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Protein partition on a derivative guar gum based aqueous two-phase system

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Abstract

A new aqueous two-phase system based on a guar gum derivative, commercialized under the trade name of Solvitose Gum Ofa, and poly(ethylene glycol) is described. Its main difference from other polymer-polymer aqueous two-phase systems lies on the nature of Solvitose Gum Ofa - a commercially available galactomannan derivative is used. Previous works have characterized almost exclusively purified carbohydrates or their derivatives and have neglected the large amount of gums and carbohydrates there are usually used in paper, food or textile industries. Two-phase aqueous polymer systems based on such polymers may have the drawback of their degree of purification, which may interfere in the separation procedure. Influence of poly(ethylene glycol) molecular weight, ionic strength and affinity ligands on protein partition were studied. The increase in the partition coefficient of a model protein, Bovine Serum Albumin, due to the presence of ligands in one of the phases was three times smaller than the corresponding increase in a traditional system. This should not prevent the successful use of such polymers on aqueous two-phase polymer extraction. The cost of the studied system is six times smaller than Reppal PES-poly(ethylene glycol) systems and of the same order of other systems formed with crude polymers. Results indicate that this is a potentialy useful aqueous two-phase system for enzyme extraction.

Introduction

Aqueous two-phase systems (ATPS) have found use in biochemical research for separation and purification of macromolecules, cells and cell organells (Walter & Johansson, 1986). In recent years the ATPS have also found applications in various areas of biotechnology. These phase systems have been used for enzyme purification in large scale (Veide et al., 1933; Tjerneld et al., 1987), affinity precipitation (Kamihira et al., 1992), affinity purification (Plunkett & Arnold, 1990) and extractive bioconversions (Andersson & Hähn-Hägertal, 1990).

ATPS provide not only a gentle environment for bioactive proteins but offer also unique possibilities for downstream processing. A very difficult mechanical separation step can be replaced by an extraction process, which allows the separation of cells and cell debris from a soluble protein by partition into opposite phases. Besides physical extraction, a reactive extraction may be applied. This is accomplished by confining a ligand into one phase by covalent binding to one of the polymers forming the system, usually poly(ethylene glycol) (PEG). It is also possible to bind the ligand to a third polymer, that preferentially favours one of the phases and has the advantage of being precipitated by a change in pH, temperature or other (Kamihira et al., 1992).

So far, most of the laboratorial work has been made with a system composed of fractionated dextran and PEG (Albertsson et al., 1990). The properties of this system are well studied but, despite the easiness of scale up, the high cost of fractionated dextran prevents the use of this system on large scale processes (Kroner et al., 1984).

As an alternative, for large scale enzyme processing, the PEG-salt systems have been used (Albertsson et al., 1990). Although inexpensive, the high salt concentration in both phases of this system limits its usefulness. PEG-salt phase forms only at rather high ionic strength, which may cause the denaturation of

sensitive biological structures and the dissociation of most ligand-protein complexes (Kula, 1989). Another problem related with the use of PEG-salt systems is waste disposal. ATPS based on dextran, starch derivatives and cellulose derivatives have the advantage of their biodegradability (Sturesson et al., 1990).

As a consequence, there is a need to develop new ATPS suitable for large scale processes. By allowing processes to be carried out at low salt concentrations, polymer-polymer systems may be more useful than PEG-salt systems. Since polymers and salts used to generate two-phase systems alone can account for as much as 75% of the total production cost of an aqueous phase extraction (Datar et al., 1986), it is necessary to find inexpensive substitutes for fractionated dextran with equivalent partition properties.

Several polymers, such as crude unfractionated dextran (Kroner et al., 1982), starch derivatives (Venâncio et al., 1993, Tjerneld et al., 1986), poly(vinyl alcohol) (Tjerneld, 1989), maltodextrin (Szlag & Giuliano, 1988) or cellulose derivatives (Skuse et al., 1992) have already been tested, indicating that they must be considered as an alternative to fractionated dextran. The utilization of polysaccharides and its derivatives used in the paper, food and textile industry, as reported by Venâncio and coworkers (1993), may have a big impact in the development of two-phase systems for large scale purification.

Guar gum is a carbohydrate polymer which is found in the seeds of two annual leguminous plants. It contains galactose and mannose as the structural building block. The ratio of the two components may vary slightly, depending on the origin of the seed, but the gum is generally considered to contain one galactose unit for every two mannose units. The structure is a linear chain of β-D-mannopyranosyl unit linked (1→4) with single-membered α-D-galactopyranosyl units occuring as side branches. The α-D-galactopyranosyl units are linked (1→6) with the main chain (Seaman, 1980).

The cyclic neutral sugar structure which makes up the polymer contain numerous hydroxyl groups which offer the possibility of reactive derivatization of the gum. All the commercially important derivatives are obtained by etherification: carboxymethylation, hydroxyethylation, hydroxypropylation or quaternization. Significant changes occur when the gum is substituted - stability in solution increases and although biodegradability decreases the substituted gum is still biodegradable.

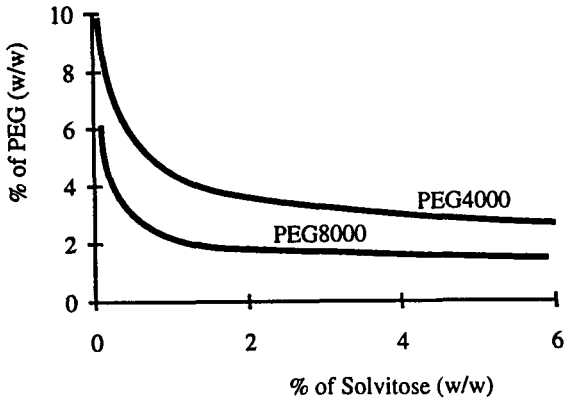

Fig. 1. Phase diagrams for Solvitose Gum Ofa/PEG systems at 19°C.

In this work we report on the use of a Guar Gum derivative, Solvitose Gum Ofa, currently used on textile industry as a thickening agent. The potential utilization of this Guar Gum ether as an aqueous-phase forming polymer, as well as its capacity for protein separation are evaluated.

Materials and methods

Polymers and ligands

Poly(ethylene glycol) (PEG8000), number average molecular weight of $(7\text{-}9)\times10^3$ was purchased from Sigma Chemical Co (St. Louis, Mo, USA), PEG4000, number average molecular weight of $(3.5\times4.5)\times10^3$ was obtained from Merck (Darmstadt, Germany). Solvitose Gum Ofa was a kind gift from Sameca (Porto, Portugal). It is a modified Guar Gum with number molecular weight of $(50\text{-}52)\times10^3$ and a mannose to galactose ratio of 1.8. Procion Navy H-ER150 (C.I. Blue 171) and Procion Yellow H-E4R (C.I. Yellow 84) were a generous gift from Zeneca (Porto, Portugal).

Proteins

Bovine Serum Albumin (BSA) with a MW of 67500 daltons was obtained from Sigma Chemical Co.

All other chemicals were analytical grade and water was distilled and passed through a mixed ion exchanger.

Table 1. Densities and viscosities of Solvitose Gum Ofa-PEG ATPS.

System	Upper Phase		Bottom Phase	
	Density (Kg m^{-3})	Viscosity (cp)	Density (Kg m^{-3})	Viscosity (cp)
0.46%Solv-8.33%PEG4000	1.006	15.5	1.030	170
0.48%Solv-4.35%PEG8000	1.010	6.5	1.023	660

Table 2. Partition coefficients of BSA, in the absence of ligand (K) and in the presence of ligand (Kl), on Solvitose Gum Ofa-PEG4000 systems.

System	pH	K	Kl[a]	$\Delta\log K$[b]
0.46%Solv-8.33%PEG4000	6.8	1.08	1.86	0.24
	8.0	1.16	1.84	0.20

[a] 1% total PEG was replaced by equal amount of PEG-Blue 171 complex.
[b] $\Delta\log K = \log Kl - \log K$

Ligand-PEG synthesis

The preparation of the conjugate Triazine dye/PEG was performed according to Johansson and Joelsson (1985). In all partition experiments the ligand was coupled to PEG8000.

Two phase systems

The systems were prepared from stock solutions of the polymers in water, 0.6% (w/w) Solvitose Gum Ofa and 30% (w/w) PEG. The polymer solutions were weighed out and mixed with water, buffer and protein sample. For pH system lower than 8.0, the phosphate buffer was used and for higher than 8.0 Tris/HCl buffer was employed. In both cases the buffer concentration was 10 mM.

The 0,6% Solvitose Gum Ofa stock solution was prepared by dissolving the powder in hot water. The solution was allowed to stay, under continuous agitation, at 80°C for 45 min, after which a 60 min centrifugation at 2000g was performed in order to eliminate insoluble material. The insoluble material that was not removed during this centrifugation step, was retained in the lower phase after ATPS formation. As a consequence the PEG top phase was clean and available for further processing. In all experiments were affinity partition was tested a small amount of total PEG was replaced with equal amount of dye-PEG8000.

Phase diagrams

After separation, each phase was weighed. The polymer composition of the top phase and of each polymer stock solution were analyzed by a combination of refractometry and a spectophotometric technique. Total polymer concentration was determined by refractometry. Polysaccharide concentration was determined by measuring reducing sugars (DNS method (Miller, 1959)) concentration after an hydrolysis step with sulfuric acid. The bottom phases of a set of three independent systems were also subjected to the described analytical procedure and the results compared to the compositions determined by a mass balance. Since the results were in agreement it was decided to determine bottom phase compositions using a mass balance instead of an analytical determination.

Phase densities and viscosities

Phase densities were measured by a picnometer. Phase viscosities were measured using a torque measuring viscometer from Viscometers UK (model TCU3 and ELV8) at 19°C±1°. Upper phase viscosities were also measured using capillary viscometer. Capillary constant of the viscometer was determined using water and glycerol as calibrants.

Table 3a. Partition coefficients of BSA, K, on Solvitose Gum Ofa-PEG8000 systems.

System	pH	K
0.48%Solv-4.35%PEG8000	6.0	0.83
	6.0 + 0.1 M KCl	0.73
	6.8	1.05
	6.8 + 0.1M KCl	0.66
	8.0	1.16

Table 3b. Partition coefficients of BSA, Kl, on Solvitose Gum Ofa-PEG8000 systems with a ligand attached to PEG.

System	pH[a]	Kl	$\Delta\log K^b$
0.48%Solv-4.35%PEG8000	6.8	1.05	
	6.8 + 1%ligand	1.67	0.20
	6.8 + 3%ligand	1.92	0.26

[a] 1-3% total PEG was replaced by equal amount of PEG-Yellow84 complex.
[b] $\Delta\log K = \log Kl - \log K$

Protein partition coefficient

The partition of proteins between the two phases was determined as described elsewhere (Venâncio et al., 1993). Protein was assayed according to Bradford (1976) and partition coefficient defined as the ratio between upper and bottom phase concentrations.

Phase separation

For phase composition determination, 30 g of the ATPS were centrifuged for 30 min at 140g and constant temperature. For partition coefficient determination, the total mass of the ATPS was reduced to 7.5 g.

Results and discussion

Phase diagram

Phase diagrams for the system Solvitose Gum Ofa-PEG-water with different molecular weight PEG are displayed on figure 1. The effect of the increase in PEG molecular weight on the binodial was, as expected (Albertsson et al., 1990) a reduction in the amount of polymer necessary for the formation of a two-phase system. It can be seen that this system forms two phases with low polymer concentrations.

Rheological properties of Solvitose Gum Ofa-PEG systems are shown on Table 1. Upper phase viscosities are comparable to other systems values (Venâncio et al., 1993), while bottom phase viscosities are much smaller for system composed exclusively with purified polymers: around 100 cp for dextran-PEG systems and 55 cp for Reppal PES-PEG systems (Tjerneld et al., 1986). However systems formed with one crude polymer, as unfractioned dextran (Kroner et al., 1982), crude hydroxypropyl starch (Venâncio et al., 1993) or hydroxypropyl cellulose (Skuse et al., 1992), exhibit higher values, 2000 cp, 1000 cp and up to 3500 cp, respectively.

Protein partition

Protein partition in such system was assayed. On Table 2 and 3, partition coefficients of a model protein, BSA, are reported. As expected (Albertsson et al., 1990), although small, an increase in partition coefficient is observed with pH increase in all tested systems. Also the presence of a neutral salt (Table 3a) has the expected effect on the partition of BSA. As the pH increases, BSA becames more negative and the effect of the salt more pronounced. For affinity partition experi-

Table 4. Cost of 1 kg of different ATPS

System	Cost (kg^{-1})	Reference
Solvitose-PEG8000	0.26	this work
Reppal PES-PEG8000	1.67	Tjerneld et al., 1986
crude HPS-PEG8000	0.20	Venâncio et al., 1993
Klucel L-Pluronic P105	0.80	Skuse et al., 1992
Maltodextrin-PEG4000	0.55	Szlag and Giuliano, 1988

ments, triazine dyes, Procion Navy H-ER150 (CI Blue 171) and Procion Yellow H-E4R (CI Yellow 84), were bound to PEG8000. The overall effect of both ligands on BSA partition coefficient was similar.

The effect of the pH on the increase in partition (ΔlogK) of BSA due to the ligand is shown on Table 2 and Table 3b shows the effect of the ligand concentration on the same affinity. It is well established (Kopperschläger & Birkenmeier, 1993) that an increase in pH usually decreases the efficiency of ligands. Such an observation was made in this system as can be seen on Table 2. Also the effect of an increase in the amount of ligand present was noticed (Table 3b). Partition coefficient increases till an upper limit value is reached.

For both ligands the increase in affinity is not very high. Parallel experiments performed in our laboratory (results not published) with the same ligands in Reppal PES-PEG systems, shown ΔlogK values for BSA ranging from 0.8 to 1.0. This last observation may be explained by the presence of some impurities in our Solvitose stocks, such as salts, which may decrease the efficiency of the ligand. Anyway, this observation does not exclude the possibility of using other ligands with an enhanced efficiency.

ATPS economics

As previously refered, one of the advantages of these systems is the low polymer concentration needed to form ATPS. This fact is expected to have a significant effect in the overall cost of a purification process. In Table 4, the cost of the ATPS (in the absence of ligand) based on Solvitose Gum Ofa is compared with recently refered alternatives to traditional Dextran-PEG systems. The cost of the tested system is of the same order of magnitude of the other referred system using an industrial crude polymer and is significantly less expensive than any other referred system.

Cost of Solvitose Gum Ofa was \$6.2 kg^{-1} (Sameca, Portugal) and of PEG was \$2.58 kg^{-1}. Values presented clearly indicate that Solvitose Gum Ofa systems are competitive alternatives for protein purification.

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References

Albertsson P-E, Johansson G & Tjerneld F (1990) Aqueous Two-Phase Separations. In: Asenjo JA (ed.) Separation Processes in Biotechnology. (pp. 287–318), Marcel Dekker, New York Basel.

Andersson E & Hähn-Hägertal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb Technol 12: 242–254

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Datar R (1986) Economics of primary separation steps in relation to fermentation and genetic engineering. Process Biochem 21: 19–26

Johansson G & Joelsson M (1985) Preparation of Cibacron Blue F3GA (Polyethylene Glycol) in Large Scale for use in Affinity Partitioning. Biotechnol Bioeng 27: 621–625

Kamihira M, Kaul R & Mattiasson B (1992) Purification of Recombinant Protein A by Aqueous Two-Phase Extraction Integrated with Affinity Precipitation. Biotechnol Bioeng 40: 1381–1387

Kopperschläger G & Birkenmeier G (1993) Affinity Partition of Biomolecules in Aqueous Two-Phase Systems. In: That T. Ngu (ed) Molecular Interactions in Bioseparations. (pp. 499–509), Plenum Press, New York.

Kroner KH, Hustedt H & Kula M-R (1982) Evaluation of Crude Dextran as Phase Forming Polymer for the Extraction of Enzymes in Aqueous Two-Phase Systems in Large Scale. Biotechnol Bioeng 24: 1015–1045

Kroner KH, Hustedt H & Kula M-R (1984) Extractive enzyme recovery: Economic considerations. Process Biochem 19: 170–179

Kula M-R (1989) Large scale protein recovery using aqueous phase systems. In: Derek Fisher & Ian A. Sutherland (Eds) Separation using aqueous two-phase systems. (pp. 295–297), Plenum Press, New York.

Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem, 31, 3: 426–428

Plunkett SD & Arnold FH (1990) Metal affinity extraction of human hemoglobin in an aqueous polyethelene glycol-sodium sulfate two-phase system. Biotechnol Technique 4,1: 45–48

Seaman JK (1980) Guar Gum. In: Robert L Davidson (Ed) Handbook of water-soluble gums and resins. (pp. 6.1–6.19), McGraw Hill, New York.

Skuse DR, *Norris-Jones* R, Yalpani M & Brooks DE (1992) Hydroxypropyl cellulose/poly(ethylene glycol)-co-poly(propylene glycol) aqueous two-phase systems: System characterization and partition of cells and proteins. Enzyme Microb Technol 14: 785–790

Szlag DC & Giuliano KA (1988) A low cost aqueous two-phase system for enzyme extraction. Biotechnol Technique 2, 4: 277–282

Sturesson S, Tjerneld F & Johansson G (1990) Partition of macromolecules and cell particles in aqueous two-phase systems based on hydroxypropyl starch and poly(ethylene glycol). Appl Biochem Biotechnol 26, 3: 281–295

Tjerneld F, Berner S, Cajarville A & Johansson G (1986) New aqueous Two-phase system based on hydroxypropyl starch useful in enzyme purification. Enzyme Microb Technol 8: 417–423

Tjerneld F, Johansson G & Joelsson M (1987) Lactate Dehydrogenase on a Large Scale. Biotechnol Bioeng 30: 514–520

Tjerneld F (1989) New polymers for aqueous two-phase systems. In: Derek Fisher & Ian A.Sutherland (eds) Separation using aqueous two-phase systems. (pp. 429–438), Plenum Press, New York.

Veide A, Smeds A-L & Enfors S-O (1983) A Process for Large Scale Isolation of β-Galactosidase from E. Coli in an Aqueous Two-Phase System. Biotechnol Bioeng 25: 1789–1800

Venâncio A, Teixeira JA & Mota M (1993) Evaluation of crude hydroxypropyl starch as a bioseparation aqueous-phase-forming polymer. Biotechnol Progr 9: 635–639

Walter H & Johansson G (1986) Partitioning in aqueous two-phase systems: an overview. Anal Biochem 155: 215–242

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