Biotechnology and Bioprocess Engineering 18: 538-545 (2013)
DOI 10.1007/s12257-012-0553-9

RESEARCH PAPER

Chymotrypsin - Eudragit® Complex Formation

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Received: 16 August 2012 / Revised: 30 December 2012 / Accepted: 4 January 2013

Abstract Eudragit® L100 (EuL) and Eudragit® S100 (EuS) are synthetic polyanions differing on their electric charge density. They interact with chymotrypsin (ChTRP), a basic protein forming soluble and non-soluble complexes. The complex formation was studied by dynamic light scattering, isothermal titration calorimetry, native fluorescence emission, circular dichroism and thermodynamical thermal stability of the enzyme. EuS was able to bind 33 ChTRP molecules while EuL, 60. The binding of ChTRP to both Eu was slightly endothermic and the entropic factor was responsible for the soluble complexes formation. The ChTRP-Eu size increases with pH and the binding of ChTRP to Eu modifies the Eu hydrodynamic radium. The interaction of ChTRP with Eu did not modify its secondary or tertiary structure. The thermal stability of ChTRP was increased when it interacted with both Eu.

Keywords: eudragit, chymotrypsin, polyelectrolytes, calorimetry

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1. Introduction

Eudragits are synthetic polymers soluble in aqueous media produced by Rohm GmbH & Co. Some of them are basic and behave as polycations, others, as Eudragit® L100 (EuL) and Eudragit® S100 (EuS) are acid and behave as polyanions. These two copolymers of 135 kDa of average molecular weight are based on methacrylic acid and methyl methacrylate. The ratio of the free carboxyl groups to the ester groups is approximately 1:1 in EuL and 1:2 in EuS. These polymers exhibit a pH-sensitive behavior due to the large quantity of carboxylic groups in their chains. Their solubility in aqueous media depends on variables such as pH, temperature and ionic strength of the dissolving medium. Both polymers can be easily dissolved in neutral or alkaline media but are insoluble in acid media. The mechanism by which these polymers become soluble in alkaline aqueous media involves the release of protons from the carboxylic groups of the polymers. The difference between both Eu lies on the fact that EuL has a higher electric density charge than EuS. This characteristic is fundamentally important since EuL and EuS present a differential interaction behavior with other electrically charged particles.

Many polyelectrolytes (PE) have been used to precipitate different proteins [1,2] through the formation of protein-polyelectrolyte complexes. Under certain conditions these protein-polyelectrolyte complexes become insoluble. In these cases, the target protein can be recovered from the solid phase by changing the medium conditions as pH and ionic strength, among others. This property allows the isolation of the protein, an interesting application being its use in bioseparation of target proteins from its natural source.

The mechanism of particle formation in protein precipitation with polyelectrolytes was initially proposed by Tsuboi *et al.* [3]. The protein-polyelectrolyte precipitate is

produced through a two-stage process, the first one being the formation of soluble protein–polyelectrolyte complexes. In this step one polyelectrolyte binds several protein molecules. Generally, this step has been studied by light scattering and isothermal titration calorimetry. The second step involves the interaction of several soluble polyelectrolyte-protein complexes to form a macro (visible) structure. In this last step electrostatic and van der Walls forces participate in the interaction. This step has been studied using turbidimetric and conductimetric measurements among others techniques [4].

Upon the addition of a polyelectrolyte of opposite electrical charge to a protein solution, the protein molecules bind to the flexible polyion via coulombic attraction. In this intrapolymer complex the polyion charges are partially neutralized by the opposite charges of the protein. Further addition of polyelectrolyte produces an increase in the concentration of the soluble complexes. When this concentration exceeds a certain threshold, larger aggregates are formed through the association of soluble complexes to form non-soluble particles.

Eudragits have been reported to interact with proteins to form soluble and non-soluble macromolecular complexes [5,6]. The properties of the proteins –as conformation, exposed surface, among others- could be modified when it is forming part of these complexes. Thus, it is interesting to investigate the effect of the complex formation on the properties of the protein to evaluate its stability and the applicability of the complexes formation as a bioseparation strategy.

Chymotrypsin (ChTRP) is a protease widely used in the food and pharmaceutical industry. It has a single polypeptide chain of 324 amino acid residues and a molecular weight of 25.7 kDa. It is one of the proteolytic enzymes of vertebrate pancreas juice, with an optimum activity of pH 8.2 and an isoelectric point of 9.1. It is widely used in biotechnology processes. We have used EuL and EuS to obtain ChTRP from its natural source: bovine pancreas [6] and we are now interested in achieving a deeper understanding of the molecular mechanism of interaction between these PE and ChTRP and the effect of the Eu on the ChTRP stability [7,8]. The aim of this work was to characterize, from a physicochemical point of view, the soluble complexes formed by two anionic Eudragits (EuL and EuS) and the basic protein ChTRP.

2. Materials and Methods

2.1. Chemicals

Crystalline ChTRP and N-benzoyl-L-tyrosine ethyl ester (BTEE) were purchased from Sigma Chem. Co. (USA). Eudragit L100 (EuL) and Eudragit S100 (EuS) were generously donated by Ethil Pharma (Buenos Aires, Argentina) and used without further purification.

2.2. Determination of the ChTRP activity

The ChTRP assay is based on the hydrolysis of BTEE. The reaction rate was determined by measuring the absorbance increase at 256 nm at 25ºC, which results from the hydrolysis of the substrate at 0.6 mM concentration in 100 mM Tris–HCl - 100 mM $CaCl_2$ pH 8.2 buffer.

2.3. Dynamic light scattering measurements

The measurements were performed at 25ºC using a Brookhaven Instrument standard setup (BI200 M goniometer, BI9000AT digital correlator) with a vertically polarized Coherent He-Ne Laser (λ = 632.8 nm) as a light source. The scattering volume was minimized by using a 0.2 mm aperture and an interference filter before the entrance of the photomultiplier. Polarized homodyne intensity autocorrelation functions $g_2(t)$ were obtained using a multi-τ mode correlator with 224 channels. The scattered light was analyzed after placing the sample cell containing the apparatus in decahydronaphthalene (Aldrich), which is an index-matching liquid.

2.3.1. Data analysis

Normalized electric field correlation functions $g_1(t)$, calculated from the intensity autocorrelation functions $g_2(t)$, were analyzed by using the regularized positive exponential sum (REPES), which employs the Laplace inversion. The resulting $A(\tau)$ is a distribution of relaxation times:

$$g_2(t) - 1 = \beta \left[\int A(\tau) \exp(-t/\tau) \, d\tau \right]^2 \quad (1)$$

In eq 1, t is the delay time of the correlation function and β is a coefficient accounting from ideal correlation. The relaxation time τ or the relaxation frequency $\Gamma(\tau^{-1})$ is associated with a diffusion coefficient D through the relation:

$$D = \Gamma/q^2 \quad (2)$$

The scattering vector q takes into account the refractive index of the solvent, n, and the scattering angle, θ, as given in eq 3:

$$q = (4\pi n / \lambda) \sin(\theta/2) \quad (3)$$

The hydrodynamic radii (R_h) were then calculated from the diffusion coefficient using the well-known Stokes–Einstein equation:

$$R_h = k_B T/(6\pi\eta D) \quad (4)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent.

2.4. Isothermal titration calorimetry (ITC)

Measurements were performed at 25ºC by using a VP-ITC titration calorimeter (MicroCal Inc., USA). The sample cell was loaded with 1.436 mL of ChTRP solution (1 mg/mL) and the reference cell contained Milli-Q grade water. Titration was carried out using a 0.3 μL syringe filled with Eu solutions. The experiments were performed by adding up to 100 aliquots of 3 mL of Eu solutions (0.1% w/v) to the cell containing the ChTRP solution. The heat associated in the interaction between ChTRP and Eu was calculated by discounting the heat of dilution of ChTRP and Eu, which was determined by the titration of Eu solutions into the buffer and the buffer into ChTRP solution, respectively. The resulting data were fitted to a single set of identical binding sites models using MicroCal ORIGIN 7.0 software supplied with the instrument. The intrinsic molar enthalpy change for the binding, ΔH°, the binding stoichiometry, N, and the intrinsic binding constant K were thus obtained. The free energy (ΔG°) and entropy changes (ΔS°) for the binding reaction were calculated by the fundamental thermodynamic equations, being R the gases constant and T the absolute temperature:

$$\Delta G^\circ = -R\ T\ \ln K \quad (5)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (6)$$

2.5. Eu effect on the ChTRP secondary structure

Circular dichroism (CD) scans of ChTRP in the absence and presence of different concentrations of Eu were carried out using a Jasco spectropolarimeter, model J-8150. The ellipticity values [θ] were obtained in millidegree (mdeg) directly from the instrument. The cell pathlength of 0.1 cm was used for the spectral range 200 ~ 250 nm and 1 cm for the 250 ~ 300 nm range. The scanning rate was 5 nm/min. Bandwidth was 0.1 nm. Five scans of each sample were made.

2.6. Eu effect on the emission of the native ChTRP fluorescence

This effect was analyzed by obtaining the fluorescence emission spectrum of the ChTRP at increasing Eu concentration. The scanning rate was 10 nm/min and the data acquisition was each 0.1 nm with a slit of 0.1 nm. The fluorescence spectra were obtained in an Amico Browman spectrofluorometer Serie 2000 using a thermostatized cuvette of 1 cm pathlength and were corrected by using the software provided by the instrument manufacturer.

2.7. Eu effect on the enzymatic activity of ChTRP

The enzymatic activity of ChTRP was determined over time (up to 26 h) when the enzyme was incubated at different temperatures (8, 25, and 37ºC) in the presence of different Eu concentrations to check if these polyelectrolytes affect the ChTRP enzymatic activity. ANOVA was used to analyze these results.

2.8. ChTRP thermodynamical thermal stability

Thermally induced unfolding was monitored by absorbance at 280 nm, as it was previously reported [9]. The analysis of the data was performed assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated and the absorptivity coefficients of both protein states were different. The unfolded protein fraction (α) was calculated from:

$$\alpha = (A_i - A_D)/(A_N - A_D) \quad (7)$$

where α is the unfolded protein fraction, A_N and A_D are the absorbancies of the native and unfolded states respectively, A_i is the absorbance at a given temperature. Least squares were used to fit the unfolded protein fraction versus temperature data and the temperature at the mid-point of denaturation (Tm) was thus determined. The equilibrium constant, K, for the unfolding process was calculated from:

$$K = \alpha / (1 - \alpha) \quad (8)$$

the free energy (ΔG^0) was calculated from equation 5. From fitting the data of ΔG^0 versus T, the unfolded entropy ΔS^0 was calculated according to:

$$\Delta S^0 = -(\partial \Delta G^0/\partial T) \quad (9)$$

The enthalpic change was calculated from the equation:

$$\Delta H^0 = \Delta G^0 + T\Delta S^0 \quad (10)$$

Absorbance measurements were recorded on a Jasco 550 spectrophotometer. The sample temperature was controlled by peltier heating and measured with a thermocouple immersed inside the cuvette. The heating rate was 1ºC/min. The data of absorbance versus temperature were collected by the software provided by the instrument manufacturer.

3. Results and Discussion

3.1. Determination of the Eu and Eu-ChTRP complexes size by dynamic light scattering

Fig. 1 shows the size distribution of EuL 0.005% w/v in a medium buffer phosphate 50 mM pH 6.80 obtained at different scattering angles and extrapolated to scattering angle 0º. The R_h of both Eu was determined at different pH conditions (from pH 6.80 to 8.50).

No changes in the R_h of each Eu were observed by varying the pH value in that range. Although the molecular weight of both Eu is the same, the R_h of EuL was higher than the EuS, as shown in Table 1.

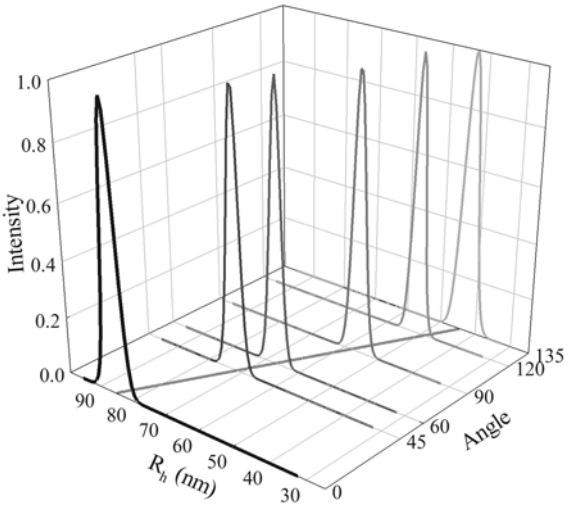

Fig. 1. Size distribution of EuL 0.005% w/v in a medium buffer phosphate 50 mM pH 6.80 obtained at different scattering angles and extrapolated to scattering angle 0°. Temperature: 25°C.

Table 1. Hydrodynamic radii (R_h) of EuL and EuS and of the soluble complexes they form with ChTRP at different pH values. Polymer concentration: 0.005% (w/v), ChTRP 1 mg/mL

	R_h EuL (nm)	R_h EuS (nm)
Eu	85 ± 5	57 ± 3
Eu + ChTRP (pH 6.80)	48 ± 4	51 ± 4
Eu + ChTRP (pH 7.20)	53 ± 3	70 ± 4
Eu + ChTRP (pH 7.90)	66 ± 4	65 ± 5
Eu + ChTRP (pH 8.50)	61 ± 4	80 ± 10

As the ionization degree of both Eu is 1 in the assayed pH range (far from the pKa of the COOH groups), all carboxylic groups are dissociated (and negatively charged) in the Eu chains. The average number of charged monomers in one EuL chain is 800 and in one EuS chain is 500, thus, the electrical charge density is higher in EuL than in EuS [10]. According to previous reports [11-13], the conformation of a PE in solution highly depends on its linear electrical charge density (since it determines the extent of the repulsion of the charges in the backbone). Taking this into account, our results are in agreement with the previous reports.

Different from the case of the free Eu, when the size of the ChTRP-Eu soluble complexes was determined, the profile of the light scattered was altered by the pH, as shown Table 1.

Complex conformation (and size) could depend on, at least, two factors [13,14]. One is the electrostatic interaction: Attraction between the Eu and the ChTRP, oppositely charged; repulsion between different ChTRP molecules bound to Eu, and between different ChTRP-free portions of the Eu chain. Another factor is the excluded volume effect (steric effect) of the bulky ChTRP molecules, which tends to expand the polyelectrolyte chain. The final structure of the complex depends upon the relative magnitude of these factors.

The R_h of the ChTRP-EuL complexes was significantly lower than the R_h of the free EuL in the entire assayed pH range. This may be indicating that the ChTRP-EuL conformation is mostly dominated by the electrostatic interaction: as the ChTRP-EuL ion pairing decreased the extent of the intrapolymer repulsion of the EuL chain, the binding of ChTRP to EuL allowed the compaction of the whole structure. The dependence of the ChTRP-EuL size on the pH may be due to the change in the ChTRP neutralization capacity of the negative charges in EuL. Even when the electrical charge of ChTRP varies approximately from +4 at pH 6.80 to +1 at pH 8.50, the electrostatic effect on the ChTRP-EuL conformation is still more significant.

The interaction of EuS with ChTRP induces a decrease of the size at pH 6.80. However, the ChTRP-EuS complex size was always higher than the size of free EuS between pH 7.20 and 8.50. As the conformation of free EuS is compact, the binding of ChTRP to EuS requires the opening of the EuS chain to allow the ChTRP to interact with it; *i.e.*, the steric hindrance may be the predominant effect on the ChTRP-EuS conformation at higher pH values. In this case, the change in the net positive charge of ChTRP effect produced a change in the factor determining the size of the complex.

The differences in size found between the ChTRP-Eu complexes formed by each Eu may depend on their electrical charge density. The electrical charge density affects the size of the free Eu and may be the determining factor of the conformation when the Eu interacts with ChTRP.

3.2. Isothermal titration of ChTRP with EuL and EuS

ChTRP was titrated with both Eu. The media conditions (pH, ionic strength, salt of the buffers) were settled up according to previous experiments. Saturation behavior was observed in all the cases as the energy released in the cell at the end of the titrations became null. In fact, as the Eu concentration increases, the total heat of interaction increases up to a constant value when the Eu concentration is high enough (data not shown). A single binding site model was fitted to the measured data to provide information about the thermodynamics of the binding interaction: the binding constant (K), the stochiometry of the complex formation (N) and interaction enthalpy (ΔH°) [15]. Table 2 shows the results. This model properly describes the complexation between ChTRP and Eu: N ChTRP molecules react reversibly with a polyanion chain in the cell.

The spontaneous character of the interactions between ChTRP and Eu is underlined by the negative Gibbs free

Table 2. Thermodynamic function values associate to the Eudragit–ChTRP complex formation

	ChTRP - EuL	ChTRP - EuS
ΔH^0 (cal/mol ChTRP)	356 ± 4	0.4 ± 0.1
N (mol ChTRP / mol Eu)	60 ± 2	33 ± 1
Number of charged monomers per Eu molecule	800	500
K (M^{-1})	(2.1 ± 0.1). 10^4	(9.9 ± 0.9). 10^5
ΔG^0 (kcal/mol ChTRP)	-5.93 ± 0.03	-8.23 ± 0.05
ΔS^0 (cal/K.mol ChTRP)	21.1 ± 0.1	27.6 ± 0.2
ΔS^0 (cal/K.mol charged monomer in PE)	1.583 ± 0.008	1.82 ± 0.01

Medium phosphate 50 mM pH 7.00.

energies. These oppositely charged macromolecules interact with high affinity, as seen from the high K values. Furthermore, the binding between the polymers is entropically driven with a weak unfavorable ΔH°.

The binding constant for the ChTRP-EuS complex was higher than EuL. This may be due to the difference in the electrical charge distribution that allows a higher flexibility, facilitating the EuS chain to adopt multiple conformations to interact with the ChTRP. Another explanation is that the separation between the charges in EuS may facilitate the ChTRP-Eu contact more than the charges separation in EuL.

The enthalpy change was expressed per mol of bound ChTRP and was slightly positive in both cases. EuL and EuS have to be ionized to interact by ion pairing with the ChTRP, and the enthalpy change associated to the ionization is positive. Thus, the global enthalpy change reflects not only the enthalpy involved in the ChTRP-Eu interaction itself but also the ionization and the proton dissociation of the PE. This fact can explain the difference observed between our results and those obtained by Romanini *et al.* [16] since they used a strong PE as polyvinylsulphonate, avoiding the endothermic PE dissociation step. The EuL-ChTRP complex formation was more endothermic than the EuS-ChTRP complex formation. This may be due to the difference in the charge amount in both Eu.

The stoichiometry of interaction was 60 ChTRP molecules per EuL molecule and 33 ChTRP per EuS molecule. The negative electric charge is almost double in EuL than in EuS, explaining the difference in the amount of ChRTP bound to each PE. On the other hand, it is remarkable that the Eu-ChTRP soluble complexes are negatively charged (considering the total amount of charges in both Eu and in the ChTRP bound to them). This net electrical charge may prevent the interaction among the soluble complexes allowing them to remain in solution due to the coulombic repulsion between the soluble complexes.

The Eu-ChTRP association to form soluble complexes produces a significant ordering in the system. However, the entropy change is positive in all the cases. This means that the complex formation process is accompanied by a disordering in the system, probably caused by the release

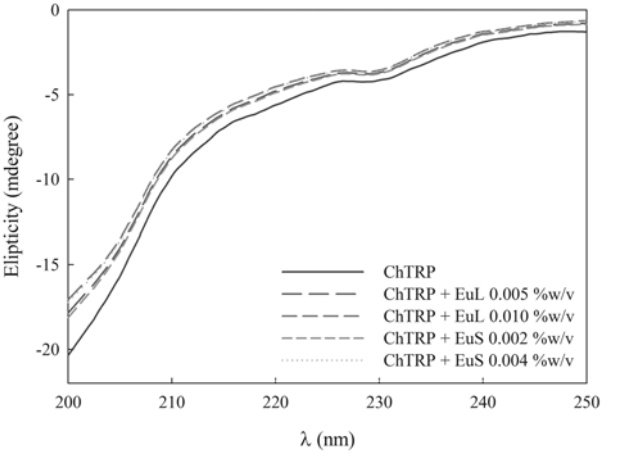

Fig. 2. Circular dichroism spectra of ChTRP in the absence and presence of EuL and EuS. ChTRP concentration: 1 mg/mL. Temperature: 25°C. Medium buffer phosphate 50 mM pH 7.00.

of counterions and the restructuring of water molecules around the charged sites during complexation [12,13,17]. Changes in the Eu conformation may also contribute to the overall entropy change [18,19]. The entropy change is higher in the case of ChTRP-EuS complex formation in agreement with an increase in the R_h value observed for this Eu when it forms the soluble complex.

3.3. Conformational changes on ChTRP induced by Eu

Circular dichroism (CD) and fluorescence spectroscopy were used to analyze possible conformational changes in ChTRP induced by its interaction with both Eu.

Fig. 2 presents the far-UV circular dichroism spectra of ChTRP in the absence and presence of different Eu concentrations. ChTRP, like all the serine proteases, has a poor content of alpha-helix. No significant modification in the CD spectrum of ChTRP was observed in the presence of both Eu in the range from 200 to 250 nm. This means that ChTRP retains its secondary structure unaltered. The CD spectrum in the range from 250 to 300 nm provides information about changes in the tertiary structure. No modifications of the spectra in this range were observed (data not shown), suggesting that conformational changes

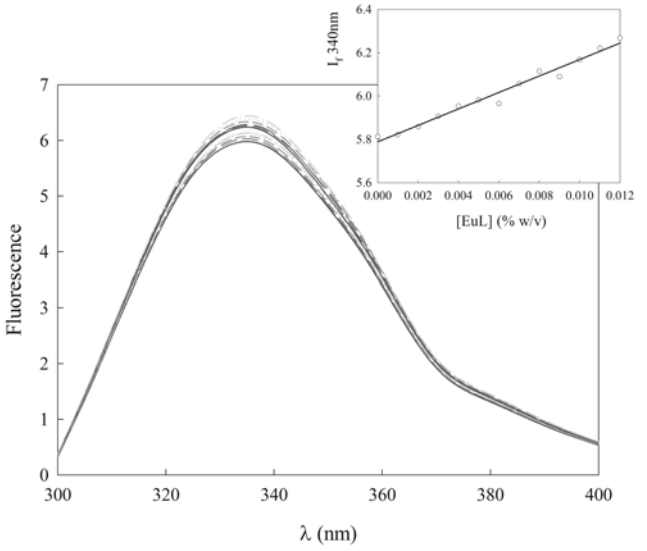

Fig. 3. Fluorescence emission spectra of ChTRP in the presence of increasing concentration of EuL. ChTRP concentration: 1 mg/mL. Temperature: 25ºC. Medium buffer phosphate 50 mM, pH 7.00. $\lambda_{excitation}$: 280 nm. Inset: fluorescence emission intensity at 340 nm *vs*. EuL concentration.

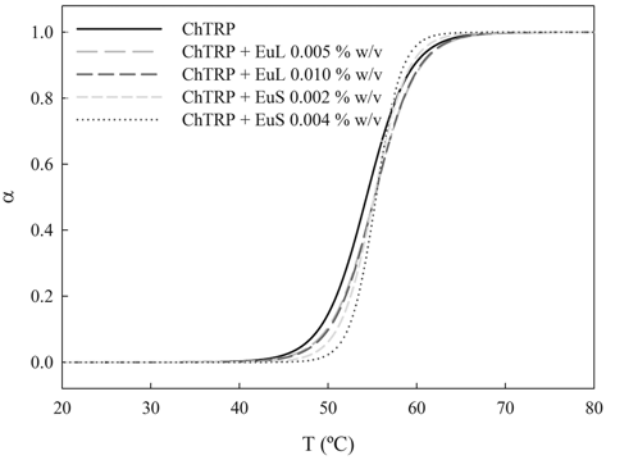

Fig. 4. Thermal stability of ChTRP in the absence and presence of Eu. ChTRP concentration: 1 mg/mL. Heating rate 1ºC/min. Medium buffer phosphate buffer 50 mM, pH 7.0.

are not produced in the protein when it is in contact with these polymers.

Fig. 3 shows the effect of increasing the concentration of both Eu on the native fluorescence emission spectra. The native fluorescence emission spectrum of a protein is sensitive to changes in the polarizability that occurs in the environment of the tryptophans; therefore, the native fluorescence signal can be used as an optical probe to analyze the effect of the interaction with Eu on the tertiary structure of ChTRP domains containing tryptophan residues [20]. ChTRP has 8 tryptophan residues, 3 of them accessible to the solvent, while the others are buried. The fluorescence band of tryptophan is centered at 340 nm. A slight enhancement in the native fluorescence emission band is observed, without any modifications of the peak position. This last finding suggests that both Eu do not change the polarizability of the environment of the tryptophan residues in ChTRP but they induce an increase in the number of particles in the excited state which may emit a photon. When the fluorescence emission intensity was plotted vs. the Eu concentration, a monotone increase in the intensity band was observed (inset Fig. 3). This is proof of a non-specific interaction of Eu with the environment of the tryptophan in ChTRP during the formation of the soluble complex.

3.4. Eu effect on the biological activity of ChTRP

The stability of ChTRP in the presence of Eu was studied by measurement of the enzymatic activity. A multivariate test was performed, including the following factors: presence of different concentrations of each Eu, temperature and incubation time. The measurements were performed at least in duplicate. The analysis of variance showed that the factors (time, temperature and Eu concentration) did not interfere with each other (time-temperature, $p = 0.96$; EuL concentration-temperature, $p = 0.96$; EuS concentration-temperature, $p = 0.89$; EuL concentration-time, $p = 0.85$; EuS concentration-time, $p = 0.84$). The incubation time ($p = 0.98$) and the temperature ($p = 0.55$) did not affect the enzymatic activity in the range tested. The presence of Eu was the only factor that significantly affected enzyme activity ($p = 0.0518 < \alpha = 0.10$ for EuL - $p = 0.0074 < \alpha = 0.10$ for EuS). In both cases, through Tukey assay [21], it was found that only the highest tested concentration of Eu significantly affected the ChTRP activity. However, the decrease in the enzymatic activity was lower than 5%. We confirmed the validity of the method: residues are normally distributed (Shapiro Wilks test: $p = 0.9683$ for EuL – $p = 0.1637$ for EuS) with constant variance.

3.5. Thermal stability of free ChTRP and ChTRP complexed with Eu

For measuring the differences in conformational stabilities of ChTRP under different conditions (free and complexed to both Eu), denaturation thermal curves were carried out. Thermal patterns of ChTRP were obtained by heating it and calculating its unfolded fraction (α) vs. temperature, as shown in Fig. 4. By non-linear fitting of the data, the midpoint temperature of the transition and the thermodynamic functions associated with the unfolding process were calculated, assuming a two-state model. The mean values for the calculated thermodynamic parameters are listed in Table 3. Both Eu induced a slight increase in the

Table 3. Thermodynamic variables calculated at 25°C associated to the ChTRP thermal unfolding in the presence of Eu

	Tm (°C)	ΔG^0 (kcal/mol)	ΔS^0 (e.u.)	ΔH^0 (kcal/mol)	$(\partial\alpha/\partial T)_{\alpha=0.5}$
ChTRP	54.3 ± 0.1	7.1 ± 0.3	201 ± 7	67.0 ± 0.4	0.102 ± 0.007
ChTRP + EuL 0.005% w/v	55.2 ± 0.1	7.3 ± 0.4	208 ± 8	69.1 ± 0.4	0.103 ± 0.009
ChTRP + EuL 0.010% w/v	55.2 ± 0.1	7.6 ± 0.4	226 ± 9	74.9 ± 0.4	0.106 ± 0.009
ChTRP + EuS 0.002% w/v	55.2 ± 0.2	9.4 ± 0.5	280 ± 10	92.0 ± 0.5	0.13 ± 0.01
ChTRP + EuS 0.004% w/v	55.4 ± 0.1	12.6 ± 0.6	370 ± 20	123.4 ± 0.7	0.17 ± 0.02

Heating rate: 1 degree/min. ChTRP concentration: 1 mg/mL. Medium buffer phosphate 50 mM, pH 7.00.

temperature at the transition midpoint (around 1°C). The last column of Table 3 shows the $(\partial\alpha/\partial T)_{0.5}$ values, *i.e.,* the derivative of the α *vs*. T curve calculated at $\alpha = 0.5$. This variable is related to the cooperative effect associated to the ChTRP thermal unfolding. Thus, an increase in this value is associated to an increase in the cooperative effect [9]. As is shown in Table 3, only EuS produces an increase in the cooperative effect, this increase being concentration-dependent. This finding agrees with the fact that EuS-ChTRP soluble complexes have a more open structure than EuL-ChTRP complexes, that structure being more sensible to the temperature increase.

From the stability curves, applying equations 7 to 10, the thermodynamic function values were calculated. They are included in Table 3. It is well known that entropic and enthalpic changes are positive for the process of thermal denaturation of proteins [9,22]. These positive changes are associated with the loss of ordered water molecules around the hydrophobic areas of the ChTRP without change in its tertiary structure. The presence of Eu interacting with ChTRP promotes the formation of a bigger and more thermally stable structure, compared to free ChTRP. This fact is reflected in the increase of the Tm value and in the significant increase in the unfolding entropic change. This can be explained considering that the final state regarding the unfolding of ChTRP when it is complexed with Eu is more disordered that the final state of free ChTRP: the unfolding of ChTRP possibly produces the release of the ChTRP from its interaction with Eu, increasing, in this way, the number of free molecules in solution.

4. Conclusion

In a previous report [6] we demonstrated the efficiency of the anionic polyelectrolyte EuL and EuS to precipitate ChTRP, partially purifying it from bovine pancreas homogenate with an appropriate recovery and purification factor. In this paper, we have studied the interaction between ChTRP and these PE to understand the mechanism of complex formation and the effect of the presence of these polymers on ChTRP stability.

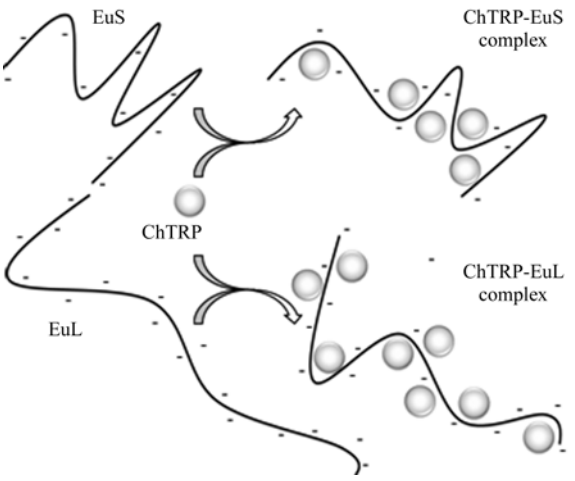

Fig. 5. Scheme of Eu-ChTRP complexes formation.

In an early work, Tsuboi *et al.* [3] proposed that the complex formation between a protein and a polyelectrolyte is composed by two sequential steps, the first one being the binding of the protein molecules to the flexible polyion via Coulombic attraction. This association results in a soluble complex, in which the polyion´s electrical charges are balanced by the protein´s opposite charges. Bohidar *et al*. [23] postulated that in this first step, all the free protein molecules are consumed and that the spacing among the electrical charges on the proteins is variable and not complementary to the very small and uniform spacing between polymer charges. Our findings are in agreement with the results reported by Cooper *et al.* [4]. As shown in the scheme (Fig. 5), our results suggest that as the electrical charges of the Eu are neutralized, due to the ChTRP binding, the Eu conformational entropy changes according to its chemical structure. Thus, in the case of EuS, which has a more open structure than EuL and in which the electrical charges are more scattered, the neutralization of the charges by ChTRP binding does not modify significantly the polymer conformation. In the case of EuL, the electrical charges are repelled and the conformation of the polymer is further extended. When the electrical charges are neutralized, the attraction of the hydrophobic chain becomes important and the result is a significant contraction in the volume of

the complex.

The difference in electrical charge density is also reflected in the stoichiometry of the soluble complexes [4,24] and in the enthalpy of Eu-ChTRP interaction.

The ChTRP complexation with both Eu did not produce any significant structural changes in the enzyme molecule, thus the secondary and tertiary enzyme structure was not modified; additionally, its biological activity remained constant at low polymer concentration. These results are important because they are demonstrating the validity of the precipitation method. On the other hand, both Eu induced an increase in the thermodynamic stability of ChTRP, which is important since the presence of these polymers may be beneficial in the case of ChTRP storage. EuS proved to be a better stabilizer of the enzyme since the enthalpic and the free energy changes of the denaturation process were increased more by this polymer than by EuL.

Acknowledgements

This work was supported by a grant from FonCyT PICT 508/2006 and CONICET PIP 0196/09-011. VB thanks CONICET for the fellowship. We thank María Robson, Geraldine Raimundo, Mariana De Sanctis, Marcela Culasso and Alicia Noguera for the language correction of the manuscript.

Nomenclature

EuL : Eudragit®L100
EuS : Eudragit®S100
ChTRP : Chymotrypsin
PE : Polyelectrolyte
ITC : Isothermal titration calorimetry
DLS : Dynamic light scattering
CD : Circular dichroism

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