

α -Chymotrypsin catalyses the synthesis of methotrexate oligomers

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ABSTRACT

The enzymatic synthesis of methotrexate (MTX) catalysed by α -chymotrypsin was studied for the first time. The proteolytic enzyme displayed activity for the synthesis of MTX oligomers composed by 6 repeating units ($DP_{avg} = 1.5$). For longer oligomers, molecular dynamics simulations confirmed that as the oligomeric chain grows its accommodation in the enzymes' active site is hindered, which is evidenced by a decrease of the binding energy associated. The full characterization of the oligomers produced was performed by nuclear magnetic resonance (NMR, 1H and ^{13}C), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), electrospray ionization (ESI) and differential scanning calorimetry (DSC).

1. Introduction

The synthesis of polymeric drugs is an area with increasing interest for the pharmaceutical industry that aims to improve their pharmaceutical performance mainly in applications requiring a drug-controlled delivery. The controlled release, the improvement of safety and the biodistribution profile, are some of the benefits associated to the application of polymeric drugs [1]. The promising results obtained so far in clinical trials using polymer-anticancer drug conjugates have been raising the interest in the development of these type of drugs [2]. The conjugation of poly(ethylene glycol) (PEG) with drugs is one of the most studied strategies for the synthesis of polymer-drug conjugates, being that some of the developed products have already reached the market [3]. Recently, Suksiriworapong et al., synthesized a methotrexate-poly(glycerol adipate) conjugate, with improved toxicity for Saos-2 cells than the unmodified methotrexate [4].

The polymer-drug conjugates or polymeric drugs are expected to be easily hydrolysed *in vivo*, depending on the type of bond established between the polymer and the drug or among the drug monomer units. Prodrugs containing amide bonds are highly stable *in vivo*, being hydrolysed by carboxylesterases [5], peptidases or proteases [6]. Proteases are enzymes commonly used for the hydrolysis of amide bonds between two amino acids. Despite their hydrolytic activity, they are also able to catalyse the inverse reaction, *e.g.* the formation of amide bonds (peptides) [7]. The chemoenzymatic peptide synthesis is considered as a green chemistry practice to replace the typical methods, liquid or solid phase peptide synthesis [8] taking advantage of the low costs associated with the use of proteases as catalyst [9]. Enzymes like papain, chymotrypsin, bromelain, trypsin and even lipase can be used

as catalysts of these type of reactions [7]. Qin et al. [9] studied the use of papain for the biosynthesis of perfectly alternating oligopeptides. They started by the synthesis of the dipeptide AG-OEt, followed by the addition of papain and a short incubation period, isolating a peptide with a maximum of 21 AG units. The authors mentioned that an accurate choice of the ideal protease is crucial to afford oligomerization. This selection is essential to avoid hydrolysis and/or transamidation reactions during the process. The protease selection should rely on the specificity of the enzyme, determined by their specific hydrolytic properties between two amino acids. Following the same approach, the same authors [10] studied the ability of α -chymotrypsin in the oligomerization between dipeptide KL-OEt. The selection of the protease relied on its high specificity for the hydrolysis between -LK- residues. After synthesis of the dipeptide KL-OEt, the α -chymotrypsin was added, forming the expected oligomer. At a specific pH the enzyme triggered the formation of an hydrogel due to the spontaneous self-assembly of the KL chains into β -sheets. When papain was used for the same purpose, no hydrogel was formed, and a random sequence of oligopeptides was produced. The low selectivity of papain for the hydrolysis of -LK- bonds was responsible for the low reactional outcome.

Methotrexate is a commercially available drug for the treatment of several cancers, auto-immune diseases, and rheumatoid arthritis [11]. However, this drug presents several limitations, such as poor solubility in water, low resistance, high toxicity, and diverse side-effects associated to its administration [12]. Due to the presence of both amine and carboxylic acid groups in its composition, it represents an excellent template for peptide bond formation. The conjugation of MTX with polymers is a well explored strategy [4,13,14], however, as far as we know, its self-polymerization has never been undertaken. The practical

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application of an oligomeric MTX is envisaged to decrease several adverse side effects associated to its administration like its toxicity and cell resistance [15].

In this work, we explored for the first time the ability of α -chymotrypsin to catalyse the oligomerization of methotrexate. The main goal is to produce an oligomeric drug with improved properties in comparison to the monomeric commercial alternative. Molecular dynamics simulations were performed to evaluate the affinity of methotrexate monomer, dimer and trimer, to the protease active site. The resulting oligomers were fully characterized by NMR (^1H and ^{13}C), MALDI-TOF, ESI and DSC.

2. Experimental

2.1. Materials

All compounds were purchased from Sigma-Aldrich or TCI chemicals, and used without further purifications. α -chymotrypsin from *bovine pancreas* type II, was purchased from Sigma-Aldrich, and used without any further treatment.

2.2. Methods

2.2.1. General procedure for the synthesis of oligomeric methotrexate

To a solution of MTX di-sodium salt (10 mg) in distilled water (1 mL) was added NaOH until pH = 9–10. α -chymotrypsin was then added to the reaction (1 mg) suspended in 200 μL of water. The solution was placed in a water bath at 50 $^\circ\text{C}$, 100 rpm for 7 days. After this time, the enzyme is removed by centrifugation at 2500g using vivaspin 10 kDa (Sartorius).

2.2.2. Activity of α -chymotrypsin

The activity of α -chymotrypsin was measured using the universal protease activity assay, with casein as substrate [16]. The activity is calculated by the tyrosine units hydrolysed by the protease, detected at 660 nm after colorimetric reaction with Folin & Ciocalteu's phenol reagent. Value of activity obtained: 0.61 U/mg. The values of the activity are given as Units (U) of tyrosine per milligram of protease. The activity of the α -chymotrypsin in the medium along time was evaluated by incubating the enzyme under the same reactional conditions for the synthesis of the oligomers, without addition of the methotrexate. At different periods of time, an aliquot of the solution was removed, and the activity was measured as mentioned.

2.2.3. NMR

^1H and ^{13}C NMR spectroscopy were performed using a Bruker Avance III 400 (400 MHz for ^1H , 100 MHz for ^{13}C), using DMSO- d_6 or D $_2$ O (Cortecnet, France) as deuteride solvent and the peak solvent used as internal reference. Heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation (HMBC) were also performed for an accurate signal assignment (Figure S4 and S5). Signal multiplicity is given as: s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet).

NMR characterization of the isolated oligomeric methotrexate: ^1H NMR (D $_2$ O): δ_{H} 1.98–2.08 (m, 1 H), 2.13–2.21 (m, 1 H), 2.32 (t, J = 8.0 Hz, 2 H), 3.21 (s, 3 H), 4.31 (dd, J = 4.4, 8.8 Hz, 1 H), 4.83 (s, 2 H), 6.92 (d, J = 9.2 Hz, 2 H), 7.75 (d, J = 8.8 Hz, 2 H) and 8.42 (s, 1 H) ppm.

^{13}C NMR (D $_2$ O): δ_{C} 28.5 (CH $_2$), 34.3 (CH $_2$), 38.5 (CH $_3$), 55.4 (CH $_2$), 55.9 (CH), 111.9 (CH), 120.7 (C $_q$), 128.4 (C $_q$), 129.0 (CH), 146.9 (CH), 147.7 (C $_q$), 151.9 (C $_q$), 155.6 (C $_q$), 163.7 (C $_q$), 169.7 (C = O), 172.7 (C $_q$), 179.3 (C = O) and 182.4 (C = O) ppm.

2.2.4. MALDI-TOF

MALDI-TOF mass spectra were acquired on a Bruker Autoflex Speed instrument (Bruker Daltonics GmbH) equipped with a 1 kHz solid-state

smartbeam laser. The samples were prepared as reported [15] using 2,5-dihydroxybenzoic acid (DHB) as matrix, and analysed in the linear negative mode. The number average molecular weight (M_n), weight average molecular weight (M_w), polydispersity ($\text{PDI} = M_w/M_n$) and average and maximum degree of polymerization (DP_{avg} and DP_{max}) were calculated using the data obtained from the MALDI spectra [17].

The conversion rate (p) was calculated based on Carothers equation, using the DP_{avg} value obtained by MALDI-TOF.

$$\text{Carothers equation: } \text{DP}_{\text{avg}} = \frac{1}{1 - p}$$

2.2.5. ESI

Electrospray ionization was performed in a mass detector Thermo Finnigan LxQ (Linear Ion Trap). The analysis was made using a mass detector susceptible of analysis in full scan mode with negative ionization. The mass spectra range was between m/z 50 and 2000 and a capillary voltage of 29 V was used. Samples were prepared using ultrapure water (Milli-Q) and methanol (HPLC grade, Fisher Chemical). Prior to analysis, the sample was filtrated using a 0.2 μm filter (Whatman).

2.2.6. DSC

All measurements were conducted on a power-compensated DSC instrument (DSC 6000 Perkin Elmer) with a nitrogen flux of 20 mL/min, using stainless steel capsules in the temperature range of 20–300 $^\circ\text{C}$ (heating rate: 10 $^\circ\text{C}/\text{min}$, sample weight: 1–2 mg). The DSC device was calibrated using indium and zinc, both of high purity. The samples were freeze-dried for 24 h prior to the analyses and each sample was measured at least three times, in order to validate the results.

2.3. Molecular Dynamics simulations and docking

Bovine α -chymotrypsin, PDB ID: 1OXG, [18] with 2.2 \AA resolution, comprising residues 1–245, was chosen for the modelling experiments in this work. The initial procedures used, as Molecular Dynamics simulations, MTXs optimization and analyses, to settle the structures to be used for docking, are described in the Supporting Information. One-to-one binding model was considered to evaluate individually each MTX molecule to the target α -chymotrypsin.

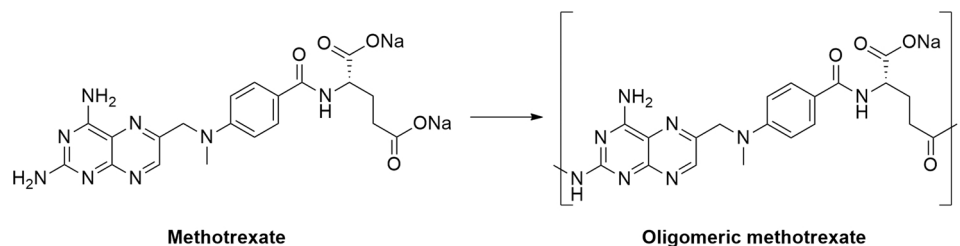
Docking experiments were performed with AutoDock 4.0 [19] and prepared with the AutoDock Tools Software [19]. The middle structure obtained for the native α -chymotrypsin, from the MD simulation at pH 9.5 and 323 K, were used as target macromolecule for the docking experiments. A grid box was set according to the size of the oligomer, ranging from 50 \times 50 \times 50–80 \times 80 \times 100 in the case of trimer, with a resolution of 0.375 \AA . The grid box was centered in the active site - catalytic triad - and grid potential maps were calculated using AutoGrid 4.0. We chose Lamarckian Genetic Algorithm (LGA) [19,20] as search algorithm. Each docking consisted of 150 independent runs, with a population of 150 individuals, a maximum number of 25 \times 10 5 energy evaluations for MTX monomer and 25 \times 10 6 energy evaluations for dimer and trimer (due the torsions of the oligomers), and a maximum number of 27,000 generations. Additionally, the binding site preference and interactions were identified through the AutoDock tools.

We analysed docking results looking at the binding energy and the Ligand Efficiency (LE), which measures the binding energy per ligand heavy-atom (kcal/HA) to understand how the increase in the oligomer size contributes to the binding. Additional information about molecular modelling studies is presented in the Supporting Information.

3. Results and discussion

3.1. α -chymotrypsin-catalysed synthesis of oligomeric MTX

The use of proteases as polymerization tools for the synthesis of



Scheme 1. Reactional scheme for the oligomerization of methotrexate.

peptides is being explored by several researchers [9,10]. Considering the chemical structure of methotrexate, composed by two carboxylic acids and two primary aromatic amines, we considered it as a promising template to evaluate the polymerase activity of α -chymotrypsin (scheme 1). Moreover, the production of a polymeric drug would represent a promising strategy for the pharmaceutical industry regarding its increased interest in the commercialization of these type of drugs [21]. As an amide prodrug, the oligomeric MTX is expected to have higher stability in the human body than the monomeric structure. Moreover, the amide linkages of the oligomeric units, being more prone to hydrolysis by enzymatic action, would improve drug metabolism and decrease dosage administration [22,23]. The improved metabolism is expected to result in a less toxic drug with lower side effects than the commercial monomeric species [22,23].

Methotrexate di-sodium salt was used as starting material to ensure water solubility and provide hydrophilic character to the produced oligomers.

α -chymotrypsin was chosen for the MTX oligomerization based on its well-known ability to polymerize peptides [10], showing its high selectivity for the hydrolysis of aromatic amino-acids, such as tyrosine, phenylalanine and tryptophan [24]. The reactional conditions, namely temperature, pH and time, were optimized to reach the maximum oligomerization degree (Table 1).

Most of the proteases have an optimum working pH between 7.0–9.0. Based on this feature, on the low basicity of the aromatic amines and on the lack of reactivity of the pteridin ring, the reactions were conducted under basic medium. Similar to what was previously observed by Qin and co-workers [10] for the synthesis of $-(KL)_x$ peptides catalysed by α -chymotrypsin, we confirmed that no oligomers are produced when the reactions were carried out at neutral pH (Table 1, entries 1–3). For an efficient reaction synthesis, it is critical to perform the reactions at alkaline pH, using NaOH as base (≈ 9.5 , Table 1, entries 4–8).

The best reactional outcomes were obtained herein when performing the reactions at 50 °C, which is in accordance with the optimum temperature postulated by other authors for the same protein

Table 1

Reactional conditions tested for the α -chymotrypsin-assisted synthesis of methotrexate oligomers.

Entry	Temperature	pH	Time ^a	Methotrexate oligomers ^{b,c}
1	RT	7	1 week	1
2	50 °C	7	1 week	1
3	50 °C	7.8	1 week	1
4	50 °C	9.5	48 h	3
5	50 °C	9.5	72 h	3
6	50 °C	9.5	96 h	5
7	50 °C	9.5	1 week	6
8	50 °C	9.5	2 weeks	6
9	50 °C	9.5	1 week	1 ^d

^a Maximum time tested.

^b Detected by MALDI-TOF, were 1 unit corresponds to the monomer.

^c Maximum oligomeric units detected.

^d Control reaction without enzyme.

Table 2

Conversion rate (%), average and maximum degree of polymerization (DP_{avg} and DP_{max}) analysed by MALDI-TOF and electrospray ionization (ESI), for the oligomerization of methotrexate, with and without α -chymotrypsin.

	With α -chymotrypsin	Control (without enzyme)
Conversion rate ^a	33.4 %	0 %
DP_{avg} ^b	1.5	1
DP_{max} ^b	6	1
ESI	975.4 g/mol ^c	453.28 g/mol ^d

^a Calculated by the Carothers equation.

^b Calculated by MALDI-TOF.

^c Most abundant specie, corresponding to the dimer. M_w dimer = 956.8, where in ESI: $975 = 956 + Na^+ - 4H^+$.

^d Monomer $M_w = 454$ g/mol or 498 g/mol (di-sodium form), where in ESI: $453 = 454 - 1H^+$.

[25].

During reaction, samples were withdrawn, and continuously monitored by MALDI-TOF, until 2 weeks of reaction (Table 1, entries 4–8). The best reactional outcome was achieved after 1 week of reaction with the formation of oligomers composed by 6 monomeric units (DP_{max}). Longer reactional times led to the recovery of similar-sized MTX oligomers however in lower amount. The DP average calculated (1.5) indicates that the most abundant specie in the reactional medium is the methotrexate dimer, confirmed by MALDI-TOF and ESI (Table 2, Figure S1). After one week of reaction, the enzyme converted 33 % of the monomer molecules into oligomers, while the reaction carried out in the absence of enzyme did not lead to the formation of any oligomeric species (Table 1, entry 9 and Table 2).

In order to ensure an efficient oligomerization, it was imperative to infer the maintenance of the basic pH during reaction. For this, the pH was also continuously monitored and after 1 week of reaction, one observed a pH decrease from 9.5 to 8, and to 7.5, after 2 weeks. We may assume that some of the NaOH used to basify the medium was consumed during the long reaction, therefore resulting in a pH decrease. As the reaction occurs the NaOH is consumed by the low basic amines in the pteridin ring, which were deprotonated, leading to a pH decrease. The addition of NaOH was crucial to increase the amines reactivity and proceed with the oligomerization.

It was noteworthy that the activity of α -chymotrypsin remained almost unaltered during the first week of reaction (activity of 0.61 U/mg), losing only 15 % of the initial activity after 2 weeks (activity of 0.52 U/mg). The high stability of the catalyst ensured the efficient catalysis for longer periods of incubation.

3.2. Synthesis of dimeric MTX – proposed mechanism

Methotrexate, mainly composed by aromatic rings, is presented herein as a suitable substrate for α -chymotrypsin. Based on the data obtained, we have proposed a mechanism for the synthesis of a dimeric unit of methotrexate catalysed by α -chymotrypsin (Fig. 1). The reaction is predicted to start by the nucleophilic attack of the OH terminal group of the serine, in the catalytic triad, to one of the carboxylic groups of MTX (step 1), leading to the formation of the tetrahedral

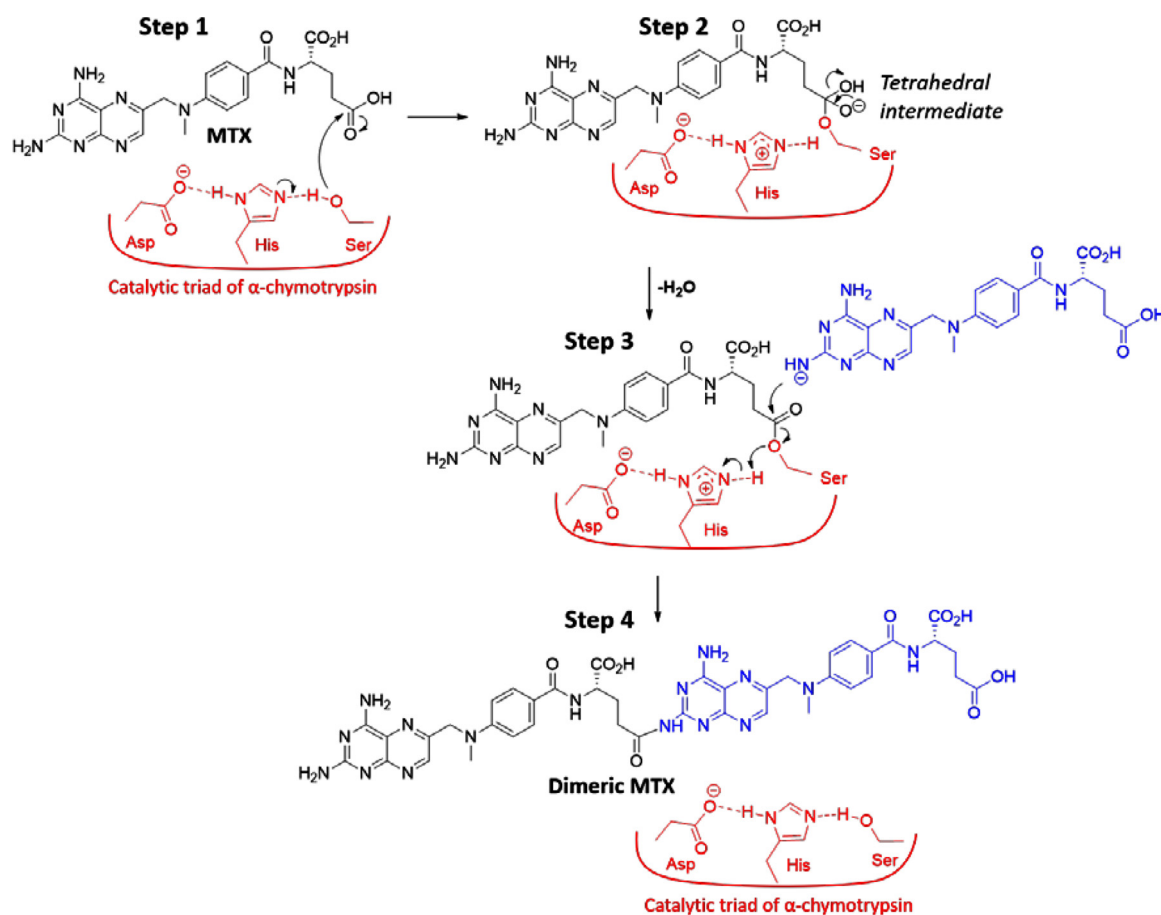


Fig. 1. Proposed mechanism for the synthesis of a dimeric unit of methotrexate catalysed by α -chymotrypsin.

intermediate (step 2). The carboxylic group in the glutamic moiety, which suffers the nucleophilic attack as depicted in Fig. 1, is reported as the most reactive [15]. This carboxylic group is also less sterically precluded, which is an important feature to afford oligomerization and an effective catalysis by the protease. After release of a water molecule, an MTX-enzyme complex is formed, through the formation of an ester bond between MTX and the serine residue of the catalytic triad. This reactive bond easily suffers a nucleophilic attack by one of the amines in the pteridin ring of another MTX unit (step 3). The dimeric MTX is then formed and released from the enzyme, where the catalytic triad of α -chymotrypsin is restored (step 4).

Considering the data previously described, we may predict that the reaction is not restricted to the synthesis of dimeric units, but it can go further to longer species ($\text{DP}_{\text{max}} = 6$). The size of the oligomers is however dependent on the affinity of MTX oligomers to the enzyme's active site. Regarding the data obtained, we may infer that α -chymotrypsin is able to synthesize species until a maximum of 6 MTX units. However, longer species are hampered to be synthesized due to stereochemical constraints resulting from the molecular weight of MTX (498 g/mol), which is comparably higher than the natural substrates of this enzyme (amino acids, peptides).

For a better understanding of these findings, molecular dynamics simulations were performed to evaluate the affinity of MTX and oligomeric MTX to the active site of the enzyme and evaluate the role of the substrate size on the enzyme activity.

3.3. Molecular dynamics simulations

Bovine α -chymotrypsin (PDB ID: 1OXG) [18] was evaluated under different conditions of temperature and pH during Molecular Dynamics (MD) simulations. Firstly, the enzyme was evaluated at 310 K and

physiological pH, then at 323 K and pH 9.5; this second simulation was performed to mimic the experimental conditions used. RMSD results (Figure S2), indicate that the experimental conditions established are the ideal to study the enzyme behaviour, since the enzyme demonstrates to be more stable at the higher temperature and pH. Figure S3 shows the α -chymotrypsin crystallographic structure (A) highlighting the active site, where the catalytic triad composed by HIS 57, ASP 102 and SER 195 takes place, and the electrostatic distribution for this enzyme (B).

MTX and MTX oligomeric structures were predicted at PM6 level [26], a semi-empirical method that performs geometry optimization and charge distribution, making the structures suitable to be used for molecular docking. In the case of MTX, the neutral and negative forms (at pH 9.5) were evaluated. The structures presented in Fig. 2 were used in Molecular Docking experiments.

Overall, monomer (MTX), dimer and trimer have an increasing flexibility due to the increasing number of torsions. Dimer and trimer (Fig. 2, C–D) optimized with PM6, can undergo more "bent" conformations when submitted to docking, due to the higher number of torsions.

Docking experiments were conducted in order to understand the interactions between MTX and α -chymotrypsin, and the role of this enzyme in the synthesis process. We select an area that enclose the active site with enough space to comprise the MTX units (monomer, dimer or trimer) to dock. Fig. 3 shows the correlation between the MTX and enzyme, and the binding energy involved for each case studied.

MTX complexed to α -chymotrypsin, present a binding energy of -6.94 kcal/mol (-29.04 kJ/mol) and a Ligand Efficiency (LE) of -0.19 kcal/HA (-0.79 kJ/HA). The monomer has hydrogen bonds with ALA56, GLY59, SER96, TYR94 and THR104, and van der Waals interactions are found with HIS57 and ASP102. For dimeric MTX, GLY59 and TYR94 are also involved in hydrogen bonds, as well as LYS90 and

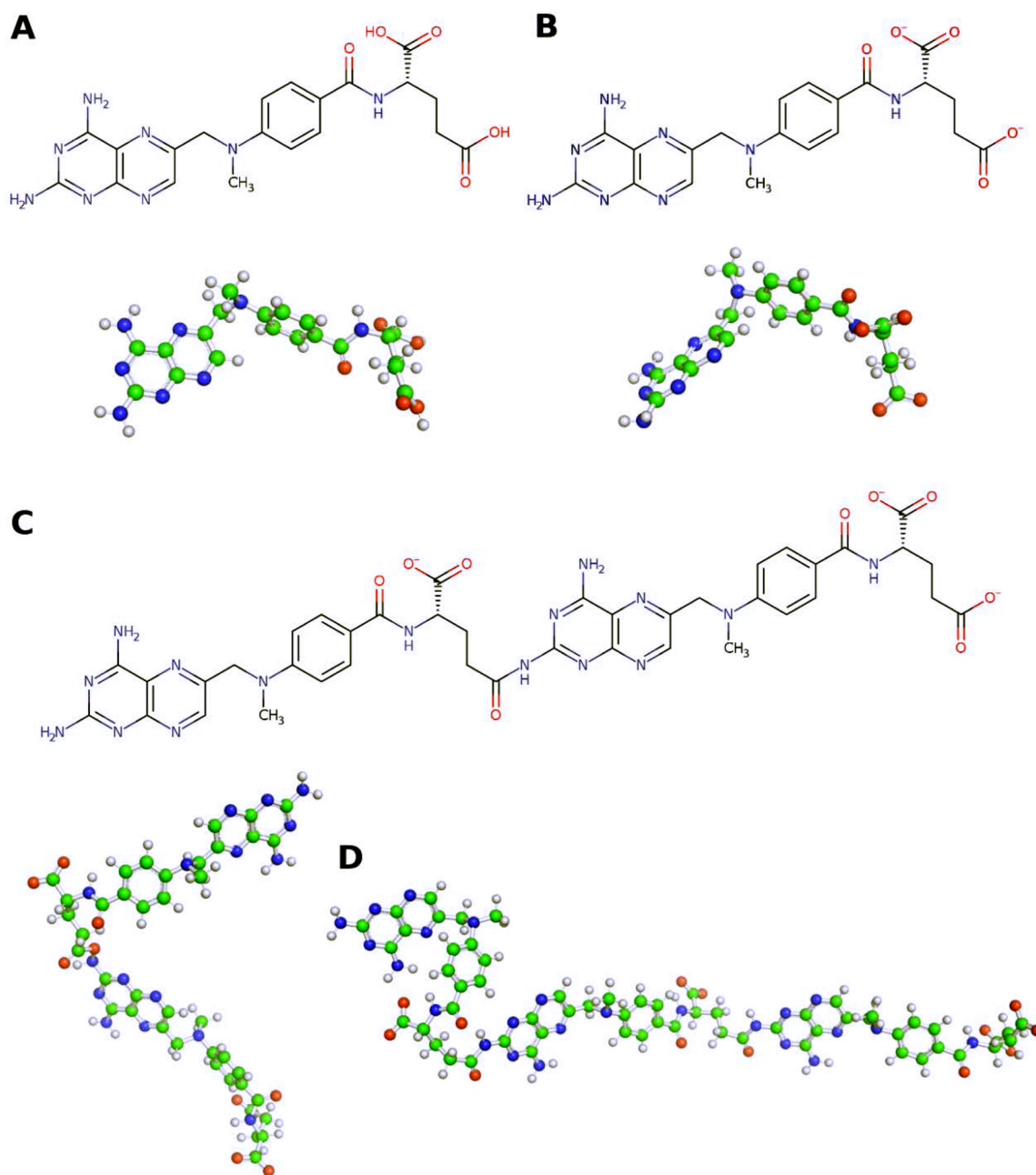


Fig. 2. (A) skeletal and 3-D methotrexate (MTX) structures at pH 7.0 and (B) the deprotonated forms at pH 9.5. (C) skeletal and 3-D structures of MTX dimer, at pH 9.5 and (D) shows the trimer 3-D representation. The colour scheme use green for carbon, blue for nitrogen, red for oxygen and white for hydrogen.

ASN95. The binding energy is -6.35 kcal/mol (-26.61 kJ/mol) in this case, and a LE of -0.1 kcal/HA (-0.42 kJ/HA). HIS57 is still a close contact for MTX dimer.

The trimer binds to the enzyme with -5.45 kcal/mol (-22.80 kJ/mol) and LE of -0.06 kcal/HA (-0.25 kJ/mol). Similarly, HIS57 and ASP102 interact with the trimer. In addition, five hydrogen bonds are found, two with SER96 and the others with LEU97, ASN91, and THR104. In all the three cases, electrostatics interactions are also present, between the carboxylic acids of MTX and the positive region observed in Figure S3 (B).

Farhadian and co-workers recently reported docking experiments with α -chymotrypsin [27,28]. However, the complexes were not formed in the active site, where the catalytic triad take place. Some other works addressing the docking near the catalytic site do not reveal the complex binding energy for the association of a molecule to the protein [29,30]. Other larger molecules have been described to

efficiently bind to α -chymotrypsin, however these molecules are used as inhibitors, and therefore cannot be compared to our work [30]. The lack of similar docking approaches makes it difficult to compare our binding preferences and energies with other studies. Moreover, the conversion of the binding energy to association constants is also hard to obtain. In docking experiments, the protein is static, the whole long-range electrostatics is not considered, and no water molecule is present. Therefore, the use of the equation $\Delta G = -RT \ln K_a$, would lead to a rough approximation.

All these interactions demonstrate the ability of MTX to bind to the protease active site, leading to the formation of oligomeric MTX. Even though being possible more interactions of longer MTX forms with the protease, the binding energy decreases discretely as well as the LE per heavy-atom. One may infer that this is due to the energy penalties resulting from the increased number of torsions and desolvation for the bulky forms of MTX (dimer and trimer), since they can undergo to more

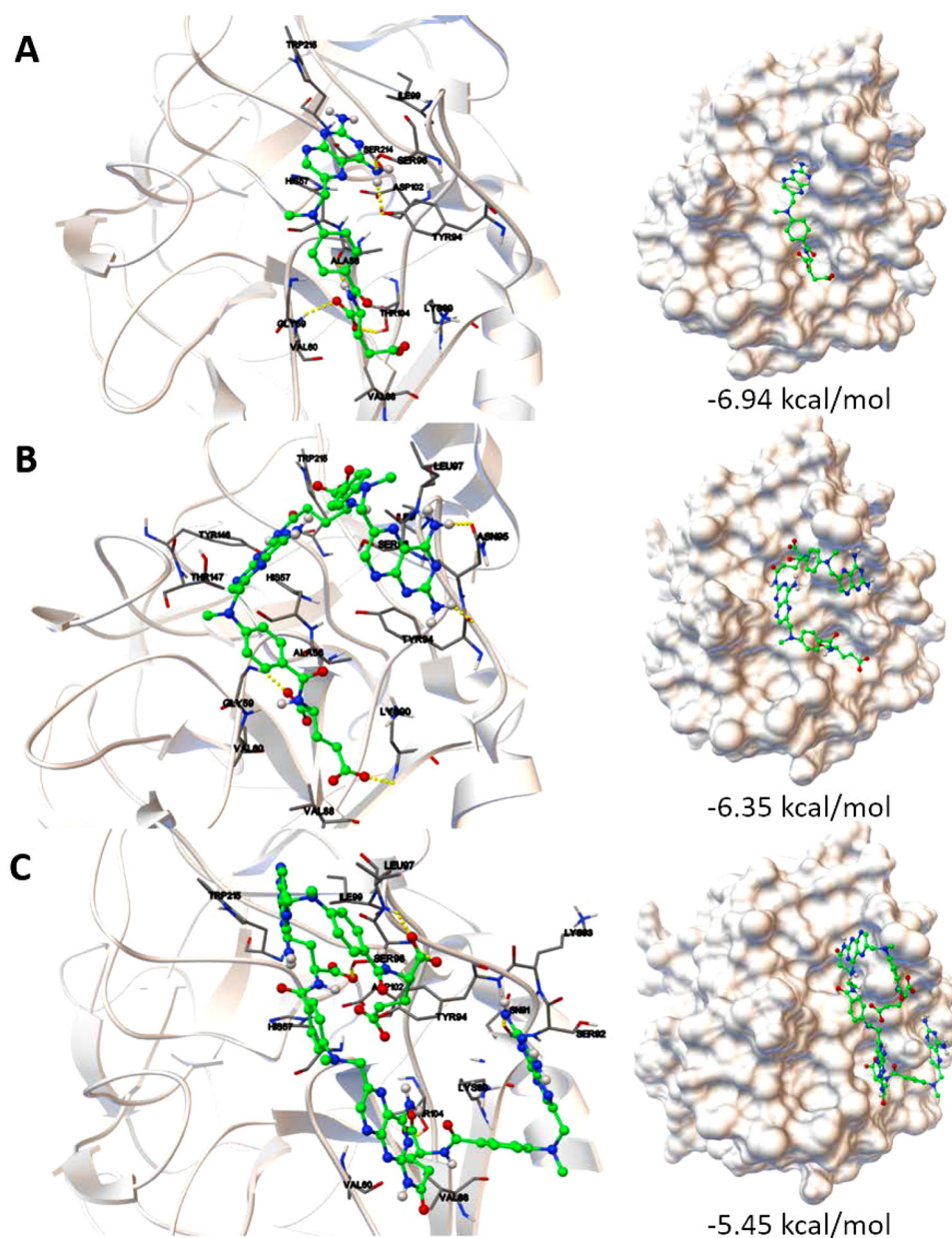


Fig. 3. Docking poses showing the interaction of α -chymotrypsin with the different methotrexate species, and the respective binding energies obtained using AutoDock 4.0: A) α -chymotrypsin with methotrexate; B) α -chymotrypsin with dimeric methotrexate and C) α -chymotrypsin with trimeric methotrexate. Surface/cavities representation are in the right panel. Enzyme is represented in light grey, hydrogen bonds in yellow dashes and methotrexates following the scheme previous described in Fig. 2. Values in SI units: A) -29.04 kJ/mol; B) -26.61 kJ/mol and C) -22.80 kJ/mol.

conformations, as previously observed during docking experiments. The results indicate a decrease of the binding energy as the oligomer chain grows, justifying that only a maximum of 6 units of MTX could be biosynthesized by α -chymotrypsin.

3.4. MTX oligomers characterization

3.4.1. NMR spectroscopy

By ^1H NMR spectroscopy, it is possible to detect differences between MTX oligomers and the starting material (monomer). In the spectra of free MTX, the signals appeared at δ_{H} 8.56, 8.13 (NH), 7.62, 7.38 (NH), 6.81, 6.58 ppm (NH_2), in the aromatic region (Fig. 4A). The spectra of oligomeric MTX (Fig. 4B) show significant chemical shifts in the pteridine ring, δ_{H} 8.15 (b), 8.14 (NH), 7.62, 6.78 ppm. The pattern of the signals remained the same as in the monomer MTX, confirming that the amide bond (near proton g) is not hydrolysed by the protease during the

oligomerization reaction. Bidimensional spectra (HSQC and HMBC) were also performed to allow an accurate signal assignment (Figure S4 and S5).

3.4.2. MALDI-TOF and ESI

MALDI-TOF spectra displays the pattern of MTX oligomers catalysed by α -chymotrypsin (Fig. 5B) and of the control reaction without enzyme, showing the peak of the monomer below m/z 500 (Fig. 5A). By ESI the ion peak corresponding to the monomer is observed at m/z 453 (Figure S1 A) and no fragmentation is detected. The most abundant specie detected by ESI on the samples catalysed by α -chymotrypsin is a dimer (Figure S1 B).

Regarding the oligomer spectrum (Fig. 5B), $(\text{MTX})_1$ is observed at 475 Da [498-Na^+], whereas a repetitive pattern of m/z of ≈ 521 Da is detected. This behaviour is observed until a maximum m/z of 3112.61 Da, attributed to the longest oligomer obtained composed by 6

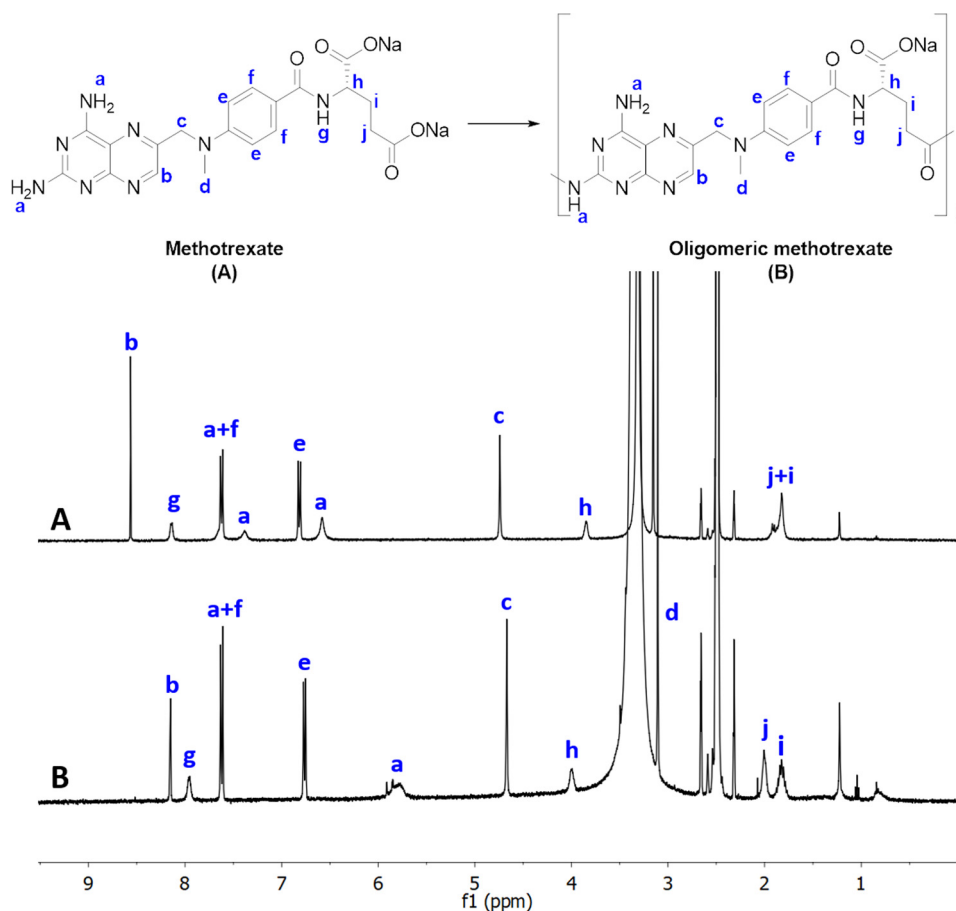


Fig. 4. ^1H NMR ($\text{DMSO}-d_6$) spectra of A) methotrexate and B) methotrexate oligomers synthesized by α -chymotrypsin.

MTX units. The repeating unit between species (≈ 521 Da) corresponds to the tri-sodium methotrexate, most probably resulting from the presence of the NaOH on the reactional medium, which may be linked to more than one amine in the pteridin ring. Additional sodium was also found on the recorded ESI spectra of the oligomer.

In Table 3 are summarized the results obtained expressed in terms of number average molecular weight (M_n), weight average molecular weight (M_w) and PDI (M_w/M_n). The oligomers produced by the protease showed a M_n value of 724.8 g/mol and a M_w of 1225.7 g/mol. The polydispersity index obtained was 1.69, showing some sample heterogeneity.

3.4.3. DSC

The DSC analysis highlights differentiated thermal behaviour depending on the size of the MTX oligomers (Fig. 6). The monomeric MTX show a melting point at 139°C (black line) and no other thermal events are observed at higher temperatures. As the chain length grows higher melting points are expected. The oligomers synthesized by the protease (red line), showed a melting point at 184°C .

4. Conclusions

In this work the synthesis of MTX oligomers assisted by α -chymotrypsin was undertaken for the first time. The oligomeric species

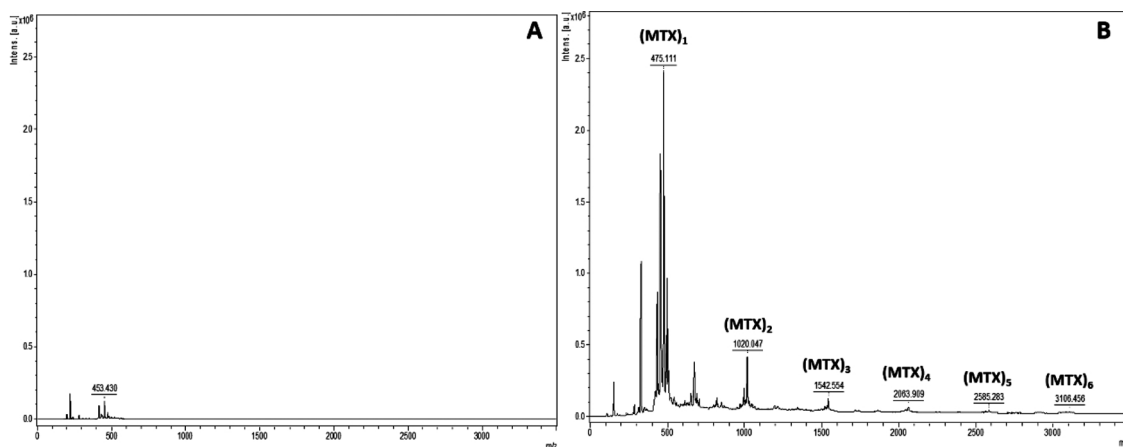


Fig. 5. MALDI-TOF spectra of A) control reaction carried without enzyme, and B) oligomeric methotrexate synthesized by α -chymotrypsin.

Table 3

Number average molecular weight (M_n), weight average molecular weight (M_w), polydispersity (PDI), average and maximum degree of polymerization (DP_{avg} and DP_{max}) of the obtained oligomeric methotrexate catalysed by α -chymotrypsin at 50 °C after 1 week.

	Oligomeric methotrexate
DP_{avg}	1.5
DP_{max}	6
M_n	724.8
M_w	1225.7
PDI	1.69

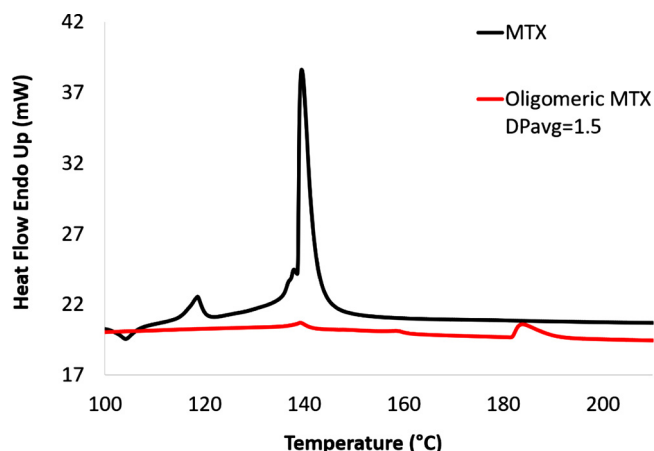


Fig. 6. Differential scanning calorimetry (DSC) curves of methotrexate (MTX) and oligomeric methotrexate; the black line corresponds to the monomer; the red line corresponds to oligomeric MTX synthesized by α -chymotrypsin.

obtained were predominantly small (dimers) due to the size of the monomer (MTX, $M_w = 498$ g/mol), which imparted stereochemical impediments to the active site. Operational conditions like temperature (50 °C) and pH (9–10) were found critical for the efficient oligomerization. Molecular dynamics simulations confirmed that as the oligomeric chain grows, its accommodation in the active site is hindered, which is evidenced by a decrease of the binding energy associated (-6.94 kcal/mol for the monomer and -5.45 kcal/mol for the trimer). The data also revealed that α -chymotrypsin is able to synthesize oligomeric species with 6 units, however in a lower extent (33 %).

α -chymotrypsin is presented herein as a versatile catalyst for oligomer synthesis, even for substrates that are not naturally catalysed by it, like methotrexate. Moreover, the oligomerization of this drug would represent an alternative route for the treatment of many diseases, by reducing some side effects associated to the monomer administration.

CRediT authorship contribution statement

Jennifer Noro: Investigation, Methodology, Validation, Writing - original draft. **Tarsila G. Castro:** Investigation, Software, Formal analysis. **Artur Cavaco-Paulo:** Resources, Supervision, Project administration, Funding acquisition. **Carla Silva:** Conceptualization, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.procbio.2020.08.004>.

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