplied by UNICER S. A., Porto, Portugal).

Whey protein isolate (WPI) powder (Lacprodan DI-9212, batch R320215) was kindly supplied by Arla Foods Ingredients (Viby, Denmark). According to the suppliers, the WPI protein content was 91 % dry basis, the moisture was 5.5 % in maximum, the ash content was 3 % and the cation content was: sodium, < 0.1 %, phosphorus, 0.2 %, chloride, 2.2 %, potassium, < 0.1 % and calcium, < 0.1 %.

Solvents for HPLC were filtered through 0.22 μm filters and degassed under vacuum for at least 15 min before use.

Purified bovine standards of β-Lg and α-La were supplied by Sigma (St. Louis, MO, USA) and dissolved in ultra purified water.

5.2.1 Trypsin Immobilization

The supports were treated as described in section 4.2.1 and the immobilization was performed as described in 4.2.2. Trypsin was immobilized on three carriers: glyoxyl-spent grain at pH 10 in the presence of benzamidine, POS-PVA activated with 1 % glutaraldehyde, and zeolite NaY, with further crosslinking with glutaraldehyde. Urea was used in the three cases to wash the carrier with the immobilized trypsin.

5.2.2 Measurement of Trypsin Activity

Trypsin activity of immobilized and native enzyme preparations was measured as described in section 4.2.3.

5.2.3 Enzymatic hydrolysis of whey protein isolate

Hydrolysis of whey protein isolate was carried out as described in section 3.2.3 with free and immobilized trypsin at different initial substrate concentration, temperature and pH values. The degree of hydrolysis was monitored by the pH-stat method and confirmed periodically by the TNBS method. Samples were collected at variable intervals of time, inactivated by decreasing the pH, immediately frozen and stored at -20 °C until further analysis.

5.2.4 Peptide profile of hydrolysates

The peptide profile of the hydrolysates was analised by RP-HPLC as described in section 3.2.5. Some samples were also analysed by RP-HPLC/ESI-MS. In this case, diluted samples were injected in a reverse phase column C18 Purospher Star from VWR, Germany (5 μm, 150 × 4.6 mm^2 i.d.) installed on a liquid chromatograph (formed by a Surveyor LC Pump Plus, a LC Autosampler Plus and a Surveyor PDA plus detector, all from Finnigan, San Jose, USA). The elution flow rate was 0.5 mL min^{-1} with the following gradient of eluents (A: 0.1 % TFA in water; B: 0.1 % TFA in acetonitrile): 0 to 30 min, 100 to 50 % A; 30 to 35 min, 50 to 20 % A; 35 to 40 min, 20 %, followed by re-equilibration to the starting conditions. Monitoring was made at 220 nm at 30 °C.

The photo diode array detector's cell outlet was connected in series to the probe of the mass spectrometer. The mass spectrometer was a Finnigan LCQ Deca XP Max (Finnigan/Thermo Unicam, San Jose, USA), equipped with atmosphere pressure ionization (API) source, using ESI interface. The capillary voltage was 3 V and the capillary temperature was 190 °C. Spectra were recorded in positive ion mode for m/z values ranging between 250 and 3000. The mass spectrometer was programmed to perform a full mass scan.

5.3 Results and discussion

5.3.1 Degree of hydrolysis with immobilized enzymes and activity retention

As already referred in Chapter 3, the immobilization activity retentions of the enzyme a) covalently immobilized on glyoxyl-spent grains and b) on POS-PVA or c) crosslinked on zeolite NaY were all high. Activity with WPI was determined as the number of peptide bonds broken per min during the first three minutes of hydrolysis. It is important to know that some differences appear if a different time is considered. For instance, if the interval used to calculate the activity retention was 20 min (instead of the 3 min used to build Table 5-1), trypsin on spent grains would have retained 26.7 % of the activity of the free enzyme, trypsin on POS-PVA would have retained 8.7 % and trypsin on zeolite would have retained only 6.2 %.

The immobilized enzymes showed a much higher activity towards low molecular weight substrates. When the macrosubstrate (WPI) was used, this activity was only 18 %, 9 % and 11 % of the free enzyme activity, respectively (Table 5-1).

Table 5-1 Comparison of activity retention (%) in the hydrolysis a micro- and a macro-substrate (BAPNA and WPI, respectively)

Carrier	BAPNA (activity retention, %)	WPI (activity retention, %)
Zeolite	63.5	10.7
POS-PVA	73.7	9.3
Glyoxyl spent grain	46.0	17.6

In general, selectivity changes due to immobilization can be controlled by the carrier (due to pores or to diffusional restrictions) or by the change of conformation of the immobilized enzyme (due to the properties of the microenvironment or to changes in the active site). As the whey proteins are much bigger molecules than BAPNA the binding to the active sites of the enzyme might be more difficult either by diffusional limitations or by partial spatial obstruction of the active site. In fact, from the analysis of Figure 5-1 it can be observed that for the free enzyme the initial rate of hydrolysis is high, suggesting that the reaction is the

rate-limiting step. As already referred in Chapter 3, at higher values of the degree of hydrolysis some kind of inhibition occurs and the reaction rate strongly decreases.

In the case of immobilized trypsin on spent grains, the initial rate of hydrolysis is much smaller than the initial rate of hydrolysis with the free enzyme indicating substrate difusional limitations. As the hydrolysis products are smaller than the substrates, product accumulation due to difusional limitations is not so likely to occur. If this was the case, the rate of hydrolysis should decrease even more because of a high concentration of the inhibiting product of reaction near the enzyme. This does not seem to be the case, as the difference between the rate of hydrolysis of free and immobilized enzymes decreases right from the beginning (after 20 min, the immobilized enzyme overall activity is already 27 % of that of the free enzyme). The introduction of a spacer might improve the results.

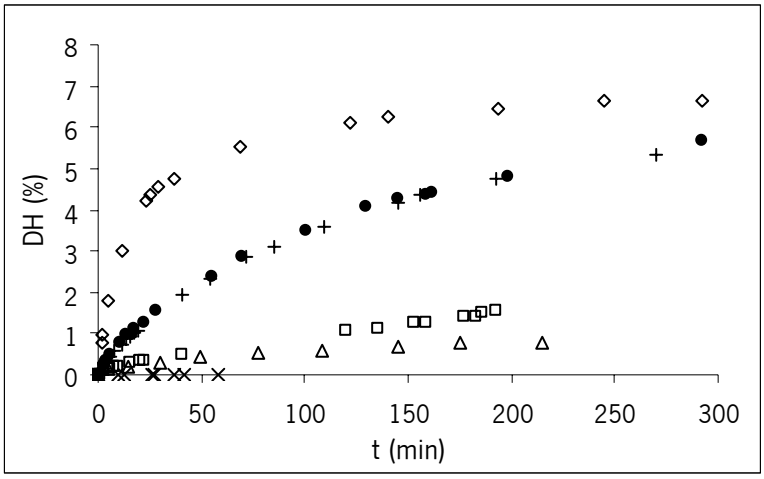

Figure 5-1 Degree of hydrolysis of whey protein isolate with free and immobilized trypsin at 37 °C and pH 8: ◊ free enzyme; + spent grain; ● spent grain (2nd test); Δ zeolite; □ POS-PVA; × control (WPC without enzyme)

The reduced activity of immobilized proteases towards high molecular weight substrates has been referred in literature. For instance, Arasaratnam and others (2000) refer that the proximity of a large internal support surface, where the enzyme is usually immobilized, may promote poor performance of immobilized proteins on macromolecular substrates. They also refer that the maximal activity of trypsin towards high-molecular weight substrate (in that case azocasein) occurs when less protein is coupled as compared to activity against low-molecular weight substrate, BAPNA. They suggest that when the support has more attached enzyme, crowding of protein molecules may occur and the accessibility to large substrates is

reduced (Arasaratnam and others, 2000). Thus, decreasing the amount of immobilized trypsin could also improve the results.

The degree of hydrolysis of the WPI with trypsin immobilized on spent grains was 4.8 % after 3 hours, against 6.5 % with the free enzyme. The enzyme immobilized on spent grain had lower activity and thus it is expected that the degree of hydrolysis achieved is lower for the same time of hydrolysis. However, the specificity of the enzyme limits the final degree of hydrolysis and it would probably be higher and closer to the value obtained with the free enzyme if a longer time had been used.

Although the initial activity of the enzymes immobilized on zeolite and on POS-PVA is less than two times smaller than that of the enzyme immobilized on spent grains (Table 5-1), the final degree of hydrolysis achieved is much smaller (0.8 and 1.5, respectively), as can be deducted from Figure 5-1. In fact, the difference between the hydrolysis rate with trypsin on zeolite or on POS-PVA and the hydrolysis rate with free trypsin increases in the beginning, which is different from what happened with the immobilized trypsin on spent grain. This suggests that diffusional limitations are in these cases much stronger and may also include product difusional limitations. In fact, at the beginning of the reaction trypsin immobilized on zeolite had a higher activity towards WPI than trypsin immobilized on POS-PVA. However, after 20 min the situation was the reverse, which may indicate stronger product inhibition in the case of zeolite. Steric hindrance problems may also be happening as well the carriers may be adsorbing the substrates or products of reaction, overcrowding their surface and not allowing the hydrolysis to proceed.

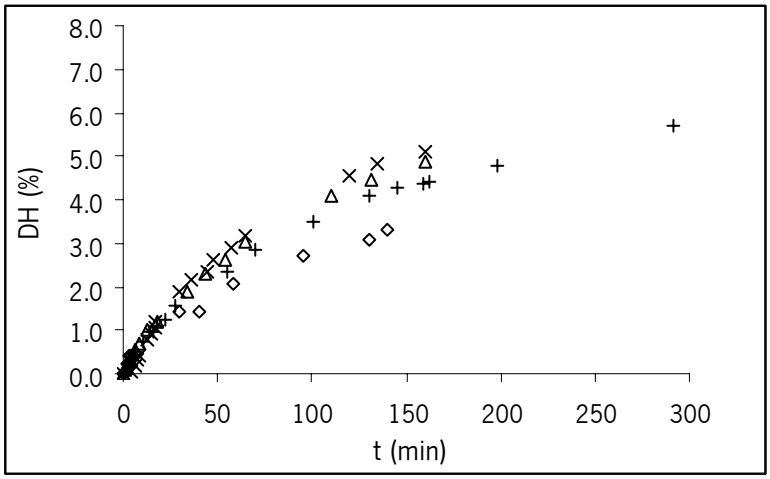

Figure 5-2 Degree of hydrolysis of whey protein isolate with immobilized trypsin on spent grains at 37 °C and 50 g/L: ◊ pH=7.5; + pH=8.0; Δ pH=8.5; × pH=9.0

As poor results were achieved for the hydrolysis of whey proteins with trypsin immobilized on zeolite and on POS-PVA studies about optimal conditions (pH and temperature) were made only with spent grains.

The optimum pH for the hydrolysis of WPI with trypsin immobilized on glyoxyl-spent grains seems to be 9 (Figure 5-2), slightly higher than for the free enzyme (8.5). However, the *DH* profiles are very similar for pH 8, 8.5 and 9 suggesting an optimal pH interval. The same behaviour was already observed for the free enzyme but for pH values ranging from 8.5 to 9.5. Higher pH values were not tested for the immobilized enzyme due to problems associated with the calculation of the dissociation constant, but the fact that immobilized enzyme is more stable at pH 8 may indicate that it is stable over a wider range of pH values.

Figure 5-3 shows the degree of hydrolysis determined by the pH-stat method for WPI hydrolysed at several temperatures with trypsin immobilized on spent grains. The activity of the enzyme increased with temperature and a maximum was found for the experiment performed at 50 °C for a hydrolysis time of 3 hours, slightly higher than the maximum found with the free trypsin: 45 °C (Section 3.3.2). The immobilized enzyme is thus slightly more stable at higher temperatures than the free enzyme. As a result, the enzyme still retains some activity after 70 min at 60 °C while with the free enzyme after 10 min at that temperature almost no activity was observed. The final degree of hydrolysis achieved for the free enzyme was 1.1 % after 10 min (and this value remained constant for the following hour) while with the immobilized enzyme it was 2.3 % after one hour and 3.0 % after three hours. Furthermore, and although the initial activity of the immobilized protein is ca. five times smaller than the initial activity of the free enzyme (Table 5-1), the degree of hydrolysis achieved after two hours at 55 °C starts to be higher in the case of the immobilized enzyme (4.3 % against 4.2 %). Thus, the immobilized enzyme can be used in a broader interval of temperatures and therefore may be useful if, for some operational reason, the hydrolysis has to be performed at higher temperatures. It has also shown to be more adequate in reactions that need longer times of hydrolysis at moderate temperatures. In all cases, at 70 °C, inactivation of the enzyme occurred rapidly and the hydrolysis reaction stopped in less than two minutes (results not shown).

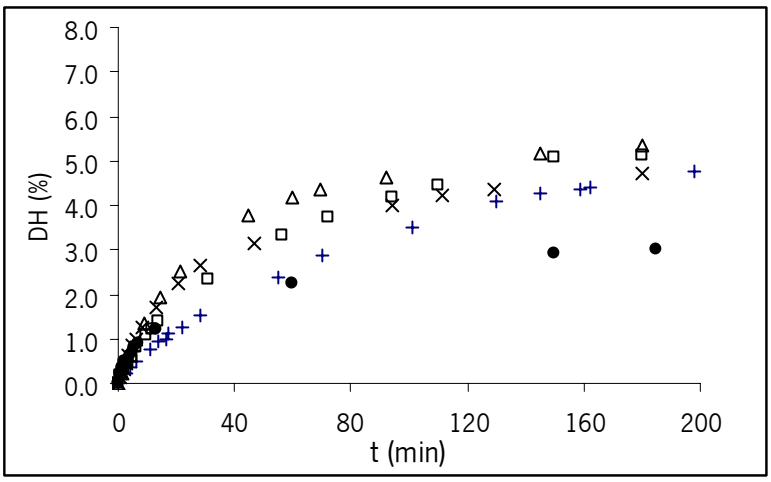

Figure 5-3 Degree of hydrolysis of whey protein isolate with immobilized trypsin on spent grains at pH 8 and 50 g/L: + 37 °C ; □ 45 °C; Δ 50 °C ; × 55 °C; • 60 °C

Several batches of the immobilized trypsin on spent grains that had been used at 37 °C were reused once with no sensible activity losses. This may indicate that the enzyme is not permanently inactivated by substrates and products of reaction and can be reused as in the case where BAPNA was used as substrate. However, further tests should be made to confirm these preliminary observations.

5.3.2 Peptide profile and composition of the hydrolysates

The evolution of the RP-HPLC profiles during hydrolysis of WPI with trypsin immobilized on glyoxyl-spent grains at 37 °C and pH 8 is shown in Figure 5-4. The peaks with an average retention time of 30.3 and 32.2 min correspond to α-La and β-Lg, respectively. The peptides formed with the free and the immobilized enzyme are essentially the same and the mechanism of peptide formation seems also to be the same with major whey protein peaks decreasing to give rise to smaller peaks, some of which appear right from the beginning and keep growing while others, more hydrophobic and, thus, probably bigger, further degradate into the smaller peptides.

However there are small differences in the chromatograms particularly in the more hydrophobic regions. In fact, higher amounts of intact proteins remain (which can be confirmed in Table 5-2) in the case of hydrolysates obtained with the immobilized enzyme. This may help to corroborate the possibility of the existence of difusional limitations for bigger substrates. When comparing the peptide profile of the hydrolysates from free enzyme and from immobilized enzyme on spent grains with the same degree of hydrolysis (4.4 % for the immobilized enzyme and 4.3 % for the free enzyme), there seems to be a preferential breakdown of peptides instead of big intact proteins. Different peptide patterns of the

hydrolysates obtained with free and immobilized proteins have been described before in literature (e.g Lorenzen and Schlimme, 1995). β-Lg for instance has ca. 29-40 Å (Yiu and others, 2001) and, although the enzyme is not immobilized on pores (because either they do not exist or they are too small), this can cause problems if the enzyme is for instance too close to the carrier and if the active site is not completely turned to the outer side of the carrier (so that the substrate can come close and rotate freely in order to fit the active site).

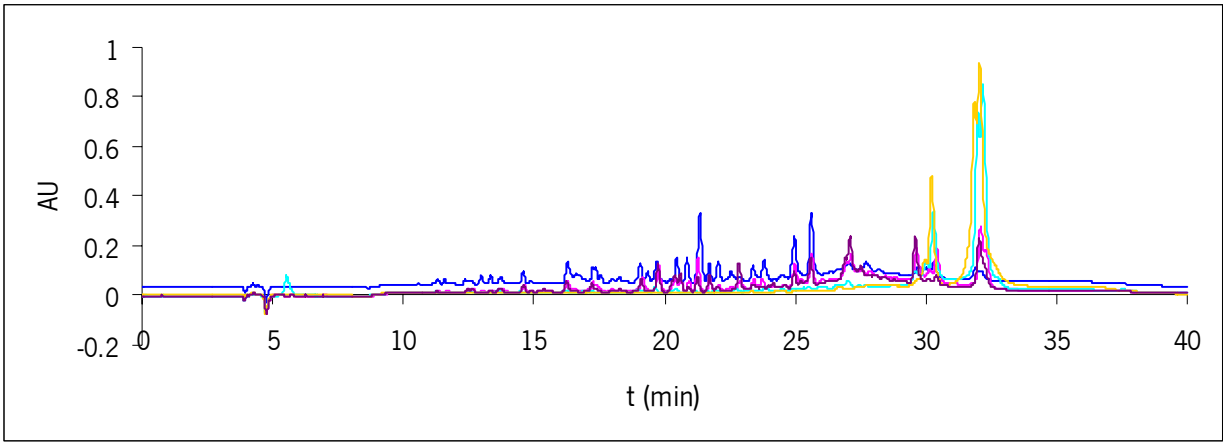

Figure 5-4 RP-HPLC profile of whey protein hydrolysates from immobilized trypsin on spent grains at pH 8.0 and 37 °C: – *DH* 0 % (*t* = 0 min); – *DH* 1.1 % (*t* = 22 min); – *DH* 4.4 % (*t* = 159 min); – *DH* 6.5 % (*t* = 498 min); – *DH* 4.3 % (free enzyme; *t* = 25 min)

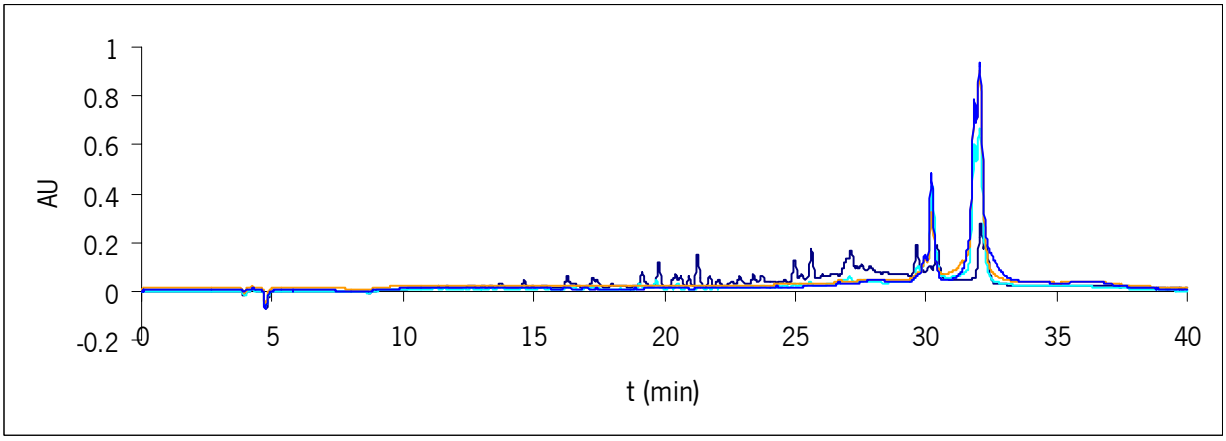

Figure 5-5 RP-HPLC profile of whey protein hydrolysates from immobilized trypsin at 37 °C, pH 8.0 and 50 g/L of WPI: – 0 (*t* = 0 min; *DH* = 0 %); – Spent grains (*t* = 159 min; *DH* = 4.4 %); – zeolite (*t* = 215 min; *DH* = 0.8 %); – POS-PVA (*t* = 190 min; *DH* = 1.5 %)

A small peak appears right next to the peak corresponding to α-La except for *DH* 6.5 % (Figure 5-4). As this peak is very similar in hydrophobicity with α-La and appears to be connected with its degradation, it can originate from the hydrolysis of α-La on the f(122) Lys. Thus, the resulting peptide only differs from the original protein in the last amino acid. This peptide has other amino acids that are breakable by trypsin and as the hydrolysis proceeds it is further degradated into smaller peptides, eventually disappearing from the chromatogram (as observed for *DH* 6.5 %).

The RP-HPLC profiles during hydrolysis of whey proteins with free trypsin and trypsin immobilized on the three different carriers are shown on Figure 5-5. They confirm the degree of hydrolysis achieved. With the enzyme immobilized on zeolite almost no α-La and β-Lg have been degradated and no minor peaks are detected. The resulting pattern is equal to the pattern of the WPI prior to the hydrolysis. After three hours of hydrolysis with trypsin immobilized on POS-PVA, a slight degradation of α-La and β-Lg is detected and smaller, more hydrophilic, peptides are formed in a very small amount. The peptide profile of the hydrolysate produced with trypsin immobilized on spent grain shows that in this case hydrolysis occurred in a much higher extension than in the other two cases.

As already happened in the case of the free enzyme, RP-HPLC peptide pattern seems to be the same for pH values between 8 and 9 (Figure 5-6). However, at pH 7.5 both α-La and β-Lg seem to be more resistant to the hydrolysis and smaller peptides are preferred by the enzyme than the intact protein. A change in the conformation of the native proteins (due to changing pH values) may be making the substrate binding to the active site even more difficult.

When the effect of the temperature on the peptide profile is analised, the main differences appear again in the native α-La and β-Lg (Figure 5-7). With the increase of the hydrolysis temperature the amount of native proteins still present at a *DH* close to 4 % decreases. This decrease is higher for α-La, which is understandable because this protein is more heat sensitive than β-Lg. Although there are differences in the amount of the native α-La and β-Lg present, at temperatures equal to or below 50 °C the peptide profiles correspondent to a degree of hydrolysis of 4.2-4.4 % are quite similar. Similarly to the hydrolysate from the free enzyme, a wide peak in the hydrophobic region appears also in the present case for temperatures above 60 °C, probably corresponding to β-Lg denaturation.

Although general peptide profiles of both free and immobilized enzyme are similar, the small differences detected in Figure 5-4 become evident when comparing Table 5-2 to Table 3-1. In fact, native proteins are

less degradated by proteolysis in all cases, confirming size (steric) limitation problems. These differences do not exist for the hydrolysis of α-La above 50 °C, *inclusive*. Hydrolysis with free and immobilized trypsin at different pH values cannot be directly compared because the profiles shown correspond to different values of degree of hydrolysis.

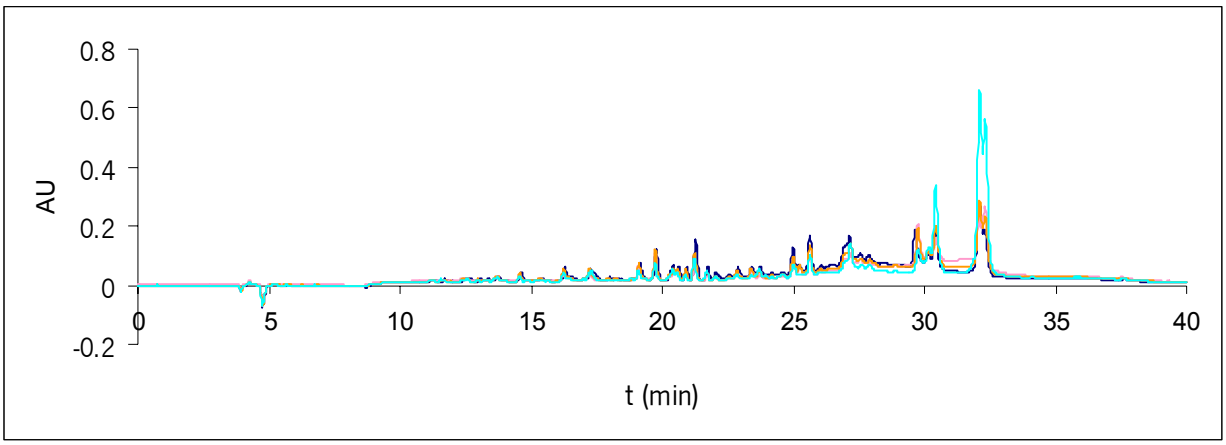

Figure 5-6 RP-HPLC profile of whey protein hydrolysates from immobilized trypsin on spent grains at 37 °C and 50 g/L of WPI: – pH 7.5 (*t* = 140 min; *DH* = 3.3 %); – pH 8.0 (*t* = 159 min; *DH* = 4.4 %); – pH 8.5 (*t* = 110 min; *DH* = 4.1 %); – pH 9.0 (*t* = 65 min; *DH* = 3.6 %)

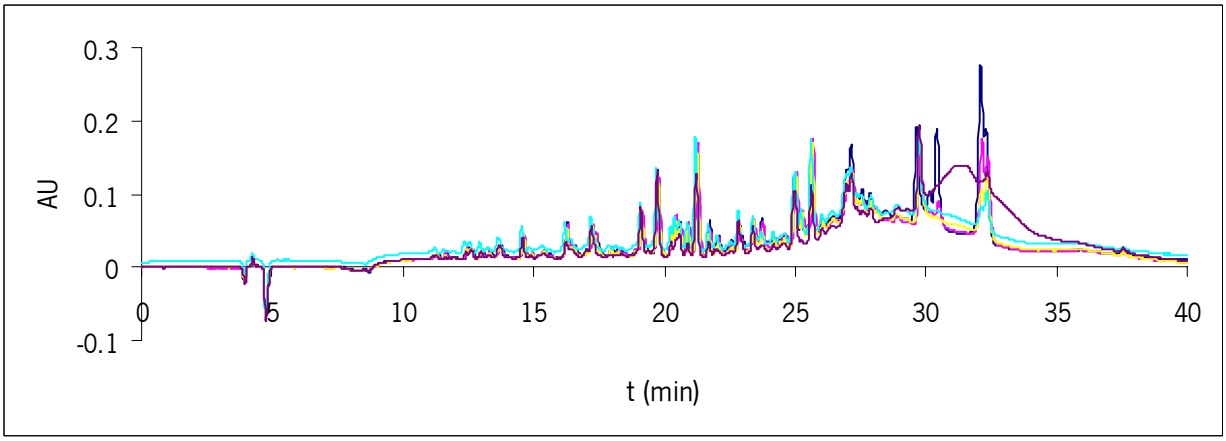

Figure 5-7 RP-HPLC profile of whey protein hydrolysates from immobilized trypsin on spent grains at pH 8.0 and 50 g/L of WPI: – 37 °C (*t* = 159 min; *DH* = 4.4 %); – 45 °C (*t* = 110 min; *DH* = 4.4 %); – 50 °C (*t* = 69.5 min; *DH* = 4.4 %); – 55 °C (*t* = 111 min; *DH* = 4.2 %); – 60 °C (*t* = 185 min; *DH* = 3.0 %)

From Figure 5-8 it can be confirmed that the peptide profiles resulting from free and immobilized enzymes are very similar. Small differences are seen in the more hydrophobic region of the chromatogram (mainly corresponding to intact proteins, that can arise from the small difference in the final hydrolysis degree of the two samples or from the difference in selectivity of the immobilized enzyme towards larger substrates). Another interesting feature is that small peaks appear in the chromatogram of the hydrolysate obtained with the free enzyme (see Figure 5-8a), e.g. in the region from 16 to 18 min and near 25 min. These peaks are not present in Figure 5-8b, which may indicate that they are originating from trypsin autolysis. Of course they can also result from small differences in the enzyme's behaviour.

Table 5-2 Degradation of α-La and β-Lg with immobilized trypsin

pH	*T* (°C)	Carrier	Hydrolysis time (min)	Degree of hydrolysis (%)	α-La concentration (% of initial α-La concentration)	β-Lg concentration (% of initial β-Lg concentration)
8	37	Spent grain	22	1.1	24.5	72.9
8	37	Spent grain	159	4.4	n.d.	18.3
8	37	Spent grain	498	6.5	4.7	14.6
7.5	37	Spent grain	140	3.3	36.6	58.8
8.5	37	Spent grain	110	4.1	17.1	20.7
9.0	37	Spent grain	65	3.6	10.1	17.7
8	45	Spent grain	110	4.4	2.8	12.2
8	50	Spent grain	69.5	4.4	0.4	6.9
8	55	Spent grain	111	4.2	0.3	3.8
8	60	Spent grain	185	3.0	n.d.	n.d.
8	37	POS-PVA	190	1.5	48.8	63.7
8	37	Zeolite NaY	215	0.8	55.4	84.4

The data on Table 5-3 confirms that the peaks with the same retention time have the same mass and thus are probably referring to the same peptide.

The binding to ACE is strongly influenced by the C-terminal sequence, whereby hydrophobic amino acids, e.g., Pro, are more active if present at each of the three C-terminal positions. In addition, the presence of the positive charge of Lys (ε-amino group) and Arg (guanidine group) as the C-terminal residue may contribute to the inhibitory power. Considering these structure-activity features of ACE-inhibitory peptides, enzymes with specificity towards the carboxylic side of aromatic or other hydrophobic amino acid residues, or towards the basic amino acids Lys and Arg might be beneficial, explaining the large number of ACE-inhibitory peptides obtained with trypsin (Lopez-Fandino and others, 2006).

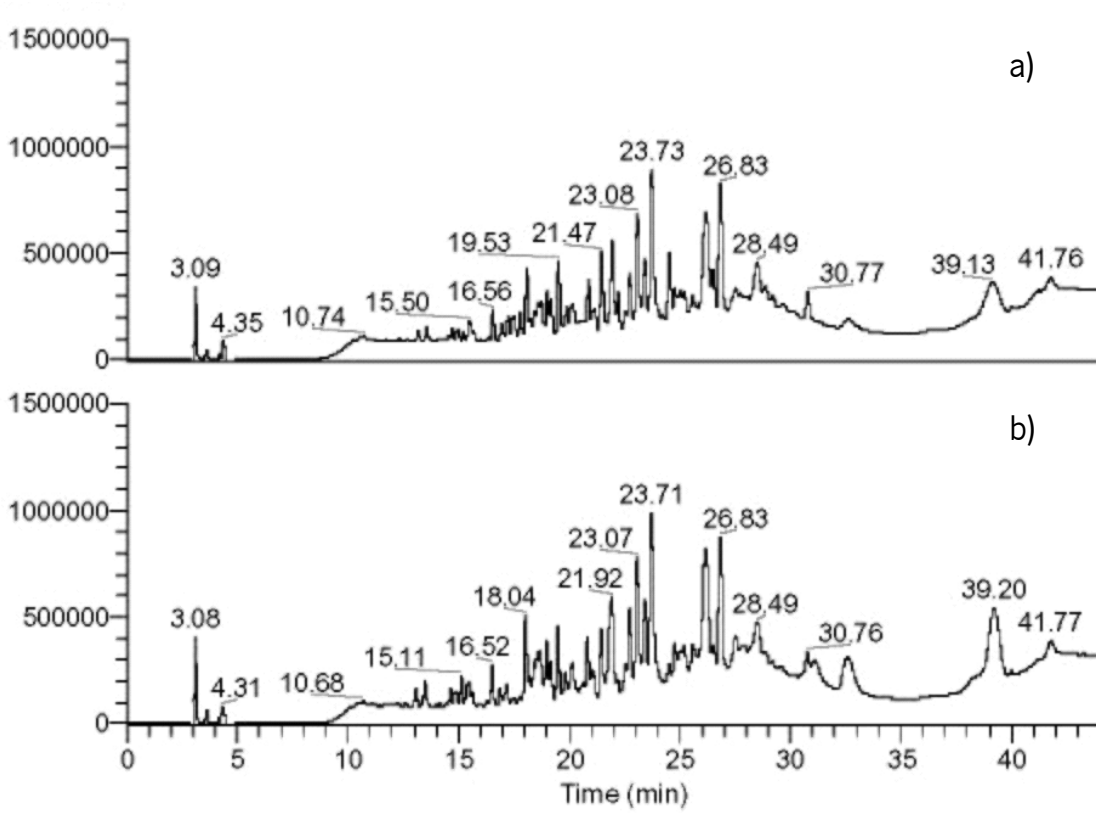

Figure 5-8 Hydrolysates' peptide profile from the RP-HPLC/MS analysis: a) free enzyme (DH=6.3 %); b) enzyme immobilized on spent grains (DH=6.5 %)

Table 5-3 Approximate *m/z* values from the RP-HPLC analysis for the peaks identified on Figure 5-5

RT (min)	m/z (free enzyme)	*RT* (min)	*m/z* (immobilized enzyme)	*RT* (min)	*m/z* (free enzyme)	*RT* (min)	*m/z* (immobilized enzyme)
16.56	587.5	16.52	587.5	23.73	711.2	23.71	711.0
18.04	933.0	18.04	932.9	26.14	1367	26.18	1367
19.53	669.3	19.53	669.2	26.83	1175	26.83	1175
20.90	950.1	20.92	950.2	28.49	1578	28.49	1578
21.47	853.7	21.47	853.8	30.77	1373	30.76	1374
21.92	834.4	21.92	834.5	39.13	290.9	39.20	290.8
23.08	1083	23.07	1083				

Refining the RP-HPLC/MS method should be the next step, in order to eliminate the interference from tri-fluoroacetic acid. Even so, treatment of this data is still needed with proper software to compare the *m/z* values obtained with those expected from a theoretical analysis (calculated considering the proteins, the available cleavable sites for the enzyme used as well as the value of *z* of each expected peptide in the environmental conditions of the analyses). However, comparing the chromatograms in Figure 5-8 with the chromatograms presented by Ferreira and others (2007) and analising the value of *m/z*, it seems probable that the peak with an elution time of 21.92 is peptide ALPHMIR from the tryptic hydrolysis of β-Lg. This peptide is known for being one of the strongest ACE-inhibitory peptides *in vitro*, although results are not very promising *in vivo* (as referred in Chapter 2).

5.3.3 Kinetics of immobilized trypsin

A simple Michaelis-Menten model was considered for the kinetic analysis. The concentration of all cleavable sites was used as the substrate concentration. The kinetic analysis showed that the apparent K_m for the immobilized trypsin was about 6 times higher than that found for the soluble enzyme (Table 5-4). This may again indicate that there are diffusional limitations due to immobilization. The ν_{max} of the immobilized enzyme is around 13 % of the ν_{max} from the free enzyme as expected from the results above. There may be substantial multi-layers of trypsin on the flat surface area of spent grains, and the internal layers of enzyme are less accessible for the substrate molecules, specially the larger ones, and therefore, a significant amount of trypsin may not be readily available to the substrate (together with the possible steric hindrance effects referred above).

Table 5-4 Kinetics of free enzyme and enzyme immobilized on spent grains evaluated at 37 °C and pH 8.

	K_m (eq_{lig}/L)	ν_{max} (min^{-1})
Free enzyme	13.6	4.89×10^{-2}
Immobilized enzyme on spent grains	86.1	6.41×10^{-3}

Changes in the kinetic constants due to difusional limitations are common in literature. For instance, Duggal and Buchholz (1982) studied the effects of immobilization of trypsin on its intrinsic kinetics. They

concluded that there are significant shifts in association constants for substrates and inhibitors due to covalent binding onto a rigid support. K_M and K_I values were changed by factors up to 6 and relative affinities were also different as compared to the native enzyme. Interference of other phenomena like adsorption or diffusion has been excluded. Protection of the enzyme by very strong inhibitors during binding can avoid such alterations. Similar findings were obtained for the maximal reaction rates.

5.4 Conclusion

Only trypsin immobilized on spent grains showed significant activity towards whey proteins. The immobilized enzyme is slightly more stable at temperatures between 50 °C and 60 °C allowing its use at a broader range of temperatures.

Peptide profile of hydrolysates from WPI with free enzymes and enzymes immobilized on spent grains were similar, which indicates that spent grains can be used as carriers for trypsin to produce hydrolysates with peptides similar to those obtained with the free enzyme (thus with similar bioactivity). However, significant differences exist in the amount of native proteins in the hydrolysates.

The peptide ALPHMIR (a strong ACE-inhibitory peptide) from the tryptic hydrolysis of β-Lg was identified.

5.5 References

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Chapter 6 Rheological characterization of gels from whey protein hydrolysates

6.1 Introduction

Hydrolysates can be characterized in several ways, being the most common the degree of hydrolysis, the molecular properties, the functional properties and the biological/biochemical properties.

Molecular characterization has already been made by the degree of hydrolysis and the RP-HPLC profile of the hydrolysates. Functional properties, namely the heat-set gelling ability, are going to be addressed in this chapter.

Rheological studies are useful to evaluate the gelling ability of biological macromolecules; in particular, they allow accessing the structure of the gel, evaluating its texture, controlling the gelling behaviour or complementing the information provided by sensory methods (da Silva, 1994). In fact, as gelation is essencially a phase transition from liquid to solid, monitoring the changes in mechanical properties is important. Small amplitude oscillatory shear techniques can be used to monitor continuously the evolution of the viscoelastic properties, avoiding any modification of the molecular structure caused by shear. This is an advantage over other rheological tests.

The gelling point can not be measured directly but the rheological properties are very sensitive indicators of the liquid-solid transition (Winter and Mours, 1997). For instance, the diverging rheological properties are an unambiguous sign of the approaching gel point. Thus one common rheological test for detecting the liquid-solid transition involves measuring the divergence of the steady shear viscosity. However it does not show the real gel point as the transition may appear early because of torque overload or may be delayed due to large strain (Winter and Mours, 1997). Another alternative is to consider that the gel point is achieved when the loss (viscous) modulus equals the storage (elastic) modulus ($G'=G''$). However the G'-G'' crossover often depends on the frequency (except when the relaxation exponent, n, is 0.5) and the gelling point cannot depend on the probing frequency. Therefore the point where G' equals G'' is usually close to the gelling point but may not be exactly identical. Alternatively it can be considered that when tan δ becomes independent of frequency the gelling point is achieved. This method is very effective but is also not universal. In fact, although tan δ is independent of the frequency when the gelling point is reached, it may also be independent in other situations (Winter and Mours, 1997).

Several other techniques have also been used to study changes in structure and molecular interactions during a gelling process. The most common are spectroscopic methods (Lefevre and Subirade, 2000;

Corredig and others, 2004; Ikeda and Li-Chan, 2004), dynamic light scattering and X-rays diffraction methods (Elofsson and others, 1996; Kavanagh and others, 2000c; Capron and others, 2001) and differential scanning calorimetry (de Wit, 1990; Hudson and others, 2000). Microscopy studies are also widely used as they allow the visualization of the gel microstructure. Scanning laser confocal microscopy is particularly useful in the study of the gelation of mixed systems and has been widely used in recent years to study the mechanism of phase separation and its implications in the functional properties of gels (Beaulieu and others, 2001; Tromp and others, 2001; Olsson and others, 2002; Gonçalves and others, 2004; Bertrand and Turgeon, 2007; van den Berg and others, 2007, to cite a few).

In this chapter the heat-induced gelling properties of WPC and whey protein hydrolysates from trypsin and pepsin with different *DH* values were studied at pH 7.0 by small deformation rheology.

6.2 Materials and methods

All chemicals used were of analytical grade and supplied by Sigma, Co (St. Louis MO, USA). Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg (one BAEE unit will produce a ΔA_{253nm} of 0.001 per min at pH 7.6 at 25 C using BAEE as substrate; in a reaction volume of 3.2 ml and 1 cm light path) was obtained from Sigma Chemical Co (ref. T7409).

A commercial whey protein concentrate (WPC) powder (Lacprodan 80, batch Q500246) kindly supplied by Arla Food Ingredients (Viby, Denmark) was used for the experiments. According to the suppliers, the WPC dry basis protein content was 82 % (5.5 % moisture), the ash content was 3.5 % max., the lactose content was 7 %, and fat content was 8 %. max.

6.2.1 Hydrolysis of WPC

Hydrolysis of WPC was performed as described in Chapter 3. The resulting suspensions were lyophilised for further analysis. As ions can strogly influence the behaviour of the partially denatured β-Lg (as referred by several authors including McPhail and Holt, 1999) peptic hydrolysates were dialysed with a 100 Da MWCO membrane (Spectrum Spectra/Por Biotech, Irvine, USA) against distilled water during 24 hours prior to lyophilization (the water was changed four times). As the amount of acid and/or alkali added to the tryptic hydrolysate during the hydrolysis procedure was lower, no dialysis was performed in this case.

6.2.2 Sodium analysis

Sodium content was determined by flame photometry with a flame photometer (JENWAY PFP7) equipped with a sodium filter (589 nm). Calibration was made with standard solutions of sodium chloride containing 3.9 to 19.7 mg/L of Na^+ (10 to 50 mg/L NaCl). All standards and samples were analysed in triplicate.

6.2.3 Chloride analysis

Chloride content was determined through ionic chromatography. A liquid chromatograph (Dionex DX-300) was used equipped with a conductivity suppressor (Anion Micromembrane Suppressor AMMS-II), a conductivity detector (Dionex CDM-II), a manual injector with a 25 μL loop, a AG4A (10-32) precolumn with 4 x 50 mm (id x L), and an Ionpac AS4A (10-32) column with 4 x 250 mm (id x L), both from Dionex. The eluent was a solution of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ and the suppressor regenerator was a solution of 50 mN H_2SO_4. Operational conditions used were: eluent flow rate of 1.5 mL/min; suppressor regenerator flow rate of 4 mL/min; background conductivity of 20.2 μS. A calibration curve was built with standard NaCl solutions ranging between 0.6 and 18.2 mg/L of Cl^- (1 and 30 mg/L of NaCl).

6.2.4 Moisture content

Moisture content was determined by heating the sample at 105 °C for 12 hours, according to Food Chemical Codex (1981) as described by Torres (2005).

6.2.5 WPC/WPH solutions

The WPC or WPH solutions were prepared into polypropylene tubes by weighting the appropriate amount of powder and adding distilled water, without reaching the final solutions weights. Sodium azide solution (2 wt %) was added to a final concentration of 5 ppm in order to prevent bacterial growth. After 2 hours of gentle mixing, the pH was adjusted to 7 with KOH 1M and/or HCl 1M. Finally, distilled water was added until the final weights were reached and the systems were kept under gentle mixing for one more hour.

6.2.6 Preliminary texture analysis

In order to determine the adequate protein concentration range to allow the study of the hydrolysates gelation ability, several solutions with different protein concentrations were prepared to establish the gelling protein concentration region. They were allowed to equilibrate for 2 hours with gentle mixing.

Heat treatment of WPC/WPH solutions

The WPI solutions were transferred to polypropylene tubes with seal caps to induce heat gelation at 80 °C during 3 hours in a controlled temperature water bath. The temperature increase rate was 2 °C/min. After heating, the tubes were allowed to slowly cool down to 20 °C.

Tube inversion

There are several "tabletop" rheological techniques that allow rapid and simple identification of the gelling behaviour, without the need of expensive equipment. These techniques include the tube inversion method (TIM) or the falling sphere method (Raghavan and Cipriano, 2005) and allow the easy construction of "phase" diagrams identyfing the conditions under which a certain substance with gelling ability will effectively form a gel (concentration of the substance with gelling ability, concentration of salts, temperature, ...). TIM is the most common diagnostic test of gelation due to its simplicity and consists in turning a test-tube or vial containing the sample upside-down. If the sample flows under its own weight the sample behaves like a liquid solution (viscous and inelastic) and if the sample does not flow it means that it has a yield stress and behaves like a gel (Raghavan and Cipriano, 2005).

Thus, several test tubes were prepared as described in section 6.2.5 with different concentrations of whey protein concentrate, tryptic whey protein hydrolysate and peptic whey protein hydrolysate, different degrees of hydrolysis and different NaCl concentrations. The tubes were then immersed in a 20 °C water bath and a ramp temperature of 2 °C/min was performed for 30 min (until 80 °C). The temperature was then maintained at 80 °C for 3 h and then the samples were cooled back to 20 °C. The tubes were gently turned upside down. By visual inspection of the tubes and the inverted tubes the samples were classified into: gel, gel with syneresis (when an observable amount of water was lying above the gel phase before the inversion), weak gel (when the sample does not flow but falls – as a whole - under its own weight, possibly due to a low value yield stress or to the presence of syneresis), solution, viscous solution (when the sample flows but at a slow rate) and two phase samples. It is to be noted that the distintion between a weak gel and a viscous solution was not always evident. These samples were both classified as pre-gels, meaning that the conditions were near a gelling point and a small change would probably lead to a "full" gel. Anyway these samples were also analysed by penetration tests (see next section) to confirm the gel/pre-gel/liquid classification. Phase diagrams were then constructed.

Penetration Tests

The contents of the tubes mentioned above were analysed in a texture analyser (TA.XT2, Stable Micro Systems, UK). Relative hardness of whey or hydrolysates' protein gels was measured with a 6 mm stainless steel cylindrical probe. A penetration speed of 0.1 mm/s and maximum penetration distance of 6 mm were used. No absolute values are possible as the diameter of the test tubes (14 mm) was not high enough (due to limitations in the amount of protein available) to eliminate tube wall interferences nor replicates were enough to allow statistical significance.

6.2.7 Rheological measurements

In dynamic oscillatory testing, samples are subjected to harmonically varying stress or strain, depending on the type of rheometer. This kind of testing is usually non-destructive and used for studying viscoelastic behaviour of food. The tests may be conducted in tension, bulk compression or shear, being this last one the predominant method (Steffe, 1996).

If a small harmonically varying strain (γ) is applied to the sample:

$$\gamma = \gamma_0 \cos(\omega t) \qquad \text{Eq. 6.1}$$

When the sample behaves in a linear form from the viscoelastic point of view (if the strain amplitude is low enough to allow the ratio of stress to strain at any particular time or frequency to be independent of the magnitude of the applied strain), the resulting shear stress can be described by a similar sinusoidal function over time. This function has the same frequency but features a phase lag (or shift) of δ degrees (phase angle or mechanical loss angle) relative to the applied strain:

$$\sigma = \sigma_0 \cos(\omega t + \delta) \qquad \text{Eq. 6.2}$$

In equations 6.1 and 6.2 γ_0 represents the maximum strain amplitude, σ_0 is the maximum stress amplitude, ω is the oscillatory frequency (rad/s) and t is the time (s). The mechanical loss angle (δ) depends on the viscoelastic properties of the sample and has two extreme cases: 1) for ideal solid (elastic) behaviour δ is zero and the shear stress wave is in phase with the shear strain wave; 2) for ideal liquid (viscous) behaviour the value of δ is 90 °. A viscoelastic material will have a phase angle between 0 and

90 °. The response of these materials to a sinusoidal strain input will have an elastic component (in phase with the shear strain) and a viscous component (90 ° out of phase with the shear strain).

Considering the vectorial representation of strain and stress, it is possible to present them as complex numbers (Ferry, 1980):

$$\gamma^* = \gamma_0\left[\cos(\omega t) + i\sin(\omega t)\right] \qquad \text{Eq. 6.3}$$

$$\sigma^* = \sigma_0\left[\cos(\omega t + \delta) + i\sin(\omega t + \delta)\right] \qquad \text{Eq. 6.4}$$

The complex modulus includes complete information of the viscoelastic properties of the material and is given by

$$G^* = \frac{\sigma^*}{\gamma^*} \qquad \text{Eq. 6.5}$$

From the manupilation of the above equations results:

$$G^* = \frac{\sigma_0}{\gamma_0}\left(\cos\delta + i\sin\delta\right) = G' + iG'' \qquad \text{Eq. 6.6}$$

Two dynamic moduli are therefore introduced: the storage modulus (G') and the loss modulus (G''). They are both functions of temperature, frequency and strain applied. However, for strain values within the viscoelastic linear domain, G' and G'' are independent of the strain. Their dependence on the frequency can be expressed in terms of the amplitude ratio and the phase shift:

$$G' = G^*\cos\delta = \left(\frac{\sigma_0}{\gamma_0}\right)\cos(\delta) \qquad \text{Eq. 6.7}$$

$$G'' = G^*\sin(\delta) = \left(\frac{\sigma_0}{\gamma_0}\right)\sin(\delta) \qquad \text{Eq. 6.8}$$

$G'\gamma_0$ can be regarded as the component of the stress in phase with the strain while $G''\gamma_0$ may be interpreted as the component of the stress 90 ° out of phase with the strain. The absolute value of the complex modulus is given by:

$$\left|G^*\right| = \frac{\sigma_0}{\gamma_0} = \sqrt{(G')^2 + (G'')^2} \qquad \text{Eq. 6.9}$$

The storage modulus and the loss modulus are objective physical parameters and can be used to interpret the gelling process. The storage modulus value is a measure of the deformation energy stored in the sample per oscillatory cycle and corresponds to the elastic behaviour while the loss modulus is a measure of the energy dissipated in the system through viscous flow (Tabilo-Munizaga and Barbosa-Canovas, 2005). The loss factor (loss tangent) corresponds to the ratio of the viscous component to the elastic component of the deformation behaviour.

$$\tan\delta = \left(\frac{G''}{G'}\right) \qquad \text{Eq. 6.10}$$

The complex viscosity η^* is another useful parameter and is given by:

$$\eta^* = \left(\frac{G^*}{\omega}\right) = \eta' - i\eta'' \qquad \text{Eq. 6.11}$$

where η' is the dynamic viscosity and η'' represents the out of phase component of the complex viscosity. They are related to the storage and loss modulus by:

$$\eta' = \frac{G''}{\omega} \qquad \text{Eq. 6.12}$$

and

$$\eta'' = \frac{G'}{\omega} \qquad \text{Eq. 6.13}$$

The loss tangent can thus be calculated as a function of these two viscosities:

$$\tan\delta = \frac{\eta'}{\eta''} \qquad \text{Eq. 6.14}$$

Dynamic oscillatory tests were performed in a controlled stress rheometer AR2000 (TA Instruments, Delaware, USA) fitted with a parallel plate geometry (40 mm diameter, gap 800 μm). A Peltier system in the bottom plate provided fast and accurate temperature control. Once placed on the measuring device,

the surface of the samples in contact with the ambient was covered with a thin layer of liquid paraffin prior to the start of the tests, in order to prevent evaporation.

Each sample was equilibrated during 5 min; this step was followed by a frequency sweep ("mechanical spectrum") from 100 to 0.1 Hz at a strain of 5 %. Then a temperature ramp from 20 to 80 °C was applied, at a rate of 2 ° C.min^{-1}, after which the temperature was maintained at 80 °C for 3 h. At the end of this time sweep the sample was cooled back to 20 °C, at the same constant rate (2 °C.min^{-1}). The mechanical properties of the resulting gel were monitored at 20 °C for 1 h. Another frequency sweep (100-0.01 Hz) was recorded at this temperature. A maximum shear strain of 0.5 % was used during the temperature, time and frequency sweep experiments after the first frequency sweep. Temperature, strain and time sweeps were performed at 1 Hz.

Finally, a strain sweep from 0.1 to 2 % was made to ensure that experiments were conducted within the linear viscoelasticity region. This deformation step was not always used to determine the critical point (the point after which the viscoelastic behaviour is no longer linear) because at high deformation values the oscillatory movements were not perfectly sinusoidal due to limited instrument resolution. Thus the strain sweep was made only until 2 %. A stress overshoot experiment (Ross-Murphy, 1995) was performed: a steady state flow step with a shear stress ramp from 0.01 to 150 Pa was used in order to determine the rupture strain (γ_r).

6.3 Results and discussion

Prior to the presentation of the main results of this chapter, the chemical characterization of the hydrolysates in relevant parameters to gelation is presented. The threshold for gelation was subsquently determined for all the hydrolysates at different ionic strength values. Finally oscillatory tests relating to gelling of whey protein hydrolysates are described.

6.3.1 Hydrolysates chemical analyses: salts

As the hydrolysis step involved the adition of either sodium hydroxide or hydrochloric acid to control the pH and/or to stop the reaction, determining the amount of sodium and choride ions was important in order to allow the standardization of the ionic strength of the samples in the heat-set gelling studies. Although a dialysis step was introduced to reduce the amount of salt in the resulting hydrolysates, a significant amount of Na^+ and Cl^- was still present as can be seen in Table 5-2. On the other side, as a

liophylisation process was used to dry the hydrolysates and to allow their storage for further studies as well as to permit the use of higher protein concentrations, the amount of water in the final hydrolysates was also determined (Table 5-2). The resulting amounts of salt can derive from incomplete dialysis (due e.g. to membrane clogging) or from binding of the ions to the protein and/or peptides.

Table 6-1 Salt and moisture analysis

	$[Na^+]$ (% w/w)	$[Cl^-]$ (% w/w)	Moisture (%)
WPC	-	-	7.2±0.3
P1.5	3.8±0.6	2.9±0.1	10.7±0.6
P2.5	2.6±0.4	0.62±0.10	9.0±0.3
P4.9	3.1±0.3	1.3±0.0	8.2±0.5
T1.0	2.1±0.3	0.14±0.00	6.6±0.2
T3.5	2.8±0.4	0.13±0.01	6.5±1.0

All values are means ± standard deviation of three determinations.

6.3.2 Gelling minimum conditions: salt and protein concentration

The results from TIM are presented in Figure 5-1. As none of the hydrolysates nor WPC were free from salts the conductivity was used as a measure of an equivalent salt concentration (and thus of the ionic strength). As a consequence, the NaCl concentrations presented in Figure 5-1 are the result of the conversion of the value of the conductivity of the solution (measured in mS) through a previously built calibration curve.

All tubes with gel or pre-gel classification (using TIM) were analized in the texturemeter. It was considered that if the maximum force achieved after a penetration distance of 3 mm was less than 0.015 N the gel was too weak and the sample was re-classified as a "pre-gel". If the maximum force achieved after a penetration distance of 3 mm was higher than 0.015 N the sample was classified as gel.

The minimum gelling concentrations achieved without salt addition (the minimum salt concentration presented for each hydrolysate and for WPC is the result of the salt already present in the hydrolysates) were 10.5 % for WPC, 7.7 % for P1.5, 7.8 % for P2.5, 16.5 % for P4.9, 10 % for T1.0 and 15.9 % for T3.5. The value obtained for P4.9 (16.5 %) corresponds to an equivalent salt concentration of ca. 0.63 (w/w),

corresponding to ca. 0.1 M. All the other hydrolysates gelled at a lower equivalent NaCl concentration. As it was not possible to gel P4.9 at lower salt concentrations, the salt concentration used in subsequent assays of all hydrolysates and WPC was 0.1 M.

Although P1.5 and P2.5 already gelled at a concentration of 6.6 % when the salt concentration was as low as 0.48 %, WPC and T1.0 only gelled at 7.5 to 7.7 % for NaCl concentration of 0.56 to 0.58 %. Thus a concentration of 7.5 % was chosen as representative of gels near the gelation critical concentration while 10 % was used for gels gelling at concentration far away from that critical point. At this salt concentration, T3.5 only gelled at 13 % and P4.9 at 16.5 %. As these concentrations were too high, these values were used only for these two hydrolysates.

An important preliminary result is that hydrolysates with a low degree of hydrolysis have a lower gelation critical concentration than intact whey proteins; such decrease is much sharper for peptic hydrolysates (P1.0 and P2.5) than for tryptic hydrolysates (T1.0). The texture analysis confirmed that these gels were also stronger for the same protein amount than the gels from WPC (results not shown).

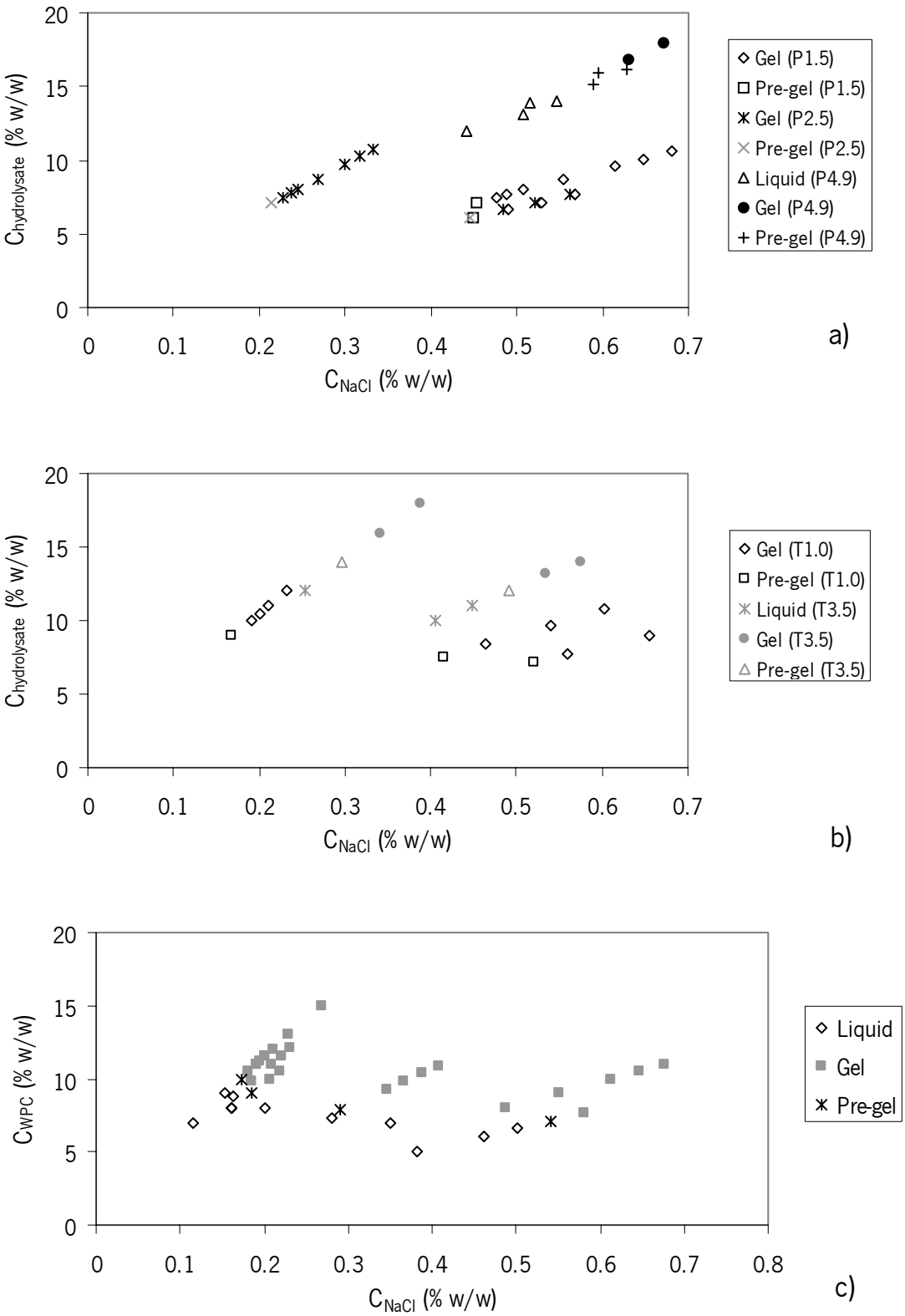

Figure 6-1 Influence of the protein and NaCl concentration on the gelling ability: a) hydrolysates from pepsin; b) hydrolysates from trypsin; c) WPC

6.3.3 Gelling ability of whey protein hydrolysates

WPC and WPH at different concentrations were subjected to heating from 20 to 80 °C in 30 min, an isothermal curing at 80 °C followed by quenching to 20 °C and by another curing of 60 min at 20 °C. The time dependence of the viscoelastic moduli (G' and G'') and of the loss angle (δ) is presented in Figure 6-2 for peptic hidrolysates and WPC and in Figure 6-3 for tryptic hydrolysates. The evolution of the parameters follows the general behaviour reported for many biopolymer heat-set gelation processes including whey proteins gelation (see for instance Paulsson and others, 1990; Hines and Foegeding, 1993; Huang and others, 1994; Lefebvre and others, 1998; Gosal and Ross-Murphy, 2000; Kavanagh and others, 2000a; Tavares and da Silva, 2003; Gonçalves and others, 2004). Initially G'' is slightly higher than G' because of the liquid nature of the sample and the absence of pre-aggregated protein molecules (Kavanagh and others, 2000b). The values at this stage are quite scattered as they are very close to the resolution limit of the rheometer. As the temperature rises both moduli decrease until the gelation treshold is achieved (either before the end of the temperature ramp or during the time sweep step). As this point approaches, a sudden increase in the values of G' and G'' is observable. However G' rises much faster and the crossover G'-G'' point is often considered the gelling point. By the same time the values of the loss angle decrease even more markedly, sign of the increase of the elastic behaviour. The increase in the storage moduli and the reduced phase angles indicate the formation of viscoelastic gels. G' continues to increase after the gel point as more and more protein reinforces the weak tridimensional network initially formed, enhancing its elasticity.

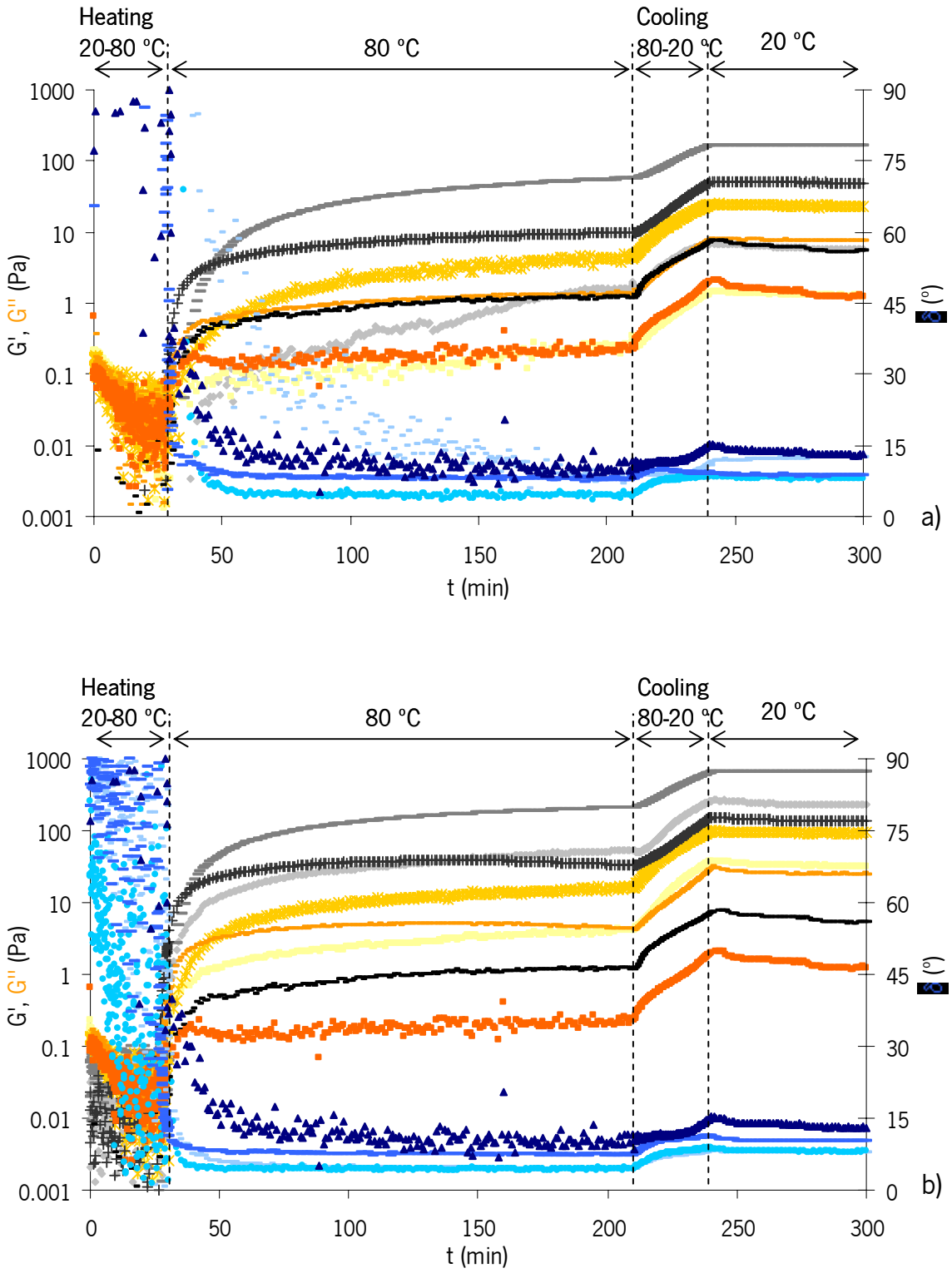

Figure 6-2 Influence of the degree of hydrolysis on the gelling ability of whey peptic hydrolysates: *G'* is presented on a grey scale, *G''* on an orange scale and δ on a blue scale; the degree of hydrolysis (0, 1.5, 2.5 and 4.9 %) is represented by the intensity of the colour (the darker the colour the higher the degree of hydrolysis) – a) 7.5 % w/w (except *DH* 4.9 % - 16.5 % w/w); b) 10.0 % w/w (except *DH* 4.9 % - 16.5 %)

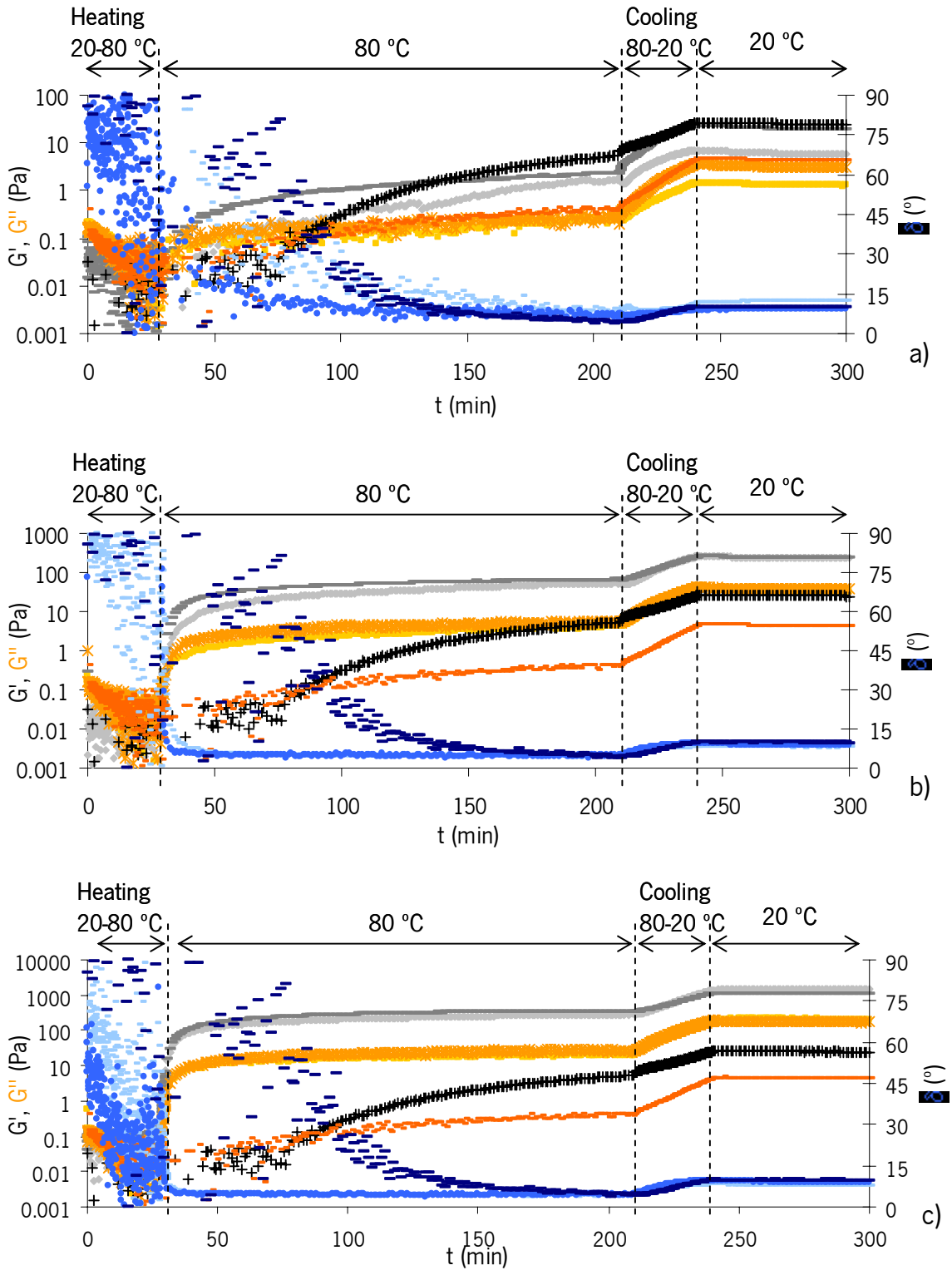

Figure 6-3 Influence of the degree of hydrolysis on the gelling ability of whey tryptic hydrolysates: *G'* is presented on a grey scale, *G''* on an orange scale and δ on a blue scale; the degree of hydrolysis (0, 1.0 and 3.5 %) is represented by the intensity of the colour (the darker the colour the higher the degree of hydrolysis) – a) 7.5 % w/w (except *DH* 3.5 % - 13.0 % w/w); b) 10.0 % w/w (except *DH* 3.5 % - 13.0 %); c) 13.0 % w/w

The increase of *G'* during the cooling period is probably due to the strengthening of the inter-particle attractive non-covalent bonds such as van der Waals interactions and hydrogen bonds, as the temperature decreases (e.g. Doucet and others, 2001; Ould Eleya and others, 2004). This indicates a significative contribution of this kind of bonds to the stabilization of the gel structure. On the other side, the loss angle seems to increase slightly during the cooling ramp which may indicate a slight decrease in the elasticity of the gel. After the cooling step, the gels were left to equilibrate at 20 ° C for 60 min. Mechanical spectra were then recorded (Figure 6-6).

Although the overall gelation patterns are similar for all tested samples the corresponding gelling parameters (*G'*, *G''*, δ, *Tg*, *tg*) are quite different. *G'* was higher for P1.5 (138 Pa) followed by P2.5 (58 Pa) and T1 (17 Pa), for a hydrolysate concentration of 7.5 % w/w. All three were stronger than WPC at this concentration (*G'* = 5.7 Pa) indicating that they were stiffer. They were also more elastic as the loss angle was smaller. However it appears that WPC gel at 7.5 % did not reach the pseudo-equilibrium plateau during the cure time. Apparently pepsin is more effective in improving the gelling ability of whey protein gels than trypsin (although *DH* is not exactly the same and higher degrees of tryptic hydrolysis could lead to better results), possibly because β-Lg (the main gelling protein) is resistant to pepsin. In fact, P1.5 still has all the β-Lg intact and P2.5 still has 96 % of intact β-Lg (Chapter 3). This improvement might be due either to the presence of low molecular weight hydrophilic peptides which can reduce electrostatic repulsions between intact β-Lg molecules enhancing protein-protein interaction (by hydrophobic and/or disulphide bonds) or to the partial unfolding of α-La and BSA exposing their hydrophobic residues, therefore improving their individual gelling ability and/or allowing for a better interaction with the intact β-Lg. In the case of tryptic hydrolysates, it has been referred that limited proteolysis releases several peptide domains from the central core domain of β-Lg (Chen and others, 1994). These domains have high hydrophobicity and interactions between these domains and intact β-Lg (chemical and/or hydrophobic) can be responsible for the improvement of the *G'* value.

Improved values of storage modulus for hydrolysed whey proteins have been referred before for several enzymes (Ju and others, 1995; Otte and others, 1996; Kuipers and others 2008).

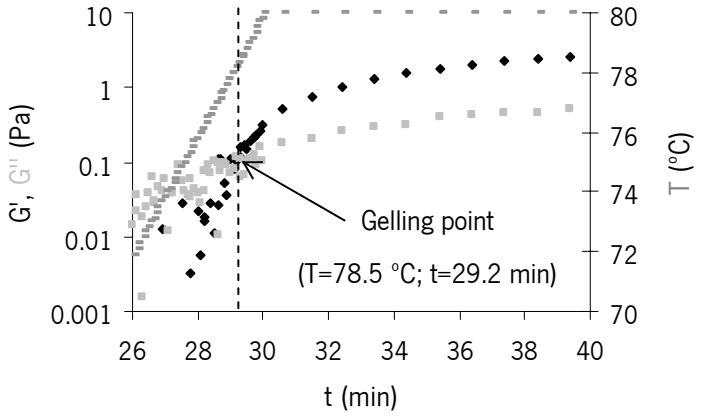

Figure 6-4 Detail of Figure 6-2; example of the determination of the gelling point considering the criteria $G' = G''$ (sample of P2.5 at 7.5 % w/w): **–** *G'*; – *G''*; – *T*

In this work, the gelling point was considered to be the point where G' equals G'', at the fixed frequency of 1 Hz (Figure 6-4). It is clear from Table 6-2 that the gelling time decreases with the increase of concentration for all hydrolysates, as expected. In fact, it has been referred that $G' \propto C^n$ (Clark and Ross-Murphy, 1987; Paulsson and others, 1990; Gosal and Ross-Murphy, 2000, among others) where *n* ranges from 1.75 to 2.25 when *C* is well above the critical value (> 5×*Cg*). However, this value (*n*) increases with the decrease of the concentration and tends to be much larger for concentrations closer to the gelling limit. Besides, the calculation of *n* supposes that the measurements are isothermal and that is rarely the case as the heating step is not instantaneous.

Table 6-2 Influence of the degree of hydrolysis and of protein concentration on the gelling ability of WPH

	WPC	WPC	WPC	P1.5	P1.5	P2.5	P2.5	P4.9	T1.0	T1.0	T1.0	T3.5
C (% w/w)	7.5	10	13	7.5	10	7.5	10	16.5	7.5	10	13	13
tan δ	0.222	0.14±0.00	0.14	0.14±0.01	0.14	0.16±0.01	0.18	0.20±0.04	0.17±0.01	0.15±0.01	0.16	0.19
Tg (°C)	80	79.5±0.0	75.8	80	80	78.4±0.1	73.7	80	80	79.7±0.1	76.6	80
tg* (s)	2059	-	-	348±31	36	-	-	0±0	296±42	-	-	3014
γc (%)	>2	1.8	>2	>2	>2	>2	1.2	1.1±0.5	>2	>2	**0.4**	>2
γr (%)	17.2	>4.0	>4.2	3.5	4.7	15.8	2.4	5.0±0.5	15.8	4.4	0.92	17.3

*Time to gelling point after the temperature ramp (after achieving 80 °C); value on bold slitghly out of the linear viscoelastic region.

The gelation time decreased with the increase of *DH* for both peptic and tryptic hydrolysates, as long as the hydrolysates kept good gelling properties (low *DH*). Reduced gelation times have been reported by several authors (Chen and others, 1994; Huang and others, 1999; Foegeding and others, 2002). This observation has been ascribed to a lower structural stability of the peptide domains when compared to that of the intact proteins (Chen and others, 1994) leading to a lower denaturation temperature. Barbeau and others, 1996 have also referred a lower denaturation temperature in mixtures β-Lg/β-Lg hydrolysates (which is the case of hydrolysates with low degree of hydrolysis), although they report that the presence of some isolated fractions of the hydrolysates can have the reverse effect and stabilize β-Lg structure against heat treatment. However this decrease of the gelation time held true only for low protein concentration, near the critical gelling point of WPC. For higher protein concentration this behaviour tends to invert (Table 6-2). Although the total protein concentration (in mass) of WPC and of hydrolysates is the same it is possible that there are small peptides unable to gel in the hydrolysates. Thus, the concentration in gel-forming proteins and peptides may be lower in the hydrolysates. Therefore, although at low concentrations this effect may not be visible, the increase in the concentration leads to a higher increase of gelling fragments in the WPC than in the hydrolysates.

This concentration effect may also explain the differences in the behaviour of G', G'' and δ during the gelling process at higher protein concentrations (Figure 6-2; Figure 6-3; Figure 6-5). For peptic hydrolysates it explains also why the slowing down of the increase of G' with the increase of the concentration is higher for P2.5 (with a higher amount of smaller peptides, possibly with non-gelling properties) than for P1.5. Besides, whereas for WPC and P1.5 the tangent of the loss angle is 0.14 (it is higher for WPC at 7.5 %, but probably because it is too close to non-gelling conditions), for all other hydrolysates this value is slightly higher (Table 6-2) indicating the presence of a slightly stronger viscous character relatively to elastic character.

For higher degrees of hydrolysis the amount of protein that can effectively take part in the gelling process decreases dramatically and thus the values of both the storage and loss moduli strongly decrease.

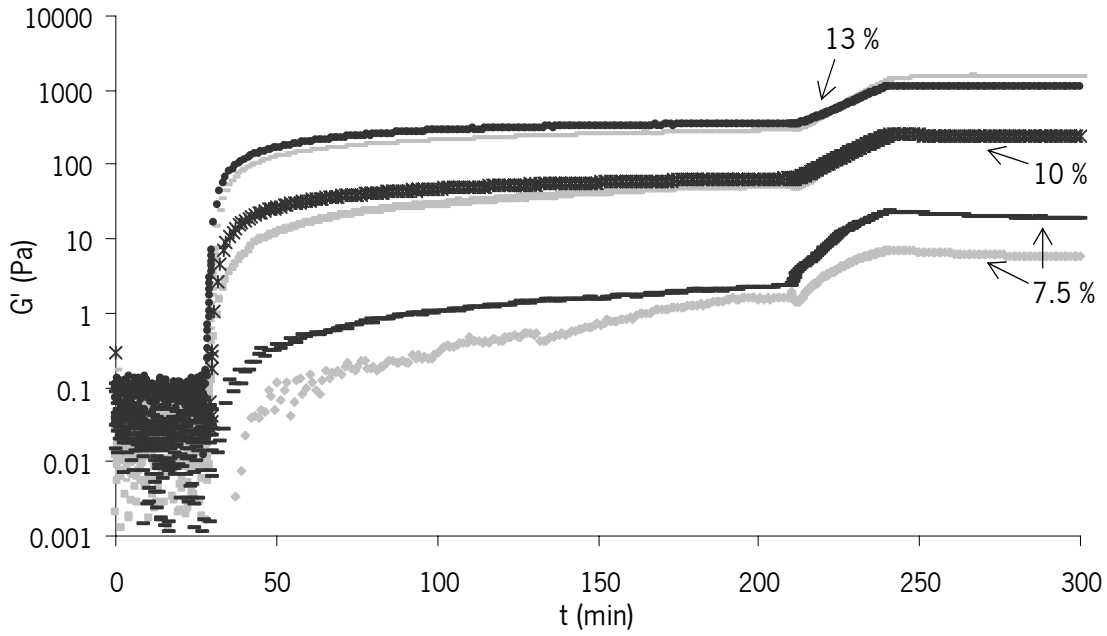

Figure 6-5 Influence of the protein concentration (% w/w) on the gelling ability of WPC and T1: – WPC; **–** T1

Critical deformation was determined considering that above the deformation point at which *G'* varies more than 5 % relatively to the initial constant *G'* value, the viscoelastic linearity is no longer true. This criterium has been widely applied and is reffered for instance by Lefebvre and others (1998), Pouzot and others (2004), and Ould Eleya and others (2004). As the strain sweep was only made until 2 %, in most cases the critical deformation value (γc) was not achieved. However it can be concluded that generally the γc value decreases when protein concentration increases. This fact has already been mentioned in the literature (Lefebvre and others, 1998) and can be attributed to the increase of the heterogeneity of the gel as its concentration increases. Nevertheless, for WPC, the value of γc reverts the trend and increases again when the protein concentration is further increased from 10 to 13 %. In fact, it has been referred (Shih and others, 1990; Foegeding and others, 2002) that γc can rise with a further increase of the concentration due to a strengthening of the gel network (reflected on a large increase in the values of *G'*). The decreasing of γc with the increasing network concentration is usually indicative of a strong-linked network (Shih and others, 1990; Foegeding and others, 2002).

Gels from hydrolysates rupture at lower strains than gels from WPC (for the same values of protein concentration). This may be indicative of more particulate gels with a coarser network structure (Ould Eleya and others, 2004). Several authors consider that whey proteins hydrolysates form particulate gels (e.g. Huang and others, 1999).

The spectra for protein and hydrolysates solutions showed typical liquid behaviour in all cases, with G'' larger than G' (in the sensibility range of the rheometer) and loss angles well above 45 °, near 90 ° over a wide range of frequencies (results not shown). The slope of the G' curve was also higher than the slope of the G'' curve (log scale), as expected for liquids.

The mechanical spectra for G', G'' and δ are presented in Figure 6-6. The wide zone with both moduli nearly independent on the frequency is tipical of a gel character (Ross-Murphy, 1995). A flat slope is indicative of a strong, elastic gel (Foegeding and others, 2002). The extreme values can also be indicative of a nonlinear viscoelastic behaviour. When the gel network bonds are of purely chemical nature G' and G'' are independent of the angular frequency. When physical bonds are also involved then $G' \propto \omega^{n'}$ holds true. In the case of protein gels this dependence exhists but is minimal (Clark and Ross-Murphy, 1987; Doucet and others, 2001). As can be seen in Figure 6-6 the values of n' ranges from 0.07 to 0.10. These values indicate that although chemical bonds exist, physical bonds also play a role in the gel structure. It has been often referred that chemical bonds are formed in heat-induced reactions of proteins, namely intermolecular-disulphide bonds and sulphydryl/disulphide bond exchange reactions (e.g.Roefs and de Kruif, 1994; Swaisgood, 2005). Also non covalent bonds such as those created by van der Waals attractive forces, electrostatic interactions, hydrogen bonds and hydrophobic interactions are usually present (e.g. Verheul and others, 1998; Galani and Apenten, 1999; Totosaus and others, 2002; Ould Eleya and others, 2004). Verheul and Roefs (1998) and Doucet and others (2001) mentioned a value of n' of 0.07 for WPI gels while Stading and Hermansson (1990) quoted values of 0.06 for particulate gels and 0.04 for fine-stranded gels of β-Lg. However it is likely that WPC has a higher n' than WPI and β-Lg. WPC is not a pure gelling protein but a misture of gelling proteins and it has still a small amount of lactose among other constituents. These constituents probably interfere with the structure of the network, increasing the physical contribution or inhibiting the chemical contribution.

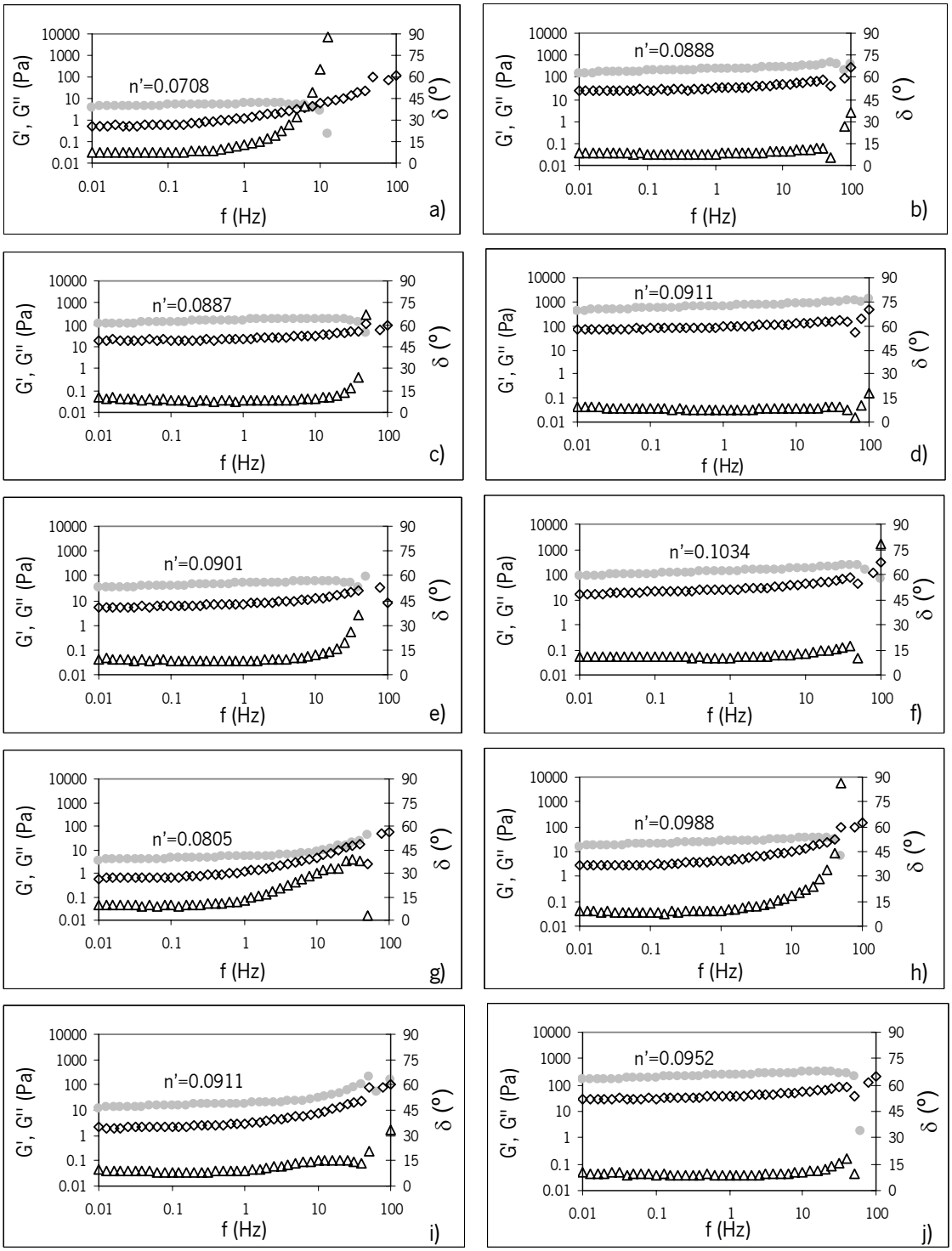

Figure 6-6 Influence of the degree of hydrolysis on the frequency spectra of whey protein hydrolysate gels (● - *G'*; ◊ - *G''*; △ - *δ*): a) WPC 7.5 % (w/w); b) WPC 10 % (w/w); c) P1 7.5 % (w/w); d) P1 10 % (w/w); e) P2.5 7.5 % (w/w); f) P2.5 10 % (w/w); g) P4.9 16.5 % (w/w); h) T3.5 13 % (w/w); i) T1 7.5 % (w/w); j) T1 10 % (w/w)

An increase in the loss moduli of the gels closer to the gelling point (Figure 6-6 a), g), h), i)) was observed at higher frequencies. This was also referred by other authors (e.g. Pouzot and others, 2004) and is caused by the relaxation of the solution phase and the gel phase on small length scales (smaller than the aggregates that form the network).

The different *n'* values for the hydrolysates indicate that the decreasing amounts of β-Lg in the native form and the increase of the amount of released peptides through hydrolysis change the balance between the forces that support the tridimensional structure of the gel. The physical contribution to the gel structure seems to increase with the degree of hydrolysis, except for P4.9 (probably because it is too close to the critical point). This may be due to both the exposure of hydrophobic whey protein residues, which contributes to increase the hydrophobic interactions, and to the decrease of electrostatic repulsions owed to the presence of small hydrophilic peptides.

6.4 Conclusion

The gelling ability of whey proteins can be changed by limited hydrolysis. Depending on the environmental conditions it can either be improved or impaired.

At WPC concentrations close to the gelling point, stronger gels with lower gelation temperatures can be achieved with limited hydrolysis of whey proteins. However, at higher protein concentrations this effect is impaired. There is an increase of the gel strength with the increase of the protein concentration, as expected, but this increase is smaller for the hydrolysates than for the intact proteins. In fact, a similar increase in the protein concentration corresponds to a lower increase in the amount of protein with effective gelation ability in the case of the hydrolysates. The relative importance of non-covalent interactions in the structure of whey protein gels seems to increase with the degree of hydroslysis.

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Chapter 7 Rheological characterization of gels from whey protein hydrolysates/locust bean gum mixed systems

7.1 Introduction

The functionality of whey proteins can be changed by the presence of other components. Proteins and polysaccharides are two important types of food macromolecules and are the most important structure forming ingredients in foods (Tolstoguzov, 1992). In fact, protein-polysaccharide complexes exhibit many functional properties able to provide new food texturization and stabilization methods (Schmitt and others, 1998).

Synergistic effects have been found between whey proteins and several polyssacharides such as galactomannans, xanthan, pectin or carrageenan (Ndi and others, 1996; Capron and others, 1999a; Croguennoc and others, 2001; Turgeon and Beaulieu, 2001; Baeza and others, 2002; Tavares and da Silva, 2003; Gonçalves and others, 2004; Bertrand and Turgeon, 2007 and many others). The effect of limited proteolysis in the interaction with polyssacharides is hardly ever mentioned.

Locust bean gum (LBG) is a galactomannan (non gelling neutral polysaccharides found in the endosperm of *Leguminosae*) widely used in the food industry as a thickening agent (Pollard and Fischer, 2006). It is also known as carob gum or carubin. It has a linear main chain of (1→4)-linked β-D-mannopyranosyl residues partially substituted with single α-D-galactopyranosyl residues grafted by (1→6)-linkages to the main chain (McCleary and others, 1985). The mannose to galactose ratio is ca. 3.5-4. Usually at high to intermediate mannose to galactose ratios (low degree of substitution), as is the case of LBG, the galactomannan is only partially soluble in water, while at very high levels of substitution the polymers are fully soluble (Pollard and Fischer, 2006).

In this chapter the effect of LBG on the gelation of aqueous whey protein hydrolysates from trypsin and from pepsin was assessed at pH 7.0. Confocal laser scanning microscopy was used to analyse gels microstructure. Two different studies of the mixed systems were made: one with three different fractions of LBG (with different degrees of substitution) and another one with different LBG concentrations.

7.2 Materials and methods

All chemicals used were of analytical grade and supplied by Sigma, Co (St. Louis MO, USA). Trypsin from porcine pancreas with an activity of 1800 BAEE units/mg (one BAEE unit will produce a ΔA_{253nm} of 0.001 per min at pH 7.6 and 25 °C using BAEE as substrate; in a reaction volume of 3.2 mL and 1 cm light path) was obtained from Sigma Chemical Co (ref. T7409).

A commercial whey protein concentrate (WPC) powder (Lacprodan 80, batch Q500246) kindly supplied by Arla Food Ingredients (Viby, Denmark) was used for the experiments. According to the suppliers, the WPC dry basis protein content was 82 % (5.5 % moisture), the ash content was 3.5 % max., the lactose content was 7 %, and fat content was 8 %. max. The whey protein hydrolysates used in the work described in this chapter were the same as in Chapter 6. Locust bean gum (> 75 % galoctomanan content) was kindly supplied by Danisco Portugal (Faro, Portugal).

7.2.1 Purification and fractioning of the LBG

Locust bean gum was purified by precipitation with isopropanol as described by da Silva and Gonçalves (1990). A LBG solution was prepared by gradually adding the gum to strongly stirred distilled water (1.5 % w/w). The dispersion was stirred during 1 h at room temperature and then heated at 80 °C for 30 min. After cooling, the solution was centrifuged at 35000 g at 20 °C to remove the insoluble material. The solubilized LBG was recovered from the solution by slowly pouring (drop by drop) into a two volume excess of isopropanol with gentle mixing. The white fibrous precipitate was collected by vacuum filtration and washed consecutively with isopropanol, acetone and diethyl ether. Purified LBG (LBGP) was then dried under vacuum for 24 hours and ground to a fine powder.

For the LBG fractioning, a LBG solution was prepared as described above. The dispersion was stirred at 20 °C for 2 h and centrifuged at 35000 g to remove insoluble material. The solubilized LBG was treated as described above resulting in the LBG fraction soluble at 20 °C (LBG20). The insoluble material was redispersed in water and stirred during 1 h at room temperature. It was then heated at 80 °C for 30 min and then treated as described for LBGP and LBG20. LBG fraction soluble at 80 °C (LBG80) resulted from this procedure.

7.2.2 LBG mannose-galactose ratio

The galactomannans were hydrolysed releasing the monosaccharides that were then converted into alditol acetates. These alditol acetates were identified and quantified by gas-chromatography. The procedure was based on that described by Blakeney and others (1983) as described by Coimbra and others (1996). A small amount of LBG (2-3 mg) was dispersed in 0.2 mL of 72 % H_2SO_4 in a capped glass tube for 2-3 h at 20 °C. This sample was further diluted to 1M H_2SO_4 (2.2 mL H_2O) and hydrolysed for 2.5 h at 100 °C. It was then cooled in an ice bath and 200 μL of internal standard (2-deoxy-glucose, 1 mg/L) were added. An aliquot (1 mL) of the resulting solution was transferred to a test tube and neutralized with 200 μL of 25 % NH_3. Subsequently, the sugars were reduced with 100 μL of sodium

borohydride (15 % w/v in 3 M NH_3; freshly prepared) for 30 min at 30 °C. The solution was then cooled in an ice bath and the reaction was stopped by two consecutive additions of 50 μL of acetic acid. An aliquot of 300 μL was transferred to a SOVIREL test tube and placed in an ice bath. 450 mL of 1-methylimidazole and 3 mL of acetic anhydride were added, mixed in a vortex and incubated for 30 min at 30 °C. The remaining acetic anhydride was decomposed by adding 3 mL of water and the alditol acetates were extracted with 2.5 mL of dichloromethane. The dichloromethane phase was then washed three times with water (each time the water was added, the resulting mixture was well stirred and centrifuged at 3000 rpm) and then evaporated to dryness under a stream of nitrogen at 40 °C. The resulting alditol acetates were stored in a desiccator until further use. Mannose and galactose content were then determined by resuspending the resulting alditol acetates in 50 μL of anhydrous acetone and injecting them in a DB-225 capillary column (30 mm length; 0.25 mm internal diameter; 0.15 μm film thickness) installed in a gas chromatograph (Hewlett Packard 5890) equipped with a flame ionization detector. The carrier gas was helium and the operating conditions were: 2 μL of injection volume; helium flow rate: 1.2 mL/min; injector temperature: 220 °C; detector temperature: 230 °C; oven temperature: 220-230 °C; temperature gradient: 4 min at 220 °; 25 °C/min until 230 °C; 6.5 min at 230 °C. Myo-inositol was used as internal standard.

7.2.3 LBG intrinsic viscosity

A Cannon-Fenske viscometer for transparent liquids (according to ASTM D-2515) was used to measure intrinsic viscosities.

LBG solutions were prepared to obtain relative viscosities from 1.2 to 2.0 (approximately), to allow linear regression and extrapolation to zero (Sittikijyothin and others, 2005). After filling, the viscometer was placed into a water bath at 25±0.5 °C and the solution was allowed to equilibrate for 5 min. It was then pulled under vacuum to the upper mark of the Cannon-Fenske viscometer and allowed to flow. The time that the solution took to flow from the upper to the lower mark was measured five times. The average time value was used to calculate the relative viscosity (η_{rel}):

$$\eta_{rel} = \frac{\eta}{\eta_s} = \left(\frac{\rho}{\rho_s}\right)\left(\frac{t}{t_s}\right) \qquad \text{Eq. 7.1}$$

As LBG concentration of the used solutions was very low, differences in density were not considered when calculating relative viscosities. η, ρ and t represent the viscosity, the density and the time of flow

of the polysaccharide solution, respectively, and the subscript *s* refers to the same properties relating the pure solvent (water, in this case).

The intrinsic viscosity ($[\eta]$) can be given by:

$$[\eta] = \lim_{c \to 0} \left(\frac{\frac{(\eta - \eta_s)}{\eta_s}}{c} \right) \qquad \text{Eq. 7.2}$$

In practice, to obtain the intrinsic viscosity, the combined Huggins and Kramer extrapolation was used:

$$\frac{\eta_{sp}}{C} = [\eta] + k'[\eta]^2 C \qquad \text{Eq. 7.3}$$

$$\frac{\ln \eta_{rel}}{C} = [\eta] + k''[\eta]^2 C \qquad \text{Eq. 7.4}$$

where η_{sp} and η_{rel} represent the specific and relative viscosities (both dimensionless), respectively, *k'* is the Huggins' coefficient, *k''* represents the Kramer's coefficient and *C* is the concentration of the solution.

$$\eta_{sp} = \left[\frac{\eta - \eta_s}{\eta_s} \right] = \eta_{rel} - 1 \qquad \text{Eq. 7.5}$$

Viscosity average molecular weight ($\bar{M}_v$) for each LBG sample was calculated using the Mark-Houwink relationship given by Doublier and Launay (1981) and modified by Gaisford and others (1986) to take into account the different D-mannose to D-galactose ratios (*M/G*) of the LBG (Sittikijyothin and others, 2005):

$$[\eta] = 11.55 \times 10^{-6} \left[(1 - \alpha) \bar{M}_v \right]^{0.98} \qquad \text{Eq. 7.6}$$

where $\alpha = \frac{1}{(M/G) + 1}$ Eq. 7.7

7.2.4 WPH/LBG solutions

LBG stock solutions were prepared by gradually adding the gum to strongly stirred distilled water (1.3 % w/w). Sodium azide (5 ppm) was added to each solution in order to avoid bacterial growth. The

dispersion was stirred during 1 h at room temperature and then heated at 80 °C for 30 min. After cooling, the solution was centrifuged at 35000 g to remove the insoluble material. The final concentrations of LBG solutions were determined from their dry matter contents.

To prepare the mixed systems, the required amount of WPC or WPH was weighted and distilled water was added to pre-solubilize the protein. Then the required amount of LBG stock solution (ca. 1.3 % (w/w)) was added. NaCl (20 % w/w) was added to a final salt concentration of approximately 0.1 M to ensure constant ionic strength and the final mass was completed with distilled water. The solution was then gently stirred for 1 h, at room temperature. The pH was adjusted to 7.0 and the solution was stirred for 2 h more. The final systems had a concentration of LBG ranging from zero to 0.8 % w/w.

7.2.5 Experimental design (factorial planning)

The effect of predefined mutually independent variables (factors) thought to influence the response parameter(s) of interest can be studied using a regression analysis. The set of factors is varied in a structured manner in order to study the effects of the factors on the response variable. If all variables have to be optimised consecutively many experiments are needed, especially when variables have interactive effects. Therefore, experimental designs are developed for efficient experimentation (van der Ven, 2002). Factorial designs are used primarily for screening significant factors, but can also be used sequentially to model and optimize a process. The design generated will include all possible combinations of the factor levels. Factorial designs of a high number of factors can result in a very high number of experiments. In this case, other experimental designs (such as fractional factorial design) should be used to reduce the number of experiments to a reasonable value.

Usually the outcome of an experiment is dependent on the experimental variables and can be described as:

$$y = f(x_1, x_2, ..., x_i) \qquad \text{Eq. 7.8}$$

This function can be approximated by a polynomial function representing a good description of the relationship between the experimental variables and the responses within a limited experimental domain (Lundstedt and others, 1998). In the case of two independent variables three empirical polynomial models are often used: a linear model, a linear model with an interaction term between the two variables or a quadratic model. This last one allows the determination of non-linear relationships between the experimental variables and the responses and the identification of an optimum point (Lundstedt and others, 1998). It can be represented by:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2 + residual \quad \text{Eq. 7.9}$$

An analysis of variance is helpful in determining the fitness of a model. If several replicates are available, it is also possible to determine if the"lack of fit" is significant or not.

In this work a full factorial design was firstly used considering two factors (LBG type, considering its mannose to galactose ratio, and degree of hydrolysis) and three levels for each factor. Two replicates of each experiment were used to estimate errors and determine if the lack of fit of the chosen model is significant. The replicate LBG and hydrolysates solutions were freshly prepared, but the hydrolysate used was from the same batch. Thus the estimated errors do not include the error from the hydrolysis process, as all the conditions for the experiments were re-created except the hydrolysis process. Two sets of experiments were performed: one with hydrolysates from trypsin and other with hydrolysates from pepsin. The results were analysed with Design Expert 6.0.6 (Stat-Ease, Inc. Minneapolis). Further refinement of the empirical model was made by excluding the factors that were found to be insignificant, one at a time, as the exclusion of one factor may influence the other (as suggested by Lundstedt and others, 1998).

Another set of experiments was made based on a full factorial design considering again two factors (LBGP concentration and the degree of hydrolysis) and three levels for each factor. Two replicates of the experiments with WPC (*DH* = 0) were used to estimate errors and determine if the lack of fit of the chosen model is significant.

7.2.6 Rheological measurements

Dynamic oscillatory tests were performed in a controlled stress rheometer AR2000 (TA Instruments, Delaware, USA) as described in 6.2.7.

The rheological behaviour of each LBG stock solution was analised to verify whether it maintained its viscous properties from test to test. A LBG80 stock solution was prepared each day, a LBGP stock solution was prepared every other day and the stock solutions of LBG20 were prepared for 3 days. Within these periods it was considered that the rheological properties of the LBG solutions did not change significantly. A cone and plate geometry (40 mm diameter, angle of 2° and truncation of 54 μm) was used and a frequency sweep test (mechanical spectrum) was performed from 100 to 0.1 Hz, followed by the determination of the flow curve (shear ramp from 0.01 to 100 s^{-1} followed by a shear ramp from 100 to 0.01 s^{-1}).

7.2.7 Microscopy study of the mixed gels

Mixed solutions were prepared as described in 7.2.4. Proteins were stained with Rhodamine B Isothiocyanate (RBITC) through covalent linking between RBITC goups and the amino groups of the protein molecules. The solutions were placed on a concave slip, covered with a slide and hermetically sealed to prevent evaporation. They were then placed in a water bath at 20 °C. A temperature ramp of 2 °C/min was applied for 30 min followed by 3 h at 80 °C, after which the samples were allowed to cool to 20 °C and stored in the absence of light. They were observed under a confocal laser microscope (Leica TCS SP2 AOBS, Leica Ltd., Heidelberg, Germany) in the fluorescence mode, excited at 591 nm with a laser, and the emission fluorescence was recorded between 570 nm and 688 nm. Objectives with a magnification of 10× (standard) and 63× (only occasionally) were used. Images were analysed with the software Image J 1.38W (National Institutes of Health, USA).

7.3 Results and discussion

Prior to the presentation of the main results of this chapter, a brief characterization of the properties of the locust bean gum is herein reported. Subsequently, oscillatory tests and microscopy results relating gelling of whey protein hydrolysates and mixed systems of whey protein hydrolysates and locust bean gum are described.

7.3.1 LBG characterization

The intrinsic viscosity and *M/G* values obtained for the purified gum (Table 7-1) are in accordance with results found in literature (da Silva and Gonçalves, 1990; Alves and others, 1999; Monteiro and others, 2005; Sittikijyothin and others, 2005). In fact, these authors report *M/G* values ranging from 3.5 to 4.0 and intrinsic viscosities ranging from 11.8 to 13.8 dL/g.

The yields of purification of LBGP and of fractioning of LBG20 and LBG80 are presented in Table 7-1.

Table 7-1 LBG characterization

	Yield of recovery (%)	Ratio D-mannose:D-galactose	Intrinsic viscosity (dL/g)	k'	$\bar{M}_v$
LBG20	26.0	2.72±0.15	13.1±0.2	0.58	2.07×10^6
LBGP	76.5	3.57±0.02	13.5±0.2	0.54	1.98×10^6
LBG80	37.8	4.35±0.03	14.9±0.1	0.41	2.11×10^6

All values of *M/G* and intrinsic viscosity are means ± standard deviation of three determinations.

The yield of purification for the LBG was 76.5 %, in accordance to those achieved by da Silva (1994) and da Silva and Gonçalves (1990) - 78 and 76.9 %, respectively. For the fractioning of the LBG the fraction which is soluble at 20 °C represented 26 % of the initial unpurified LBG and the fraction which is soluble at temperatures between 20 and 80 °C represented 37.8 % of the unpurified LBG. These values correspond to 34 and 49 % of the purified gum, respectively, also in accordance to the values presented by da Silva and Gonçalves (1990): 38 and 52 %. The *M/G* ratio and the intrinsic viscosity increased with the increase of the solubilization temperature, as expected. The higher the *M/G* ratio the less residues of galactose are linked to the mannan chain and the more insoluble is the galactomannan. This difference was not detected for the viscosimetric mean molecular weight but a less substituted chain does not necessarily have a higher or lower molecular weight.

7.3.2 Gelling ability of mixtures LBG/hydrolysates - rheological study of the influence of the concentration of LBGP

Thermodinamic incompatibility between whey proteins and neutral polysaccharides has been widely reported (e.g. Syrbe and others, 1995; Syrbe and others, 1998; Grinberg and Tolstoguzov, 1997). In the case of native whey proteins this incompatibility may be restricted to a specific pH range (e.g. 5-7) and to high concentrations of the polysaccharide (Syrbe and others, 1998). Phase separation in mixtures of gelling proteins with gelling polysaccharides increases the effective concentration of both biopolymers by confining them to a smaller volume. This can lead to a significant enhancement in *G'* and *G''* (Clark and Ross-Murphy, 1987; Fitzsimons and others). In the case of a mixture of a gelling biopolymer and a non-gelling biopolymer, segregative interactions can also lead to higher gel strength. In fact synergistic effects have been referred for guar gum (Fitzsimons and others), xanthan (Bryant and McClements, 2000), cassia gum (Gonçalves and others, 2004), tara gum (Sittikijyothin and others, 2007), pectin (Beaulieu and others, 2001) or locust bean gum (Tavares and da Silva, 2003). At low polysaccharide concentrations the segregative phase separation can also give rise to an increment of the protein concentration, increasing the elastic response if the connectivity between the protein aggregates is not affected. Thus the effect of a polysaccharide on the gelation of a protein depends on the nature of the polysaccharide, on the pH, on the ionic strength, on the temperature treatment and on the concentrations used (Olsson and others, 2002a).

The time evolution of the viscoelastic moduli and of the loss angle for mixed systems hydrolysates/LBGP followed the general behaviour described for hydrolysates alone. The profile of the storage modulus is presented in Figure 7-1 and Figure 7-2. In all cases it is notorious that for higher amounts of LBGP (≥

0.55 %) the increase in the storage modulus during the transition from the "solution" state to the "gel" state is much smaller than for lower amounts of LBGP. Besides, the resulting gels have a less pronounced elastic character as the final values of tan δ are higher (Table 7-2).

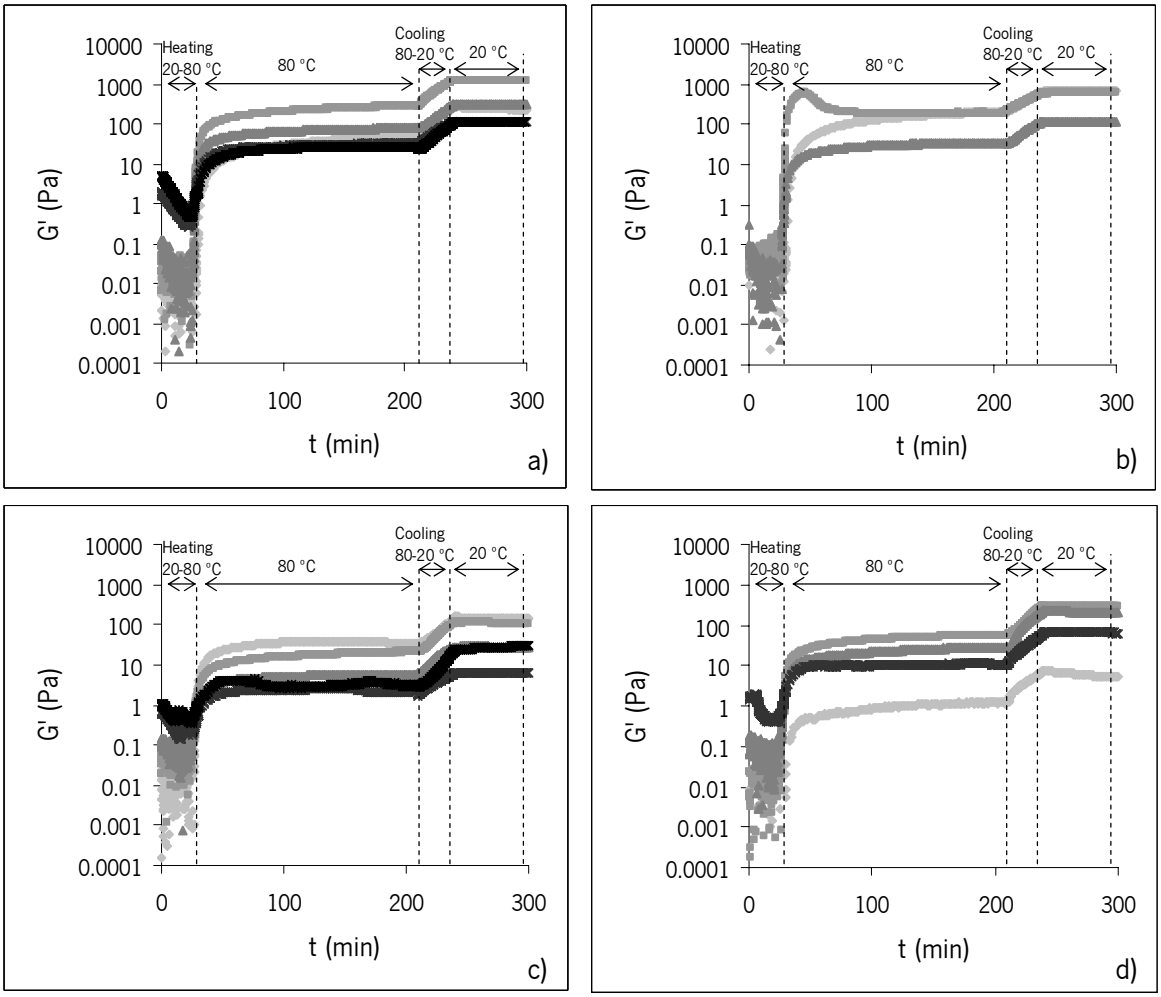

Figure 7-1 Influence of the LBGP concentration on the gelling ability of whey peptic hydrolysates: the darker the colour the higher the LBGP amount (0, 0.1, 0.3, 0.55, 0.8): a) WPC 10 % (w/w); b) P1.5 10 % (w/w); c) P2.5 10 % (w/w); d) P4.9 16.5 % (w/w)

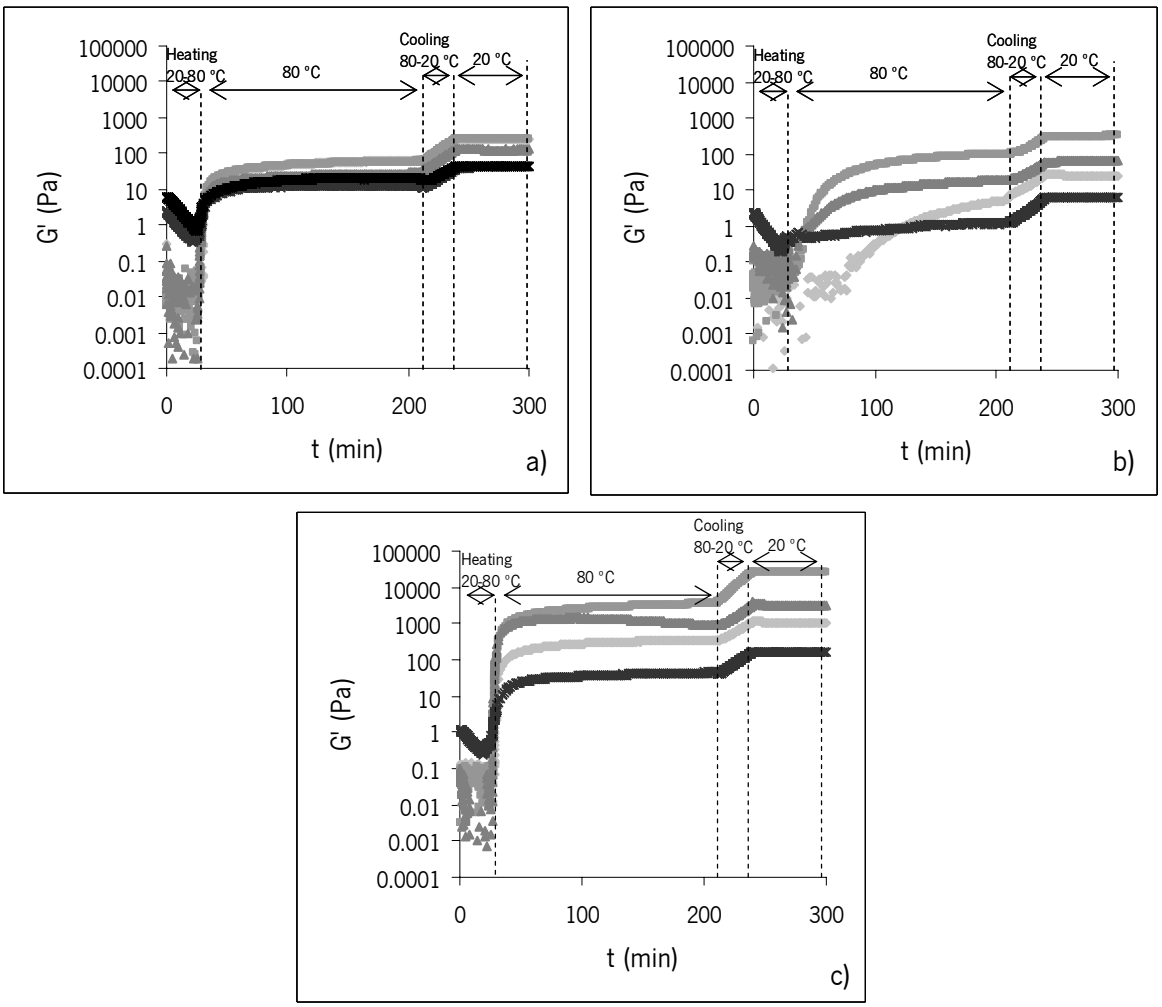

Figure 7-2 Influence of the LBGP concentration on the gelling ability of whey tryptic hydrolysates: the darker the colour the higher the LBGP amount (0 – lighter gray, 0.1, 0.3, 0.55, 0.8 - black): a) T1.0 10 % (w/w); b) T3.5 13 % (w/w); c) T1.0 13 % (w/w)

For WPC at 10 % (w/w) a small amount of LBG was highly beneficial from the storage modulus point of view. However, for tryptic hydrolysates with a low degree of hydrolysis the influence of LBG is almost null and for peptic hydrolysis with low degree of hydrolysis (P1.5 and P2.5), the presence of LBG always decreased the *G'* value although this decrease was gentle for 0.1 % and sharper for 0.3 %.

The microstructural analysis revealed the phase-separated character, as expected. The inclusion LBG led always to some degree of phase separation (Figure 7-3, Figure 7-4, Figure 7-5, and Figure 7-6) with LBG promoting protein aggregation. One phase is rich in protein and the other is protein-depleted and possibly enriched with polysaccharide. All micrographs presented were taken with the 10× lens or with the 63× lens and were magnified digitally four times.

Table 7-2 Influence of the LBGP concentration and hydrolysis degree on the gelling ability of whey protein hydrolysates

Hydrolysate (% w/w)		LBGP (% w/w)	*DH* (%)	*G'* (Pa)	*G''* (Pa)	*tan δ*	*Tg* (°C)	*tg** (s)	*γc* (%)	*γr* (%)
WPC	10	0	0	204±33	28.6±3.8	0.14±0.00	79.5±0.0	-	1.8	>4.0
WPC	10	0.1	0	1436±334	207±47	0.14±0.00	75.2±0.1	-	2.0	>2.8
WPC	10	0.3	0	596±420	97.9±69.1	0.17±0.00	76.0±1.4	-	1.6	10.7
WPC	10	0.55	0	119±7	30.7±1.1	0.26±0.03	78.9±0.6	-	1.0	12.0
WPC	10	0.80	0	108	39.0	0.36	80	326	1.7	23.4
P1.5	10	0	1.5	657	92.8	0.14	80.0	36.0	>2	4.7
P1.5	10	0.1	1.5	588	105	0.18	77.6	-	**0.2**	7.0
P1.5	10	0.3	1.5	118	19.4	0.17	77.9	-	0.8	28.7
P2.5	10	0	2.5	138	24.8	0.18	73.7	-	1.2	2.4
P2.5	10	0.1	2.5	107	16.2	0.15	72.6	-	1.9	8.0
P2.5	10	0.3	2.5	27.8	5.00	0.18	72.4	-	0.8	6.0
P2.5	10	0.55	2.5	29.5	11.4	0.39	78.7	-	0.7	45.0
P2.5	10	0.8	2.5	31.5	13.0	0.41	80	745	1.0	625
P4.9	16.5	0	4.9	18.4±18.4	3.35±3.04	0.20±0.04	80.0±0.0	0.0±0.0	1.1±0.5	5.0±0.5
P4.9	16.5	0.1	4.9	284	43.2	0.15	74.9	-	1.4	7.7
P4.9	16.5	0.3	4.9	207	37.3	0.18	72.8	-	0.8	7.0
P4.9	16.5	0.55	4.9	76.0±17.1	23.8±2.3	0.32±0.04	80.0	35.9±0.1	0.5	130
T1	10	0	1	225±25.1	34.2±5.2	0.15±0.01	79.7±0.1	-	>2	4.4
T1	10	0.1	1	245	42.8	0.17	77.7	-	1.4	3.9
T1	10	0.3	1	126	20.3	0.16	76.2	-	1.9	5.6
T1	10	0.55	1	42.8	15.2	0.35	80.0	0.0	1.0	15.2
T1	10	0.8	1	45.6	18.7	0.41	80.0	505	1.1	26.2
T3.5	13	0	3.5	46.8	9.05	0.19	80.0	3014	>2	17.3
T3.5	13	0.1	3.5	312	57.8	0.19	80.0	565	>2	68
T3.5	13	0.3	3.5	63.7	13.0	0.20	80.0	1282	>2	-
T3.5	13	0.55	3.5	12.2	5.75	0.55	80.0	2656	1.7	65.8
T1	13	0	1	1080	171	0.16	76.6	-	**0.4**	0.92
T1	13	0.1	1	25530	3953	0.15	74.2	-	2.0	>2.0
T1	13	0.3	1	3327	617	0.19	74.6	-	**0.2**	1.3
T1	13	0.55	1	164	55.9	0.34	80.0	86.8	0.6	51.6

Values on **bold** are slitghly out of the linear viscoelastic region.

*Time to gelling point after the temperature ramp (after achieving 80 °C); value on bold slitghly out of the linear viscoelastic region.

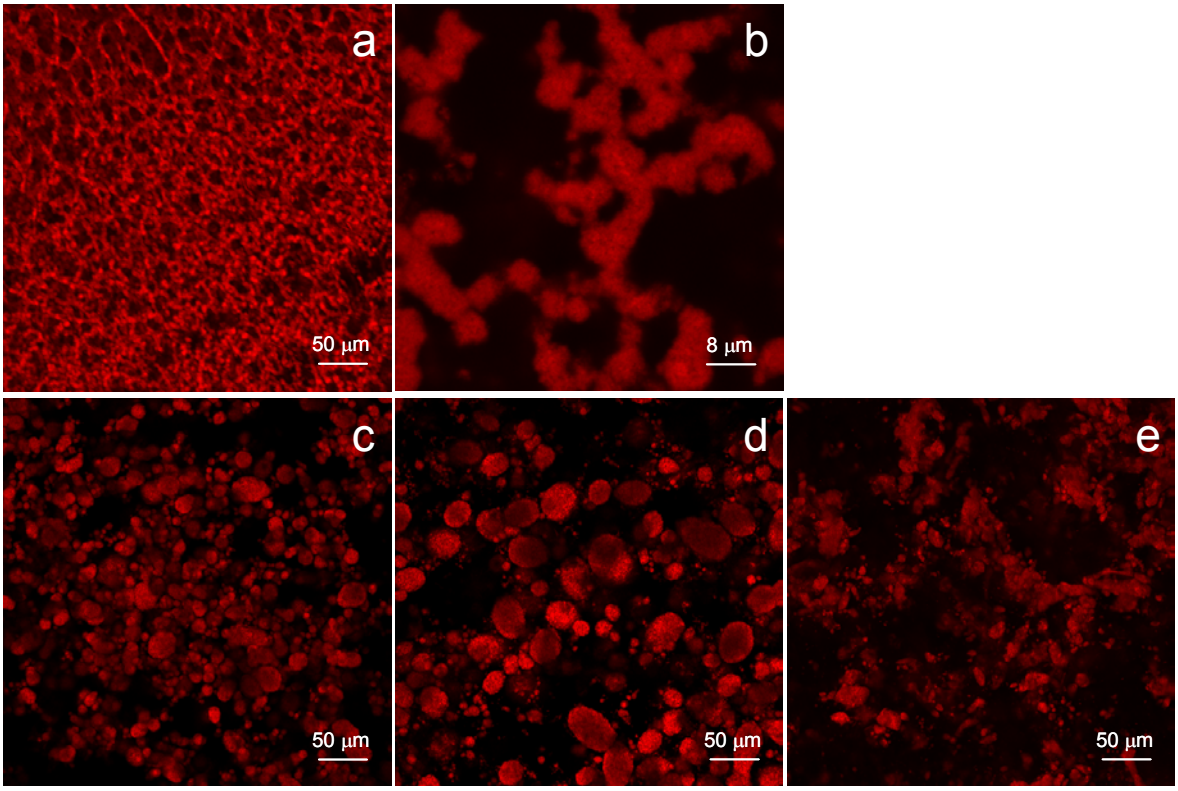

Figure 7-3 Influence of the LBGP on the structure of mixed WPC/LBGP gels (10 % protein): a) 0.1 % of LBGP with the 10× lens; b) 0.1 % LBGP with the 63× lens; c) 0.3 % LBGP; d) 0.55 % LBGP; d) 0.8 % LBGP

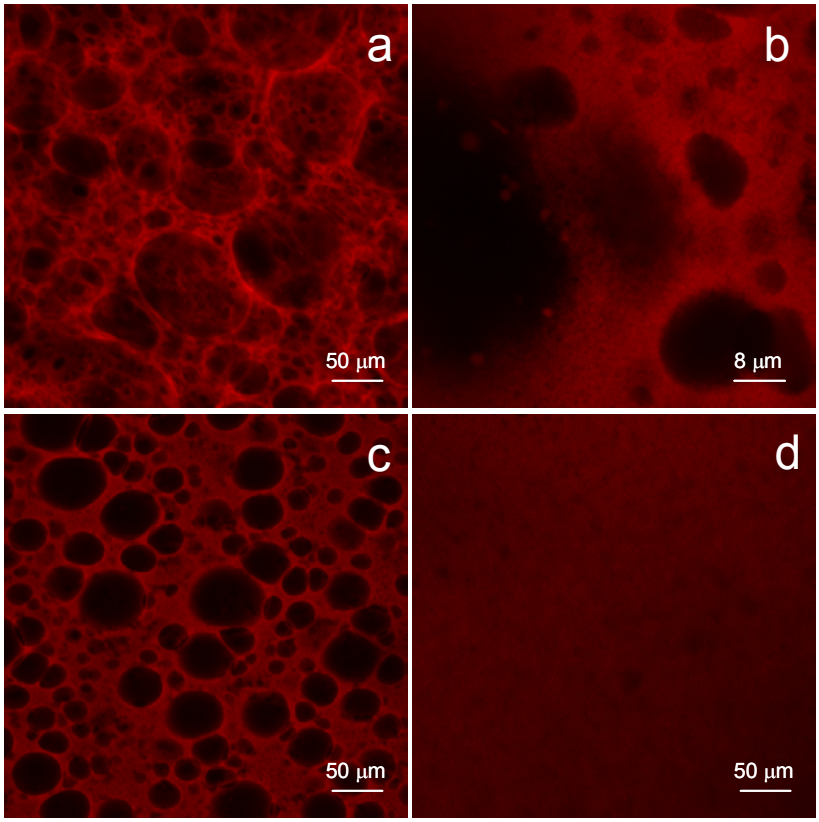

Figure 7-4 Influence of the LBGP on the structure of mixed P1.5/LBGP gels (10 % protein): a) 0.1 % of LBGP with the 10× lens; b) 0.1 % LBGP with the 63× lens; c) 0.55 % LBGP; d) 0 % LBGP

For WPC with 0.1 % LBGP a bicontinuous microstructure was observed (Figure 7-3). As the LBGP concentration increased the microstructure became more coarse-stranded and the protein aggregates bigger and more spherical. The phase separation was more evident and the volume occupied by the enriched protein phase decreased. The aggregates are grouped in large clusters first and then, with a further increase of LBG, in smaller clusters but with bigger aggregates. Further increases of LBG resulted in the shrinkage of the spherical protein domains with higher protein concentration. Even further increases would probably lead to the collapse of the gel structure. This is reflected in the rheological properties of the final gels. For very low LBG content (0.1 %), the phase separation caused an increase in the concentration of protein in one of the continuous phases that subsequently increased the elastic response of the network. For 0.3 % LBG, although the protein concentration is probably further increased, the connectivity of the network is worsened and the storage modulus decreased even though the resulting G'' is still higher than that of the gel with no polysaccharide. For the same amount of LBG, the loss tangent starts increasing indicating an increment in the viscous character relatively to the elastic character. At 0.5 % LBG the protein aggregates are very big and the connectivity of the structure was further reduced, as well as the storage modulus, and at 0.8 % the big aggregates seem to start collapsing into numerous smaller aggregates. The viscous character was further increased, though the storage modulus was not significantly reduced. The gelation temperature follows a similar behaviour. It decreases from 0 to 0.1 % LBG corresponding to the increase of protein concentration in the network and then continuously decreases following the decrease in the network connectivity. The critical strain also decreases whereas the rupture strain increases (at least for higher LBG amounts), indicating a more plastic character of the gel. These findings are in agreement with the weakening of the gel microstructure. Eventually at higher LBG concentrations the system would fully collapse and no gel would form.

A maximum storage modulus for very low concentrations of non-gelling polyssacharides was also reported by other authors. Fitzsimons and others found an optimum concentration of 0.1 % (w/w) guar gum with a 12-fold enhacement in gel strength in comparison with WPI alone. de Jong and van de Velde (2007) found maximum values for all large deformation parameters around 0.1-0.15 % (w/w) for mixed systems with guar or LBG. A similar trend is also reported for κ-carrageenan at pH 7 and an ionic strength of 0.1-0.15 M by Capron and others (1999b) and Neiser and others (2000), with a maximum gel strength for 0.1-0.3 % κ-carrageenan. Although this is a gelling anionic polysaccharide the behaviour seems to be ruled also by aggregation and demixing kinetics as in the present case.

Tavares and da Silva (2003) report a different trend with the increase of LBG concentration. In fact, they refer a continuous increase of the WPI gel strength with the increase of LBG concentration at pH 7. Nevertheless, no salt was added and the ionic strength was probably much lower. It remains to enlighten if at a higher LBG concentration the effect would reverse or if there is no optimum for very low ionic strength. For κ-carrageenan, an increase in the optimum polysaccharide concentration with the decrease of ionic strength was already reported (Neiser and others, 2000), but again this is a gelling polysaccharide. The presence of salt screens electrostatic repulsion between the protein aggregates and allows further aggregation and larger clusters. Thus phase separation is further promoted. The absence of salt will probably delay the phase separation (in comparison with the former case), and the maximum gel strength will probably be achieved for much higher LBG concentrations. In fact, micrographs presented later by the same group of authors (Monteiro and others, 2005) show that phase separation is very low for 0.25 % LBG and more significant for 0.70 % LBG. Even so, this phase separation is only comparable to the phase separation at 0.1 % LBG in the presence of 0.1 M salt. Similar results are achieved by Sittikijyothin and others (2007) with β-Lg (6.5 % w/w) and tara gum (0-0.56 % w/w) at pH 7 and low ionic strength.

Gels from whey protein peptic hydrolysates with 1.5 % of hydrolysis had a different microstructure (Figure 7-4). Although phase separation was also present, a continuous phase enriched in protein and a discontinuous phase (possibly enriched in LBG) were observed. The visible micro-phase separation was lower and the protein phase seems to be highly stranded or with very small aggregates both not visible at a micrometer scale (a TEM microscope would have been more suitable). The inclusion of LBG probably "cleaved" some strands resulting in a more "porous" protein phase but the phase separation also probably increased the protein phase concentration. The change in the gel strength in the presence of 0.1 % LBG was not significant (Table 7-2). However the critical strain was highly decreased and the measurements were made out of the viscoelastic region and are not meaningful. The system was very sensible to strain changes and the 0.5 % strain value used probably affected the microstructure of the gel, breaking some protein aggregates and resulting in a G' corresponding to a lower "measured" gel strength than the effective one. For a higher LBG concentration the boundaries of the discontinuous phase became sharper. The area of the protein network decreased as well as the gel strength.

Peptic hydrolysates with a degree of hydrolysis of 2.5 % led also to a bicontinuous microstructure in the presence of 0.1 % (w/w) LGB as in the case of WPC (Figure 7-5). However the protein strands are thinner and shorter, appearing that the structure was partially broken by the "phase separation". Thus

the resulting storage modulus decreased slightly in comparison to the G' of the P2.5 gel in the absence of polysaccharide and the concentration effect was not observable. It seems quite possible that lower LBG amounts would lead to lower phase separation and to a strengthening of the gel microstructure. Increasing the LBG amount to 0.3 % led to the formation of big spherical protein microdomains with very sharp boundaries. These aggregates seem to be roughly connected with each other forming clusters that sustain the coarse gel structure. Further increase in the LBG concentration resulted in the shrinkage of the big aggregates due to further depletion of LBG, causing further increase of protein concentration inside the microdomains. The volume occupied by the protein-enriched phase was highly reduced (Table 7-3). The gel coarsened and became even weaker. A further increase of LBG to 0.8 % causes the collapse of the clusters. As the LBG phase is supposedly non-gelling, a system with these characteristics should show a liquid behaviour. However the mechanical behaviour of P2.5 with 0.8 % LBG seems to be typical of a gel, although with a strong viscous character with a high value of tan δ, a low storage modulus and a high rupture strain. The storage modulus had even a very slight increase (although this increase is probably related to the increase in the viscous character as tan δ also increases). Neiser and others (2000) also referred a minimum of the storage modulus at 0.6 % carrageenan followed by a slight increase. Either the system is a liquid with a very large relaxation time causing the G' curve to cross G'' for very low frequencies and behaving like a gel at the tested frequency (1 Hz) or the system is indeed a very weak gel. In this case, this could mean that LBGP could form weak gels under the present environmental conditions. In short, for P2.5 the strength of the gel and the gelation time decreased with the increase of the LBG amount (except for the highest value where other phenomena seem to be involved).

Tryptic hydrolysates with a degree of hydrolysis of 1.0 % had a similar behaviour. At 0.1 % LBG a bicontinuous microstructure was observed. Connectivity was severely damaged with the additional increase of LBG concentration. The small spherical protein-enriched microdomains connected in coarse clusters. Higher LBG concentrations (0.55 %) decreased the microdomains size; the clusters became denser and the volume of the phase between the clusters had a minor increase; the protein concentration within the microdomains probably also increased. Increasing LBG concentration to 0.8 % resulted in slightly larger aggregates hardly connected with each other.

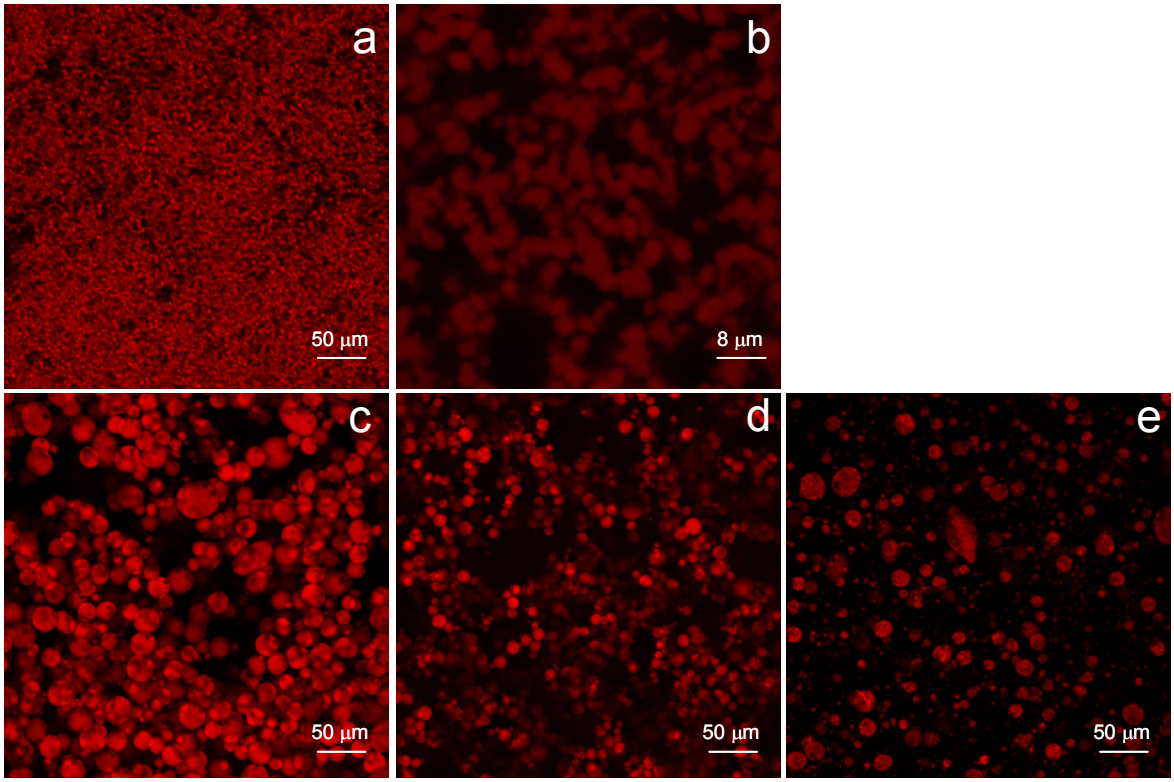

Figure 7-5 Influence of the LBGP on the structure of mixed P2.5/LBGP gels (10 % protein): a) 0.1 % of LBGP with the 10× lens; b) 0.1 % LBGP with the 63× lens; c) 0.3 % LBGP; d) 0.55 % LBGP; d) 0.8 % LBGP

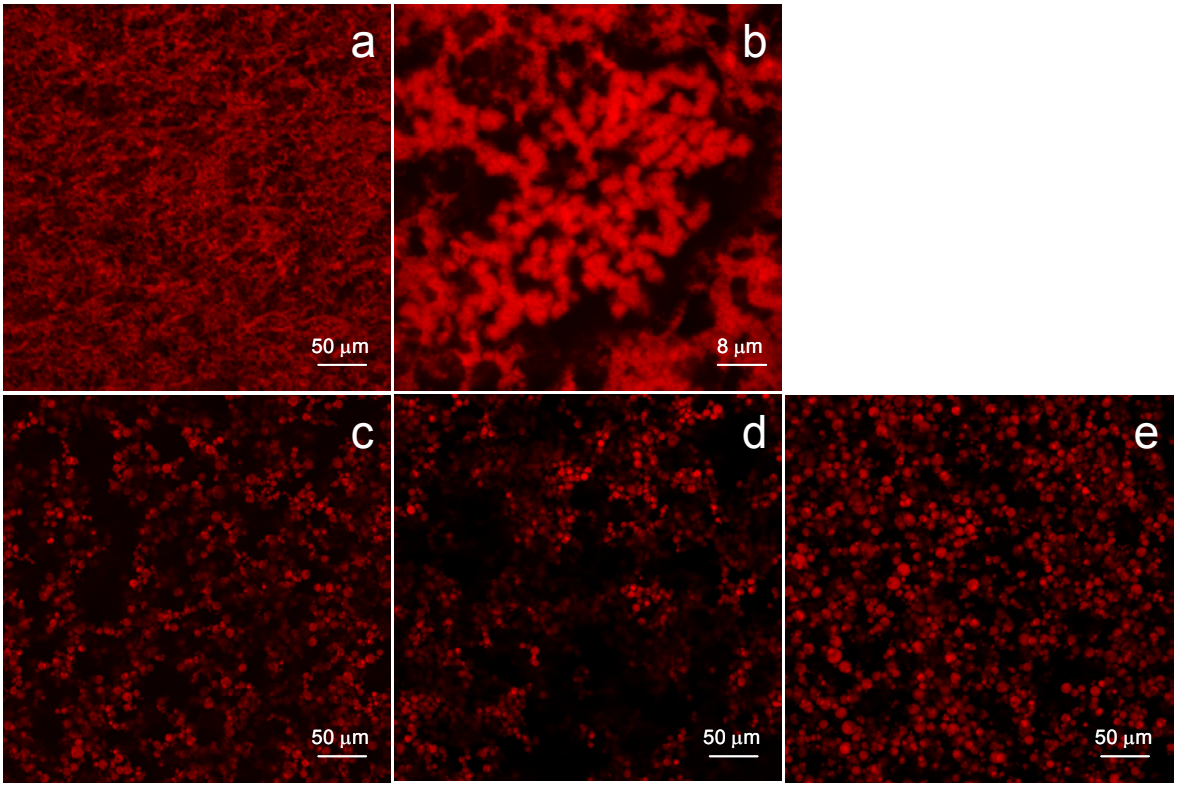

Figure 7-6 Influence of the LBGP on the structure of mixed T1/LBGP gels (10 % protein): a) 0.1 % of LBGP with the 10× lens; b) 0.1 % LBGP with the 63× lens; c) 0.3 % LBGP; d) 0.55 % LBGP; e) 0.8 % LBGP

Although no influence in the gel strength was visible for T1.0 with 0.1 % (w/w) LBG (only a slight increase, within the experimental error), the gelation time, the critical strain and the rupture strain were all reduced (Figure 7-2; Table 7-2). Further increases in the LBG concentration caused weakening of the gels, increased viscous character (increased tan δ) and increased rupture strain. However, when the structure changed from coarsely bicontinuously stranded to highly particulated, the gelation time was further decreased and only then it started to increase, probably because the increase in the viscous character only then prevailed over the reducing effect of LBG on the time for gelation.

P2.5 and T1.0 microdomains were more spherical than WPC microdomains when the structure was highly particulated. WPC and P2.5 spherical microdomains were much bigger and with a broader size distribution than T1.0 spherical domains. Phase separation was more severe in P2.5 mixed systems. Although no microstructure was analized for P4.9 and T3.5, their general rheological behaviour is quite similar to the behaviour of WPC, although weaker gels were always obtained. In fact, the maximum gel strength was found in both cases for 0.1 % LBG. The viscous character increased consistently after 0.1 % LBG, the critical strain generally decreased and the rupture strain generally increased. The gelation time increased after 0.3 % LBG for P2.5 and after 0.1 % LBG for T1.0. The lower value of γc for higher amounts of LBG can indicate that the system is approaching a colloidal behaviour, with bigger clusters as referred by Clark and Ross-Murphy (1987).

Table 7-3 Influence of the LBGP concentration on the relative volume of the enriched phase in protein in mixed whey protein or hydrolysates (10 % w/w)/LBGP heat-set gel systems

	Relative volume of the enriched phase in protein (%)			
LBGP (% w/w)	0.1	0.3	0.55	0.8
WPC	40	31	27	23
P1.5	46	-	38	-
P2.5	45	41	23	16
T1.0	43	22	20	27

The behaviour of the more hydrolysed proteins towards the presence of LBG was more similar to that of the WPC when compared to the behaviour of the less hydrolysed proteins. However the protein concentration used in these systems was much higher. To check if the different behaviour could be

ascribed to differences in concentration values or if the differences resulted from differences in the gels' microstructure, another set of mixed systems was prepared with 13 % (w/w) T1.0. In this case, notorious maximum gel strength, minimum tan δ and minimum gelation time were found for 0.1 % LBG. The critical strains were generally lower than for WPC, reflecting a stonger gel character. The rupture strain also increased for the higher LBG concentration. A stronger viscous character was found at 0.55 % of LBG for T1.0 than for WPC, with higher tan δ and much higher rupture strain. Thus it can be stated that the rheological behaviour of P1.5, P2.5 and T1.0 did not show well defined maximum gel strength for 0.1 % possibly because the concentration was not high enough. As explained for the hydrolysates alone, the hydrolysates have probably a mixture of gelling peptides/proteins and non-gelling peptides. Thus, to achieve a similar concentration in gelling agents and, thus, a similar behaviour, it is necessary to have a higher concentration of these hydrolysates in comparison with WPC.

The experimental error associated with strain fracture measurements may be high due to the sensibility of the fracture to the presence of defects that differ between samples. This fact is more important for small volumes and is a handicap of the plate and plate geometry (Pouzot and others, 2006). This may justify small differences in behaviour verified in Table 7-2.

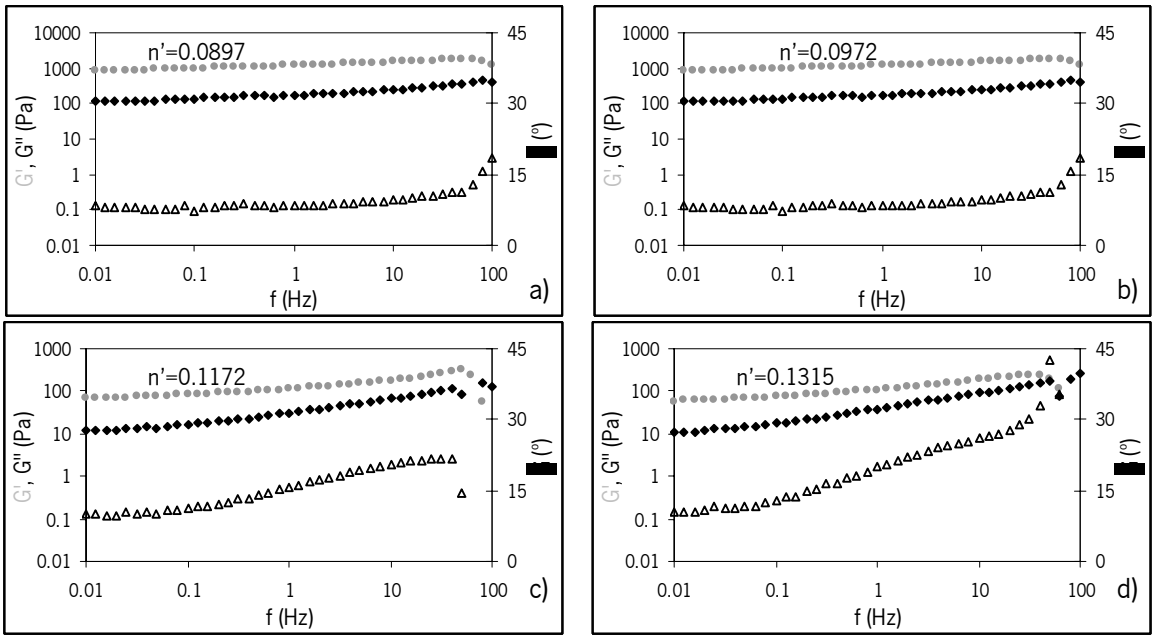

Figure 7-7 Influence of the LBGP concentration on the frequency spectrum of whey protein concentrate gels (● - G'; • - G''; Δ - δ): a) 0.1 % (w/w); b) 0.3 % % (w/w); c) 0.55 % (w/w); d) 0.8 % (w/w)

Both *G'* and *G''* spectra have the characteristic form of a gel with non-covalent bonds, with *n'* higher than zero. At small enough frequencies both viscoelastic moduli are usually strongly independent of the

frequency (Figure 7-7). This stands for both strong and weak gels (Ross-Murphy, 1995). However, for high amounts of LBG, the slope of the storage modulus profile increases for higher frequencies (Figure 7-7 c) and d)). Thus, in these cases, *n'* was calculated only considering the points for lower frequencies (< 1 Hz). This slope increase may be caused by the weaker character of the gels. Furthermore tan δ strongly depends on the frequency which is indicative of an increasing viscous character. It is also possible that the LBG concentrations used do not lead to "real" gels but pre-gels with a high viscous character. The tan δ dependence on the frequency increased with the increase on LBG concentration. This is in agreement with the microstructure described for these systems.

Nevertheless, the value of *n'* increases with the increase of the LBG concentration, indicating a possible increased importance of non-covalent interactions.

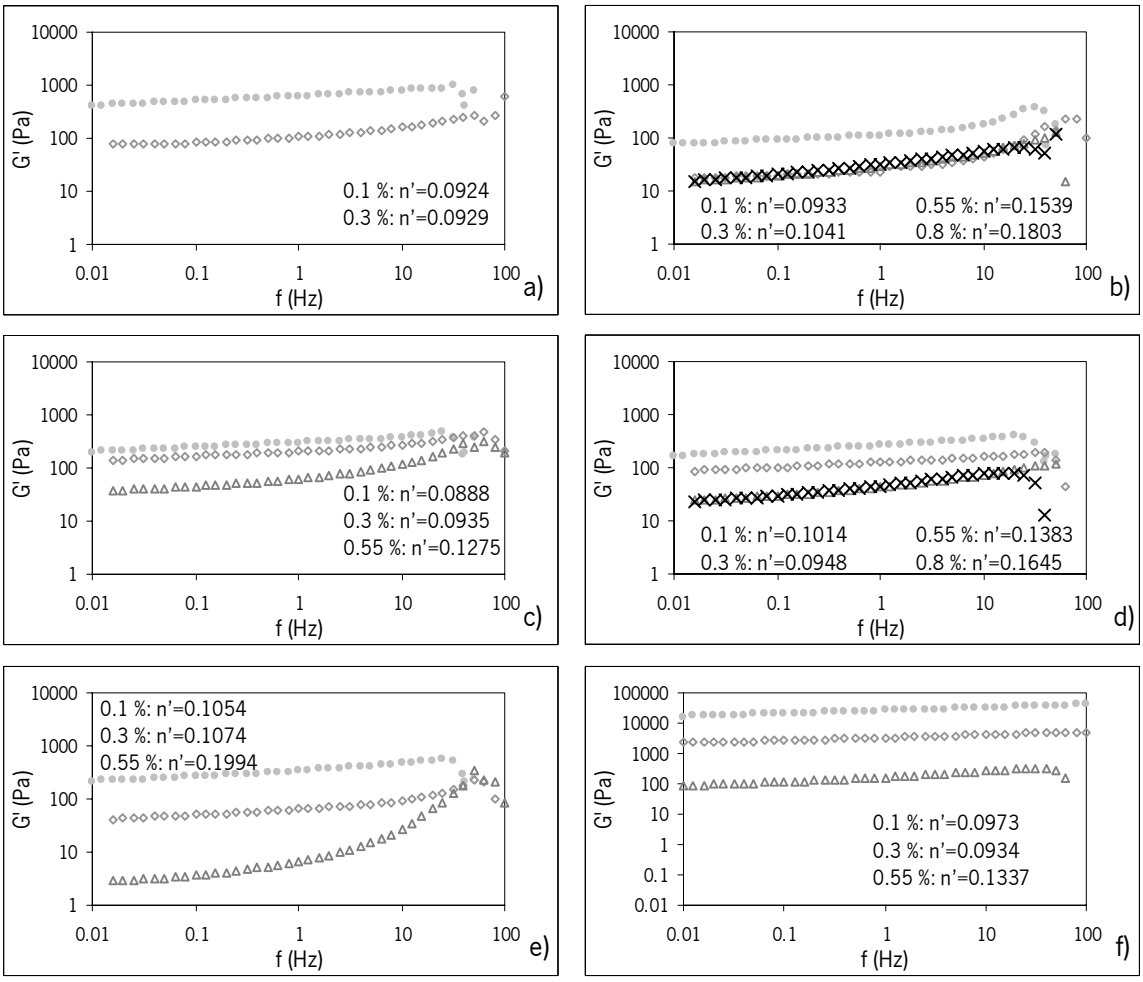

Figure 7-8 Influence of the LBGP concentration on the frequency spectrum of whey protein hydrolysate gels (• - 0.1 %; ◊ - 0.3 %; △ - 0.55 %; × - 0.8 %): a) P1 10 % (w/w); b) P2.5 10 % (w/w); c) P4.9 16.5 % (w/w); d) T1.0 10 % (w/w); e) T3.5 13 % (w/w); f) T1 13 % (w/w)

Gels from hydrolysates with LBG showed similar frequency spectra. *G'* and tan δ were almost constant over a wide range of frequencies. The slope of the *G'* spectrum was not zero (though very low) indicating that non-covalent interactions play a role in the gel structure. Once again, for higher LBG concentrations, *tan* δ increased with the frequency. The values for *n'* are presented in Figure 7-8. For lower LBG concentration (0.1 to 0.3 % w/w) *n'* was approximately constant indicating that, although the microstructure of these gels changed, the relevance of non-covalent forces was the same.

When analysing the influence of the concentration of LBG on gelling properties (*G'*, *G''*, tan δ and *Tg*), the differences from the hydrolysates with no polysaccharide and the hydrolysates with 0.1 % of LBGP were usually very sharp; this difficults modelling with a simple quadratic function. Therefore the statistical analyses and empirical modelling were performed as described in 7.2.5 only with the data from mixed systems for the LBGP range of 0.1 – 0.55 %. The regression coefficients and significance of the adjusted model are presented in Table 7-4 for peptic hydrolysates and in Table 7-5 for tryptic hydrolysates. LBG concentration corresponds to *x1* and *DH* to *x2* in Eq. 7.9. Empirical models were adjusted to the experimental value considering that the adusted parameters (*G'*, *G''*, tan δ and *Tg*) are dependent on the LBG concentration and on the *DH* (used as variables of the model). *Tg* stands for the temperature at which the system gelled during the temperature ramp from 20 to 80 °C at a rate of 2 °C/min. An equivalent temperature value was calculated for the samples that gelled above 80 °C, assuming that the equivalent time was an exponential function of the difference between the actual temperature and 80 °C.

By means of an analysis of variance it was concluded that all the adjusted models are significant. *PF* represents the probability of the calculated model F-value occuring due to noise and not by the identified influence of the studied variables and is in all cases very low. The significance of the "*lack-of-fit*" was also tested by an F-test with data from the replicates.

Table 7-4 Statistical analysis of the influence of the LBGP concentration and hydrolysis degree on the gelling ability of 10.0 % (w/w) whey peptic hydrolysates

Variable	Regression coefficients						Regression quality	
	b0	*b1*	*b2*	*b11*	*b22*	*b12*	*PF* (%)	Lack-of-fit
G' (Pa)	1619	**-2886**	-618	-	-	1086	0.20	Not significant
G'' (Pa)	235	**-393**	-87.3	-	-	146	0.31	Not significant
tan δ (°)	0.193	-0.470	-0.012	1.09	-	0.102	0.05	Not-significant
T_g (°C)	75.8	-10.4	5.40	31.3	-2.50	-	0.51	Not-significant

Items in **bold** correspond to non significant model terms that could not be withdrawn from the model because they where required to support hierarchy.

The effect of LGB concentration and of the degree of hydrolysis on peptic hydrolysates gelation was negative for *G'* and *G''*, though an interaction factor has also to be considered (Table 7-4). For tan δ and the temperature of gelation a minimum value exists in the studied range of LBG concentration, while a maximum exists in the studied range of *DH* for the temperature of gelation. For tryptic hydrolysates a maximum for *G'* and *G''* maximum was found in the *DH* range, while a negative influence was observed in the range of the LBGP concentrations used. For tryptic hydrolysates, tan δ has a minimum value in the LBGP concentration range and the gelation temperature has a minimum value in the *DH* values range.

Table 7-5 Statistical analysis of the influence of the LBGP concentration and degree of hydrolysis on the gelling ability of 10.0 % (w/w) whey tryptic hydrolysates

Variable	Regression coefficients						Regression quality	
	b0	*b1*	*b2*	*b11*	*b22*	*b12*	*PF* (%)	Lack-of-fit
G' (Pa)	1238	-1591	-765	-	169	-	0.90	Not significant
G'' (Pa)	183	-218	-113	-	25.2	-	0.79	Not significant
tan δ	0.169	-0.189	-0.022	0.580	-	0.186	0.03	Significant
Tg (°C)	74.6	7.23	-3.25	-	1.51	8.89	0.01	Significant

The lack of fit was significant for tan δ and *Tg* in the case of tryptic hydrolysates. However, for tryptic hydrolysate T3.5, as no gels were achived at 10 % (w/w), the amount of protein used was 13 % (w/w). In the case of peptic hydrolysates, there were some adjusted coefficients which were not significant. Thus, there are probably other functions, possibly more complex, that would provide a better fit of the available data. However it can be concluded from this analysis that differences are significant, that the adjusted functions effectively describe the behaviour of the data obtained (although other functions might do it better) and that LBGP concentration and the *DH* significantly influence the gelation of the hydrolysates.

7.3.3 Gelling ability of mixtures LBG/hydrolysates - rheological study of the influence of the type of LBG

For these experiments, the time evolution of the viscoelastic moduli and of the loss angle followed the general behaviour described for hydrolysates alone. They are not presented as the moduli values are of the same order of magnitude as the general range of experiences with different *M/G* ratios and no differences were easily observed. However, the magnitude of the moduli was significantly reduced for all hydrolysates and highly improved for P4.9, as detected in the last sections for higher protein concentrations (Table 7-6). For WPC the behaviour was different: while 0.55 % of LBGP for 10 % WPC

impaired the moduli of the resulting gels (Table 7-2), 0.55 % of LBGP for 7.5 % WPC improved the same parameters when compared with the same gel without galactomannan. These results provide further evidence that extrapolation for higher amounts of LBG cannot be made, as had already been concluded in the previous section for 0.8 % of LBG. Besides, it also reinforces the idea that the ratio LBG/protein is also an important parameter.

The mean values of the measured parameters after the gelling process are presented in Table 7-6.

Table 7-6 Influence of the LBG type (0.55 % w/w) and hydrolysis degree on the gelling ability of 7.5 % (w/w) whey peptic and tryptic hydrolysates (except P4.9 and T3.5: 16.5 and 13.0 % w/w, respectively)

	LBG fraction	*M/G*	*DH* (%)	*G'* (Pa)	*G''* (Pa)	tan δ	*Tg* (°C)	*tg* (s)
WPC	-	-	0	5.68	1.26	0.222	80.0	2059
WPC	Soluble at 20	2.72	0	31.8±7.2	11.3±0.7	0.37±0.11	79.0±1.5	
WPC	Purified	3.57	0	31.6±12.1	11.4±1.1	0.38±0.11	79.6±0.5	
WPC	Soluble at 80	4.35	0	14.7±8.5	8.60±1.98	0.65±0.24	80.0	582±399
P1.5	-	-	1.5	138±37	19.4±4.6	0.14±0.01	80.0	348±31
P1.5	Soluble at 20	2.72	1.5	20.8±6.3	6.05±0.21	0.31±0.10	79.2±0.4	
P1.5	Purified	3.57	1.5	20.1±10.2	6.90±1.13	0.38±0.14	80.0	169±115
P1.5	Soluble at 80	4.35	1.5	12.0±8.8	6.10±2.55	0.59±0.22	80.0	192±106
P2.5	-	-	2.5	58.0±12.2	9.00±2.26	0.16±0.01	78.4±0.1	
P2.5	Soluble at 20	2.72	2.5	5.40±3.39	3.30±1.41	0.67±0.16	80.0	834±380
P2.5	Purified	3.57	2.5	7.85±0.64	4.20±0.28	0.53±0.01	80.0	805±169
P2.5	Soluble at 80	4.35	2.5	3.20±0.57	3.10±0.42	0.97±0.06	80.0	1192±42
P4.9	Soluble at 20	2.72	4.9	53.1±6.9	15.7±2.4	0.30±0.01	79.0±0.2	
P4.9	Purified	3.57	4.9	76.0±17.1	23.8±2.3	0.32±0.04	80.0	36.0±0.1
P4.9	Soluble at 80	4.35	4.9	60.4±11.6	16.4±0.6	0.28±0.06	77.8±0.9	
T1.0	-	-	1.0	16.6±3.3	2.75±0.49	0.17±0.01	80.0	296±42
T1.0	Soluble at 20	2.72	1.0	14.8±6.2	5.25±2.33	0.34±0.02	80.0	117±42
T1.0	Purified	3.57	1.0	19.8±2.8	7.60±0.14	0.39±0.06	80.0	326±254
T1.0	Soluble at 80	4.35	1.0	9.65±0.07	4.95±0.92	0.51±0.09	80.0	685±760
T3.5	Soluble at 20	2.72	3.5	3.20±0.71	3.95±0.21	1.25±0.21	80.0	6749±1056
T3.5	Purified	3.57	3.5	12.2±7.9	5.75±0.92	0.57±0.29	80.0	2656±3125
T3.5	Soluble at 80	4.35	3.5	7.90±0.00	6.10±2.12	0.78±0.27	80.0	2657±3041

All values are means ± standard deviation of two determinations.

Statistical analyses were performed as described in section 7.2.5 using the data from Table 7-6, excluding the samples without LBG. *tg* represents the gelation time after the ramp of temperature from 20 to 80 °C at a rate of 2 °C/min. A negative equivalent time value was calculated for the samples that gelled before 80 °C. It was assumed that the equivalent time was an exponential function of the

difference between the sample gelling temperature and 80 °C. LGB type (*M/G*) corresponds to *x1* and *DH* to *x2* in Eq.7.9. The regression coefficients (*b0*, *b1*, *b2*, *b11*, *b22*, *b12* from Eq. 7.9) obtained for each variable are presented in Table 7-7 and Table 7-8. Again the analysis of variance led to the conclusion that all the adjusted models are significant. The lack-of-fit was not significant in all cases, meaning that experimental errors cannot not be responsible for the detected variations.

Table 7-7 Statistical analysis of the influence of the LBG type and hydrolysis degree on the gelling ability of 7.5 % (w/w) whey peptic hydrolysates

Variable	Regression coefficients						Regression quality	
	b0	*b1*	*b2*	*b11*	*b22*	*b12*	PF (%)	Lack-of-fit
G' (Pa)	47.06	-5.64	-8.01	-	-	-	0.04	Not significant
G'' (Pa)	-7.70	11.28	-2.76	-1.68	-	-	0.01	Not significant
tan δ	2.91	-1.62	-0.226	0.255	0.1315	-	0.07	Not significant
tg (s)	-8.84	213	395	-	555	-	0.01	Not significant

Bidimensional surface responses that reproduce the influence of *M/G* and *DH* on the gelation process were built from the obtained equations (Figure 7-9 and Figure 7-10).

In the case of peptic hydrolysis the adjusted models seem to describe well the behaviour of the system. Besides, the lack of fit is not significant in all cases indicating that for all adjusted variables the experimental error is lower than the variations suggested by the models. For none of the four studied parameters the interaction between *DH* and *M/G* was significant indicating that their influence is independent from each other. The influence of both *M/G* and the degree of hydrolysis is generally negative on the value of *G'*. The higher degree of hydrolysis available (4.9 %) was not used in the statistical studies because the experiences were made at a different concentration (16.5 %).

Although the influence of the degree of hydrolysis is also negative on the values of *G''*, an optimum (maximum) seems to exhist in the case of *M/G* (Table 7-7, Figure 7-9).

Analysing the results on Table 7-6, it seems probable that the negative influence of *M/G* on *G'* is only valid for higher values of *M/G* (that is to say that it also has an optimum value). In fact, the values of *G'* for WPC with LBG20 and LBGP are statistically equal and for P2.5 and P4.9 they seem to be the highest for a *M/G* experimental value of 3.57. This would mean that the influence of the two variables on *G'* and *G''* was similar. These adjusted models can be used as indicative but there may be other models that provide a better fit of the results. Anyway, it can be surely concluded that there is an influence of these two variables on the studied parameters though the choosen model may not be the best one. These

results reveal a different behaviour of the system with LBG in comparison with the hydrolysates alone (Figure 6-2). In fact, for the hydrolysates alone, there is a clear maximum of *G'* for a low degree of hydrolysis (P1.5).

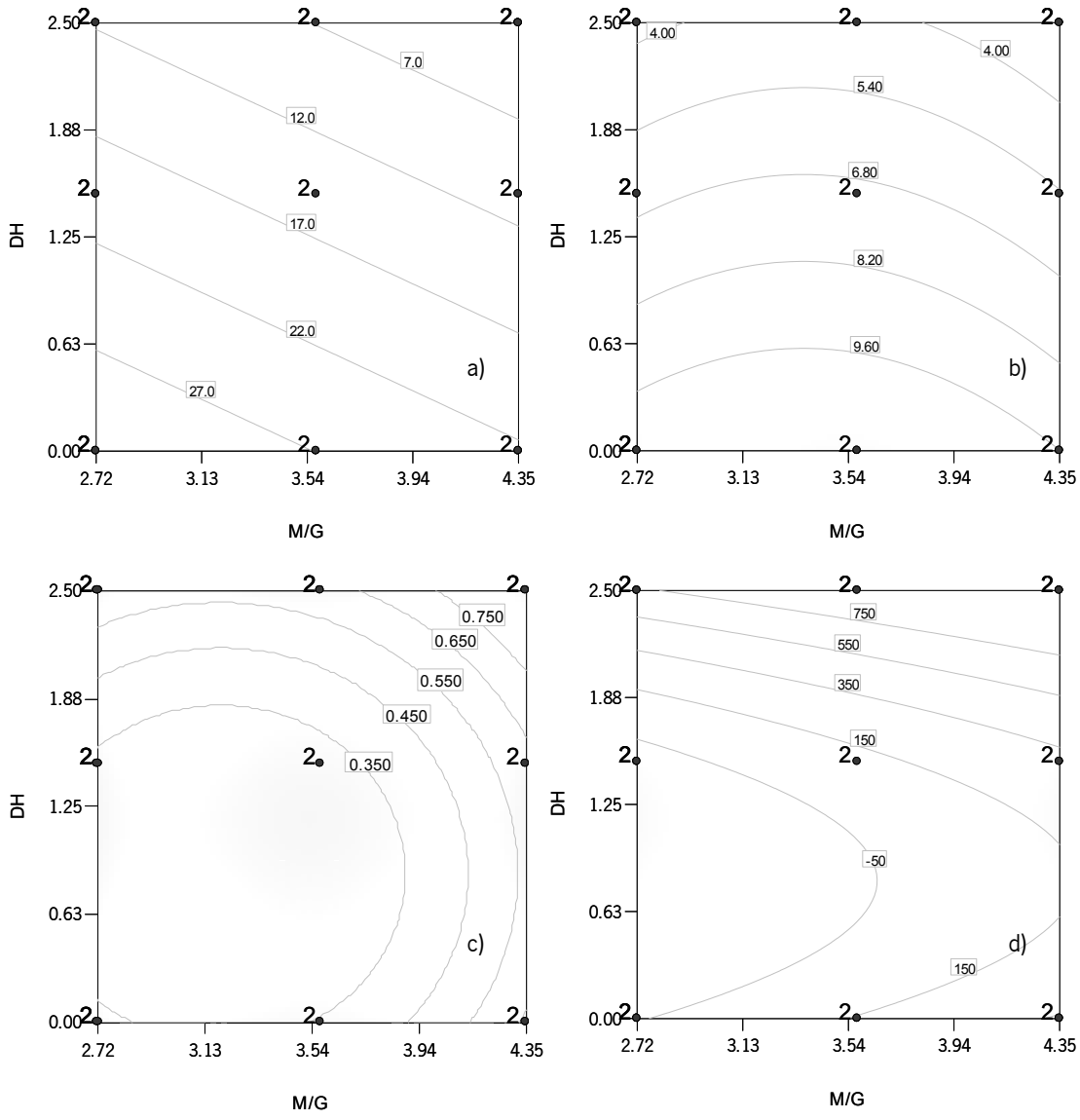

Figure 7-9 Isoresponse lines for the influence of the LBG type (0.55 % w/w) and the degree of hydrolysis on the gelling ability of whey peptic hydrolysates (7.5 % w/w): a) *G'* (Pa); b) *G''* (Pa); c) *tan* δ; d) *tg* (s) at 80 °C after a 30 min temperature ramp from 20 to 80 °C; the symbols • correspond to experimental data points and the number adjacent to them corresponds to the number of replicates of that data point

In the case of tan δ, minimum values for both the *M/G* and the *DH* influence occur. Again, the differences between LBG20 and LBGP for lower degrees of hydrolysis (WPC and P1.5) are not evident (Table 7-6).

Finally the influence of *M/G* on the gelation time was positive and the gelation time had a minimum value in the *DH* range used (unlike the case of hydrolysates alone for the same range of *DH* and for the same hydrolysates concentration). Again this tendency is only seen for LBG80 values in Table 7-6.

Table 7-8 Statistical analysis of the influence of the LBG type and hydrolysis degree on the gelling ability of 7.5 % (w/w) whey tryptic hydrolysates

Variable	Regression coefficients						Regression quality	
	b0	*b1*	*b2*	*b11*	*b22*	*b12*	*PF* (%)	Lack-of-fit
G' (Pa)	-81.4	72.6	-26.3	-11.5	2.45	3.54	0.08	Not significant
G'' (Pa)	-13.2	**15.3**	-8.53	-2.36	1.21	0.796	0.01	Not significant
tan δ	3.43	**-1.96**	**0.373**	0.307	0.067	-0.139	0.27	Not significant
tg (s)	-2925	**756**	4375	-	-	-903	0.06	Not significant

Items on **bold** correspond to non significant model terms that could not be withdrawn from the model because they where required to support hierarchy.

In the case of the tryptic hydrolysates, some information must be considered when analysing the results. Firstly, the T3.5 concentration (13 %) was not the same as for T1.0; it was chosen because the *G'* values obtained were of the same order of magnitude (but lower, as it would be expected for the same concentration) of WPC and T1.0. Secondly, T3.5 with LBG20 did not gel at all (at some point *G'* crossed *G''* but after curing and cooling times this was again inverted). Besides, for T3.5 with LBGP and with LBG80 one of the replicates hardly gelled and *G'* was very near to *G''* (though slightly higher: 6.6 to 5.1 and 7.9 to 7.6 Pa, respectively). The gelation time for these three cases was very high and may have had an exarcerbated influence on the results. Moreover, the variability in tan δ and *tg* final values in these cases was very high, probably due to the fact that they were very close to the gelation threshold where slight differences in the concentration values could have a high influence on the final result (at the gelling point the gelation time diverges to infinity). However, the use of all the available data in the statistical study resulted in a variation that is statistically significant and not ascribable to noise, although the models fitted may not be the best ones, as some model terms were not significant. This means that the degree of hydrolysis of the protein and the LBG type had a significant influence on the gelling ability of the mixed system.

The presented graphics with the isoresponse lines are standard error shaded. This means that for trypsin the differences between the estimated and the experimental values are more pronounced than for pepsin (Figure 7-9 and Figure 7-10) as expected from the discussion above, and can also indicate that there might be other functions providing a better fit of the results.

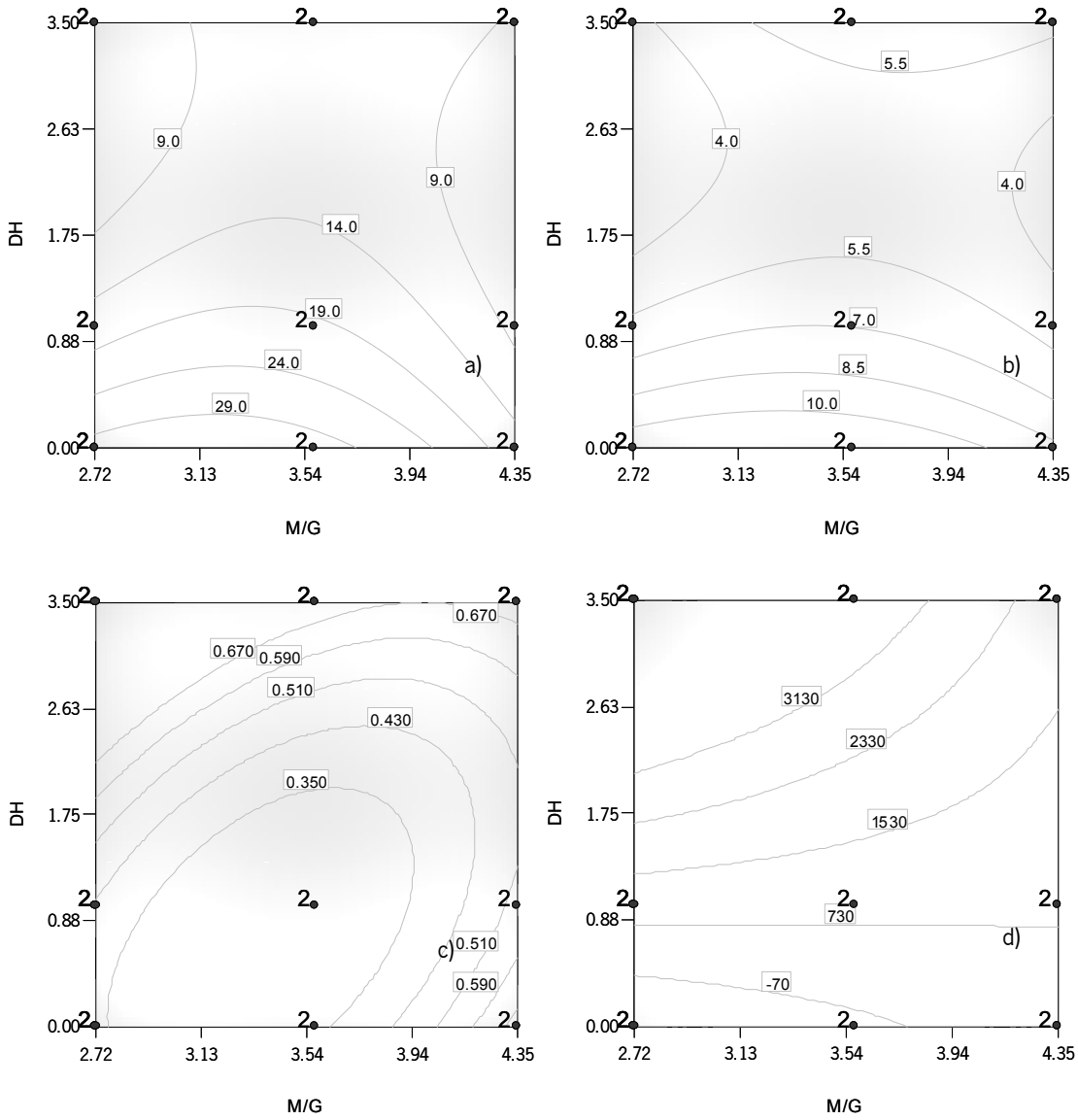

Figure 7-10 Isoresponse lines for the influence of the LBG type (0.55 % w/w) and the degree of hydrolysis on the gelling ability of whey tryptic hydrolysates (7.5 % w/w, except for T3.5 – 13.0 %): a) *G'*; b) *G''*; c) tan δ; d) *tg* (s) at 80 °C after a 30 min temperature ramp from 20 to 80 °C; the symbols • correspond to experimental data points and the number adjacent to them corresponds to the number of replicates of that data point

Even so, some conclusions can be drawn. *G'* and *G''* have a maximum value for LBGP and decrease generally with the *DH* (Table 7-6 and Figure 7-10). The value of tan δ seems to increase with *M/G* (not considering T3.5 with LBG20), decreasing the gel elasticity and increasing the liquid character. The tendency for the influence of the *DH* over tan δ is not clear. There seems to be a minimum value at *DH* 1.0%, but this is not always valid. The gelation time increases with the increase of the *DH* and increases with the increase of *M/G*, although T3.5 cannot be considered. In fact, the same happened for P4.9. The

behaviour of tan δ and *tg* with *M/G* variations was also different in this case. Either the system behaves differently for higher degrees of hydrolysis or the system behaves differently for higher protein concentrations. Anyway, the structure of the gel is probably different in these situations and extrapolations of the conclusions to other values of *DH* and concentrations are dangerous.

A significant effect of the branching degree (the higher the degree of branching the lower the value of *M/G*) on the gelling ability of whey proteins has also been reported by Tavares and others (2005). In fact, an increase in *M/G* will significantly change the properties of the galactomannan: increases viscosity, decreases solubility and even alters its gelling ability (although galactomannans with low *M/G* do not gel, an increase in the *M/G* can improve its gelling ability) and is likely to affect also its behaviour in mixed protein systems. Besides, branching usually reduces the incompatibility between protein and polysaccharide (Grinberg and Tolstoguzov, 1997) which is an important feature in the gelation of mixed systems. However, Tavares and others, 2005 only found a significant influence for low values of *M/G* (< 2.3). For *M/G* values between 2.3 and 3.7 the differences were not significant and no higher values were tested. It is important to mention that the protein concentration used was also higher (13 %) and far away from the gelling critical point. Thus extrapolations are not advisable. However these results are not inconsistent with the results presented in this chapter. In fact, a maximum in *G'* and *G''* seems to occur between these two values of *M/G*.

Table 7-9 Influence of the LBG type and hydrolysis degree on the gelling ability of 10 % (w/w) WPC, whey peptic hydrolysates and whey tryptic hydrolysates

	LBG fraction	M/G	*DH* (%)	*LBG* (%)	*G'* (Pa)	*G''* (Pa)	tan δ	*Tg* (°C)
WPC	Soluble at 20	2.72	0	0.1	1467	207	0.14	75.6
WPC	Purified	3.57	0	0.1	1436±334	207±47	0.14±0.00	75.2±0.1
WPC	Soluble at 80	4.35	0	0.1	1147	166	0.14	75.7
P2.5	Soluble at 20	2.72	2.5	0.1	68.8	11.2	0.16	75.3
P2.5	Purified	3.57	2.5	0.1	107	16.2	0.15	72.6
P2.5	Soluble at 80	4.35	2.5	0.1	249.7	38.5	0.15	74.3
T1.0	Soluble at 20	2.72	1.5	0.1	306.6	51.6	0.17	78.0
T1.0	Purified	3.57	1.5	0.1	245	42.8	0.17	77.7
T1.0	Soluble at 80	4.35	1.5	0.1	157.8	26.3	0.17	78.2

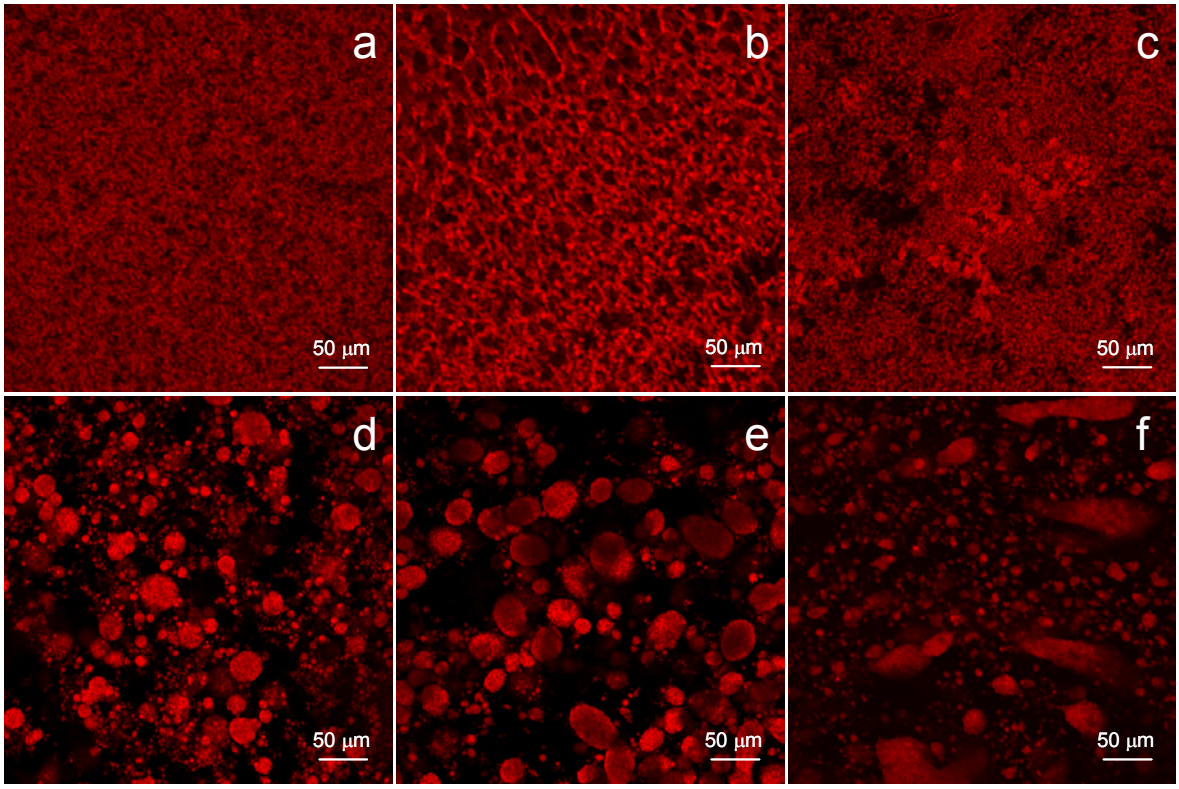

Figure 7-11 Influence of the *M/G* ratio of the LBG on the structure of mixed WPC/LBG gels (10 % protein): a) 0.1 % of LBG20; b) 0.1 % LBGP; c) 0.1 % LBG80; d) 0.55 % LBG20; e) 0.55 % LBGP; f) 0.55 % LBG80

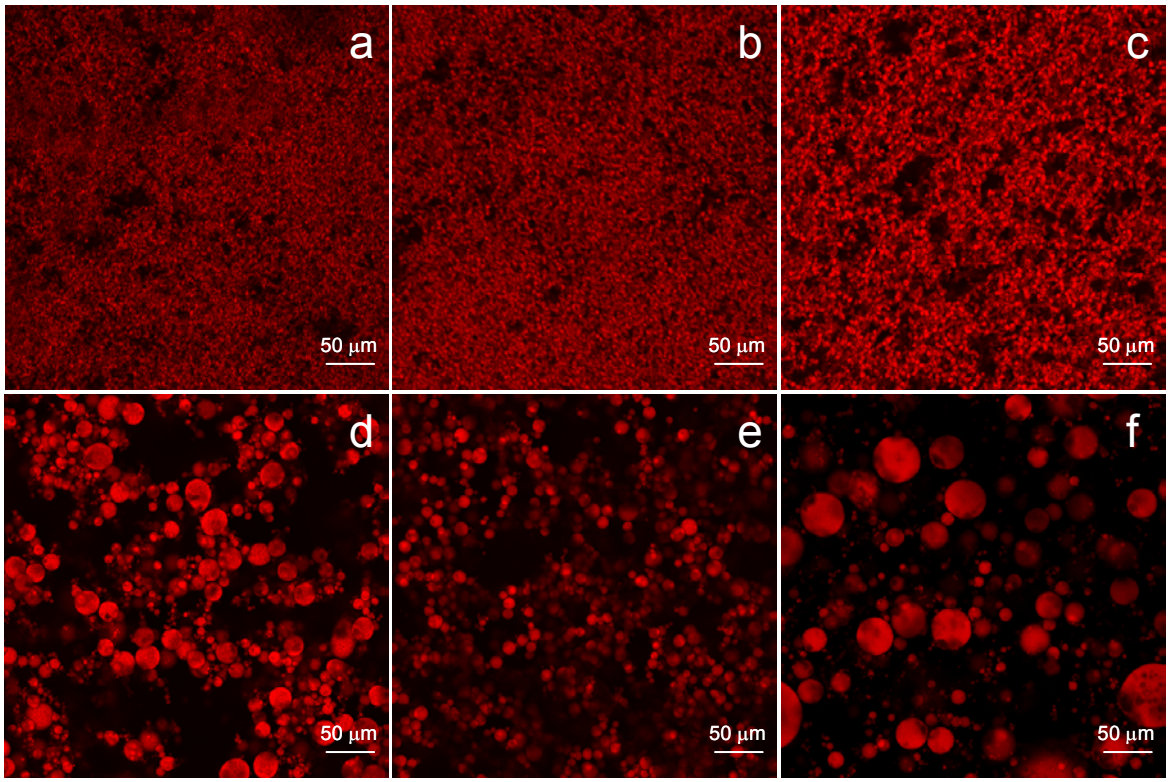

Figure 7-12 Influence of the *M/G* ratio of the LBG on the structure of mixed P2.5/LBG gels (10 % protein): a) 0.1 % of LBG20; b) 0.1 % LBGP; c) 0.1 % LBG80; d) 0.55 % LBG20; e) 0.55 % LBGP; f) 0.55 % LBG80

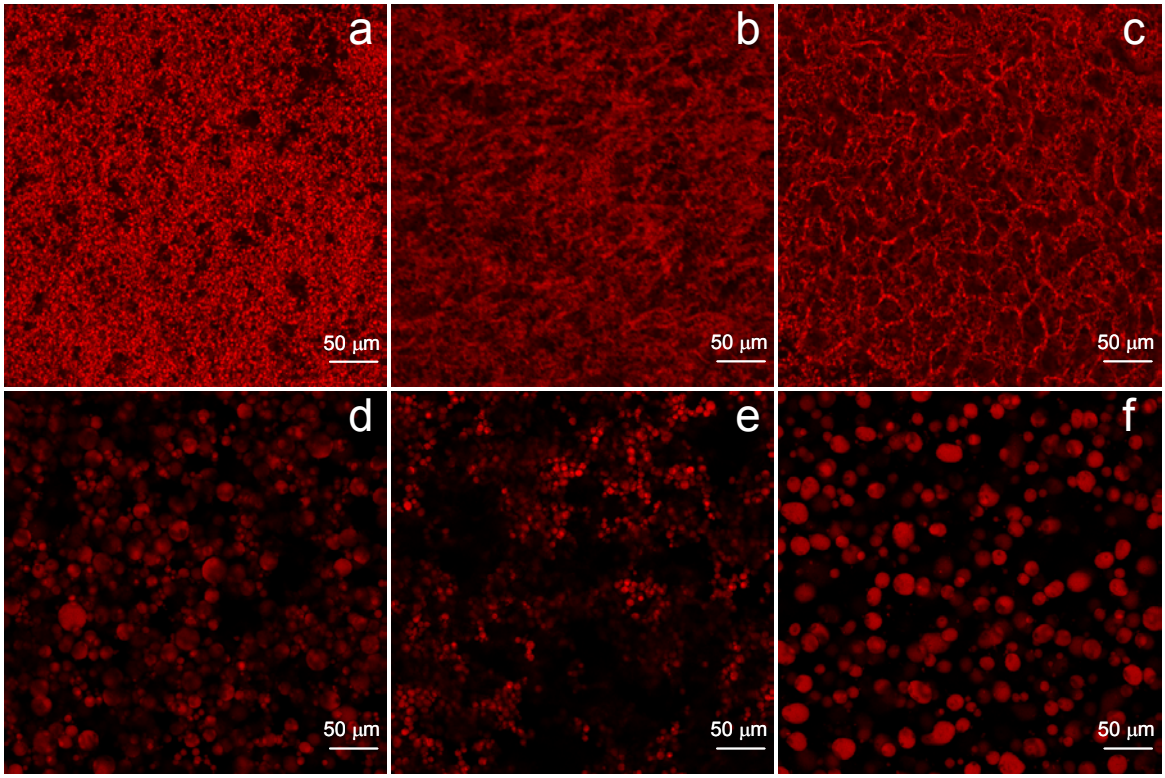

Figure 7-13 Influence of the *M/G* ratio of the LBG on the structure of mixed T1/LBG gels (10 % protein): a) 0.1 % of LBG20; b) 0.1 % LBGP; c) 0.1 % LBG80; d) 0.55 % LBG20; e) 0.55 % LBGP; f) 0.55 % LBG80

Although an increase in the viscosity of the solution may improve the gel strength, the opposed effect is observed for higher viscosities. In fact, increasing *M/G* from 3.57 to 4.35 generally resulted in a decrease of the gel strength. Possibly the correspondent increase in viscosity restricted particle aggregation and clusters formation and impaired the protein network formation. A similar effect has also been referred by Olsson and others (2002b) when analysing the effect of the viscosity of amylopectin in the gelation of whey proteins.

Differences in the gel structure at 10 % w/w of protein are also visible in Figure 7-11, Figure 7-12 and Figure 7-13, both for 0.1 and for 0.55 % of LBG.

The influence of the branching degree on the gelation parameters at 10 % is presented on Table 7-9 for 0.1 % LBG. The only consistent conclusions to be made are: the gelation temperature has a minimum value for WPC, P2.5 and T1.0 at a value of *M/G* of 3.57; and the loss angle (and its tangent) seems to be independent from the type of LBG in the used data range.

For gels at 0.1 % LBG, the volume fraction of the enriched protein phase is always lower for the fraction of LBG80 (Table 7-10). Whereas the bicontinuous protein network seems to be more open at the highest used value of *M/G* for WPC and T1.0, this is not so clear for P2.5. For WPC with 0.1 % LBG the protein strands appear to be thicker for LBGP but less interconnected than for LBG20. For LGB80 the connectivity decreases as well as the netwotk homogeneity.

Table 7-10 Influence of the LBG type on the relative volume of the enriched phase in protein in mixed whey protein or hydrolysates (10 % w/w)/LBGP heat-set gel systems

	Area of the enriched phase in protein (%)					
Type of LBG	LBG20	LBGP	LBG80	LBG20	LBGP	LBG80
LBG (% w/w)	0.1	0.1	0.1	0.55	0.55	0.55
WPC	47	40	34	25	27	22-24
P2.5	43	45	35	28	23	20
T1.0	42	43	34	23	20	22

The influence of the type of LBG is more evident at 0.55 %, where the phase separation is more notorious. In all cases the LBG80 fraction (the less branched) led to isolate protein-enriched microdomains with almost no connectivity between them. This is consistent with the fact that branching usually reduces incompatibility between proteins and polysaccharides (Grinberg and Tolstoguzov, 1997). Those microdomains were once again more spherical for P2.5 and T1.0 and with a broad size distribution for P2.5 and WPC. T1.0 with LBG80 presented regular small spherical microdomains. For P2.5 and T1 the size of the microdomains was minimum (and thus more concentrated in protein) with LBGP while for WPC it was maximum for the same branching degree. Clusters can be seen with both LBG20 and LBGP. For lower amounts of LBG (0.1 %) there was a general trend to a minimum volume occupied by the enriched protein phase for the highest *M/G* value. At 0.55 % of LBG, the differences in the volume fraction occupied by the protein-enriched fraction are small and the volume correspondent to the enriched phase in protein is almost constant, though much smaller than for 0.1 % LBG (Table 7-10).

7.4 Conclusion

LBG alters the microstructure of whey protein gels by modifying the equilibrium between aggregation and segregation. The gelation time is also decreased. The volume of the protein-enriched phase decreases with the increase of the LBG concentration and the protein concentration probably increases within that phase. The final structure of the gels is a result of the equilibrium between aggregation and segregation and of the increase of the protein concentration on the protein-enriched phase. The behaviour of gels from whey proteins or whey protein hydrolystates towards the presence of LBG is very similar. For whey proteins and for whey protein hydrolysates a small amount of LBG in the presence of salt leads to a big enhancement in the gel strength.

The gelation process is very sensible to environmental conditions and to processing and often leads to quite coarse data. The factorial planning used in this work allowed validating conclusions using fewer experiments than those needed if no planning had been used, while still getting statistical significance out of the results. However, as many factors are involved, the modelling of the process was not straightforward. A simple linear or quadratic function was generally not enough to accurately describe the system behaviour.

It is possible to make all kinds of different gels (strong, weak, stranded, particulated, ...) by manipulating the protein concentration, the degree of hydrolysis, the amount of LBG and the salt content. It is important though to master the mechanism of phase separation in order to be able to design the adequate conditions for the desired texture.

7.5 References

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Chapter 8 General conclusion

The work presented in this document is the result of a plan that aimed at studying the hydrolysis of whey proteins for food applications. In particular, the research undertaken was directed to the hydrolysis of whey proteins (aiming at changing their functional properties) and to the study of rheological interactions between whey proteins/hydrolysates and galactomannans, with the final goal of obtaining new textures, with high protein content or with bioactive peptides that can be used in existing food formulations or in the development of new food products.

The hydrolysis of whey proteins was performed with the aid of enzymes, both free and immobilized in different carriers. A comparison was established for the various conditions tested based on enzymes' activity and specificity, kinetic parameters and peptide profile of the hydrolysates produced. The gelling properties of the hydrolysates were tested and the hydrolysates were combined with a polyssaccharide (locust bean gum), in order to evaluate the interaction of those components in terms of possible new functional properties.

The paragraphs below summarize the main contributions of the present work:

- The choice of the enzyme for the hydrolysis is particularly important in determining the properties of the resulting hydrolysates;
- The selection of an adequate form of the chosen enzyme with the adequate purity and treatment (for instance, a treated trypsin with low chymotryptic activity) for the desired application is also a crucial factor, as different hydrolysates are achieved with different forms of the enzyme;
- The selection of the adequate operational conditions (time, pH and temperature) also determines the composition of the resulting hydrolysate; in fact, higher reaction times lead to higher degrees of hydrolysis and smaller peptides (usually more hydrophobic), while pH and temperature determine the resistance of whey proteins to the hydrolysis as well as the activity of the enzyme;
- The purity of the enzyme used in the immobilization process is determinant to the activity recovery;
- Trypsin was successfully immobilized on spent grains by multipoint covalent attachment using glycidol, on POS-PVA functionalized with glutaraldehyde and on zeolite through cross-linking of the enzyme;

- The immobilized trypsin with the highest activity towards low molecular weight substrates was obtained with covalent binding through glutaraldehyde to silanized zeolite followed by crosslinking with glutaraldehyde, probably due to a positive effect of the zeolite on the enzyme activity;

- Only trypsin immobilized on spent grain showed significant activity towards whey proteins; in fact, although trypsin immobilized on cross-linked zeolite NaY and trypsin covalentely immobilized on POS-PVA and glutaraldehyde have shown a high activity towards a small substrate (e.g. BAPNA), this did not happen when whey proteins were used as substrate;

- Peptide profile of hydrolysates from whey protein isolate with both free enzymes and enzymes immobilized on spent grain were similar, which indicates that spent grains can be used as carriers for trypsin to produce hydrolysates similar to those obtained with the free enzyme;

- The control of the extent of the hydrolytic reaction is extremely important to ensure that a hydrolysate with the intended properties is obtained. The immobilization allows such control by simply withdrawing the enzyme from the reaction medium, without the need of using high temperatures or considerable pH shifts. Further, immobilization also allows the reuse of the enzyme, with obvious advantages from the economical point of view;

- At WPC concentrations close to the gelling point, stronger gels with lower gelation temperatures can be achieved with limited hydrolysis of whey proteins; however, the reverse is observed at higher protein concentrations, probably due to a concentration effect;

- Locust bean gum (a non-gelling neutral polyssacharide) alters the microstructure of whey protein gels by modifying the equilibrium between aggregation and segregation. The time for gelation is also decreased. The volume of the protein-enriched phase decreases with the increase of the LBG concentration and the protein concentration probably increases within that phase. The final structure of the gels is a result of the equilibrium between aggregation and segregation and of the increase of the protein concentration on the protein-enriched phase. The behaviour of gels from whey proteins or whey protein hydrolystates towards the presence of LBG is very similar. For whey proteins and for whey protein hydrolysates a small amount of LBG in the presence of salt leads to a significant enhancement in the gel strength.

- Systems with many different textures can be tailored associating globular proteins or protein hydrolysates with locust bean gum.

In short, hydrolysates with many different functional, nutritional and biological properties can be produced by manipulating the hydrolysis conditions and the source of the enzyme (alone or in combination; free or immobilized; pure or impure; ...). The introduction of a polyssacharide allows an even bigger range of functional properties and can be used to adjust the desired property to the desired application.

Although much has been done, a work like this is never complete. Thus, some recommendations for improving present work and guidelines for future work can be given:

- During trypsin immobilization procedures the amount of enzyme (purified) should be optimized because if the surface of the carrier is overcrowded the activity performance can be poor; also, the use of spacers could be tested to improve the efficiency of the enzyme immobilized on POS-PVA and on zeolite (and even on glyoxyl-spent grains) towards high molecular weight substrates such as whey proteins; affinity ligands could also be tested to improve the enzyme stability;

- Lower gelling temperatures could be tested to confirm possible technological advantages of the use of hydrolysates: lower energy consumption and possibility of application to foods which are more sensitive to high temperatures;

- Other protein and LBG concentrations should be tested to confirm the conclusions obtained with mixed polyssacharide/protein systems and total solids content should be analysed to check if the differences in those mixed systems are due to variations in the total solid amount and/or to differences in the LBG concentration;

- The approach selected to the statistical analysis of the influence of LBG on the gelling ability of whey protein hydrolysates led to interesting results; however more complex functions should be used to better represent the system; in a simple factorial design, these functions will probably need a large number of experiments to allow the identification of the model parameters and the model estimation procedure could be improved using other experimental design tools in order to maximize the information obtained;

- The connectivity of the protein-enriched phase of the mixed systems mainly determines the rheological properties of the resulting heat-set gels from hydrolysates and LBG mixed systems. Thus, a quantitative analysis of the phase separation mechanism through a clusters size distribution analysis and through the determination of the ratio between the protein-enriched and the protein-depleted phases is advisable; further studies with laser scanning confocal microscopy are needed in order to obtain results with statistical significance;

- The biological features of the produced hydrolysates can be studied; testing different types of bioactivity (such as ACE-inhibitory activity) and identifying the hydrolysates' fraction responsible for the highest bioactivity could be interesting; further sequencing and peptide identification can be made to isolate peptides responsible for bioactivity;

- The produced gels can be tested as drug delivery systems; studying the releasing kinetics of compounds of interest could also be done, particularly for the gels with a "less" open structure and potentially (at least partial) resistant to the stomach environment (possibly gels from peptic hydrolysates with high intact β-Lg content), allowing the component of interest to get to the intestine with the bioactivity intact; a possibility is to study ALPMHIR (a bioactive peptide present in the whey protein tryptic hydrolysates) retention on whey protein hydrolysates gels;

- The methodology used could also be tested for other systems such as egg or fish protein hydrolysates;

- If an industrial application is intended, then the hydrolysates should be tested in "real" food systems and an analysis of their rheological and sensorial behaviour is necessary; particularly, hydrolysates are known for frequently having a bitter taste that may have to be corrected (e.g. using enzymes or encapsulating the interest compounds – this last suggestion is only possible when the function of interest is bioactivity and not a technological feature); furthermore, the presence of other food components is likely to affect the hydrolysates' functionality (as observed in the presence of LBG);

- Finally, the possibility of developing new food, cosmetic and farmaceutical products with the produced hydrolystates could be addressed.