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Analysis of the interactions between Eudragit® L100 and porcine pancreatic trypsin by calorimetric techniques

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ABSTRACT

Flexible-chain polymers with charge (polyelectrolytes) can interact with globular proteins with a net charge opposite to the charge of the polymers forming insoluble complexes polymer–protein. In this work, the interaction between the basic protein trypsin and the anionic polyelectrolyte Eudragit® L100 was studied by using isothermal calorimetric titrations and differential scanning calorimetry. Turbidimetric assays allowed determining that protein–polymer complex was insoluble at pH below 5 and the trypsin and Eudragit® L100 concentrations required forming the insoluble complex. DSC measurements showed that the T_m and denaturalization heat of trypsin increased in the polymer presence and the complex unfolded according to a *two-state model*. ΔH° and ΔS° binding parameters obtained by ITC were positives agree with hydrophobic interaction between trypsin and polymer. However, ionic strength of 1.0 M modified the insoluble complex formation. We propose a mechanism of interaction between Eudragit® L100 and trypsin molecules that involves both hydrophobic and electrostatic interactions. Kinetic studies of complex formation showed that the interaction requires less than 1 min achieving the maximum quantity of complex. Finally, a high percentage of active trypsin was precipitated (approximately 76% of the total mass of protein). These findings could be useful in different protocols such as a protein isolation strategy, immobilization or purification of a target protein.

1. Introduction

Synthetic and natural polyelectrolytes interact with globular proteins forming stable protein–polyelectrolyte complexes. The protein–polymer interactions result in the formation of soluble or insoluble complexes [1,2]. When polyelectrolyte–protein complex is specifically formed with one of the proteins in the crude extract followed by a phase separation, the process could be used as a convenient strategy for the isolation, immobilization and purification of the target protein [3–5].

A deeper understanding of complexation in mixtures of globular proteins and flexible polyelectrolytes is of considerable technological importance. Complexation sensitively depends on the solution pH and ionic strength. This dependence has been systematically studied by Dubin and co-workers [6,7] for mixtures of globular proteins and highly charged synthetic polyelectrolytes. Because of the strong dependence on pH and ionic strength it is usually assumed that, at least to a large extent, complexation is caused by electrostatic protein–polyelectrolyte interactions. Although we expect that generic electrostatic effects dominate the complexation behavior, however molecular details certainly do matter. It should be consider that protein and polyelectrolytes have an hydrophobic framework chain which can interact by other kinds of forces.

Isothermal titration calorimetry (ITC) is beginning to emerge as a key tool in the functional analysis of proteins, lipids and nucleic acid molecules as well as in routine binding assays in areas such as drug development [8]. This calorimetric technique has given us the direct heat associated with the complex formation and the thermodynamic parameters of this process.

Differential scanning calorimetry (DSC) is an experimental tool capable of determining the calorimetric parameters (ΔG, ΔH, ΔS and ΔCp) associated with a given conformational change of the protein induced by temperature variation. Trypsin is a very interesting model for the microcalorimetric study of a serine protease which presents two similar domains [9], its three-dimensional structure is known, it possesses high reversibility for the type of denaturation utilized (thermal), and shows great stability in its native form.

Trypsin is a serin protease of 23.3 kDa with an isoelectric point between 11.0 and 11.4 [10], produced in the pancreas of mammalians in the form of inactive zymogen, trypsinogen. It is then

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secreted into the small intestine, where the enzyme enterokinase activates it into trypsin by proteolytic cleavage. The resulting trypsin molecules activate more trypsinogens (autocatalysis), so only a small amount of enterokinase is necessary to start the reaction. This enzyme is used for numerous biotechnological and industrial processes [11].

The enteric polymer Eudragit® L100 (EL100) is an anionic copolymer based on methacrylic acid and methyl methacrylic acid [12]. This polymer which can be made reversibly soluble and insoluble by change in a particular medium parameter like pH, temperature, presence of certain ions, etc., present an interesting alternative to separation of proteins by precipitation. This polymer was used as a carrier of molecules having affinity for specific proteins, and also was used for protein purification by a technique named affinity precipitation [13,14].

We have used calorimetric and spectroscopic techniques to obtain information about the molecular mechanism of interaction between a basic model protein (trypsin) and a negatively charged polyelectrolyte (Eudragit® L100) with the aim of this information may be used in future applications of biotechnology.

2. Materials and methods

2.1. Chemical

Trypsin (TRP) from porcine pancreas was purchased from Sigma Chem. Co. (USA) and the polymer Eudragit® L100 (EL100), molecular average mass 135 kDa, was generously donated by EvonikDegussa Argentina S.A. Sodium citrate/Tris–HCl buffer solutions of different pH were prepared at concentration of 100 mM. The pH was adjusted with NaOH or HCl in each case. Stock solutions of EL100 at 2.5% (w/w) and TRP 1 mM pH 3.00 were prepared in water and the pH adjusted using HCl.

2.2. Enzymatic activity assays

Trypsin activity was determined with the substrate α-*N*-benzoyl DL-arginine-*p*-nitroaniline (BAPNA) using a method modified from Gildberg and Overbo [15]. BAPNA was used in the assay at a final concentration of 0.85 mM in buffer Tris–HCl 50 mM pH 8.20. The reaction is followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10,500 M^{-1} cm^{-1} for 90 s. Absorbance of solutions, was measured using a Jasco 520 spectrophotometer. The activities were calculated from the slope of the absorbance 400 nm vs time straight lines. One unit of activity (U) was defined as the amount of enzyme required to release 1 μmol of BAPNA per min.

In order to evaluate the enzyme stability at 25 °C in the presence of the polymer, TRP was incubated with different concentrations of EL100 and the enzymatic activity was measured. The solutions were prepared with buffer citrate/Tris 100 mM pH 5.00.

2.3. pH effects on complex EL100–TRP formation

Solutions prepared with buffer citrate/Tris–HCl 100 mM pH 8.00, EL100 at a fixed concentration (0.045%, w/w) and different concentrations of TRP (giving different molar ratio solutions) were used in this experiment. The pH of each one was varied 0.50 units using HCl or NaOH solutions and for each value of pH the absorbance at 420 nm was measured at 25 °C. Then, the phase diagram of the complex was obtained by plotting absorbance at 420 nm vs pH [16].

2.4. EL100 turbidimetric titration curves with trypsin

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration. Buffer sodium citrate/Tris–HCl solutions (8.00 mL) with a fixed polymer concentration were titrated at 25 °C with the TRP solution as the titrant at pH 4.50 and 5.00; respectively. For each aliquot of trypsin solution added, the absorbance at 420 nm (turbidity) was measured in a 1 cm path-length cuvette. The concentrations of protein and polymer solutions were 1 mM and 0.006% (w/w) respectively. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The complex formation was followed through a plot of absorbance at 420 nm vs protein/polymer molar ratio [17].

2.5. Kinetic assays

Interaction between polyelectrolytes and proteins requires time to achieve the maximum quantity of complex (maximum turbidity). Thus, solutions of EL100 with different concentration of the polymer 0.045; 0.090 and 0.180% (w/w) were prepared with buffer citrate/Tris–HCl, 100 mM, pH 5.00. Then, 300 μL of 1 mM TRP were added to these solutions and absorbance at 420 nm was measured during 5 min, thermostatizasing at 25 °C.

Finally, a plot of absorbance at 420 nm vs time was made and the time required for reaching the maximum quantity of turbidity was obtained [18].

2.6. Isothermal titration calorimetry (ITC)

Measurements were performed at 25 °C by using a VP-ITC titration calorimeter (MicroCal Inc., USA). The sample cell was loaded with 1.436 mL of trypsin solution (0.3 mM) and the reference cell contained Milli-Q grade water. Titration was carried out using a 300 μL syringe filled with polyelectrolyte solutions. The experiments were performed by adding aliquots of 5 μL of polymer solutions 0.175% (w/w) to the cell containing the protein solution.

The resulting data set was fitted using MicroCal ORIGIN 7.0 software supplied with the instrument and the intrinsic molar enthalpy change for the binding, ΔH_b, the binding stoichiometry, n, and the intrinsic binding constant, K, were thus obtained. The equation for determining the heat associated to each injection is:

$$Q = \frac{nM_t \Delta H_b V_0}{2}\left(1 + \frac{1}{nkM_t} + \frac{X_t}{nM_t} - \sqrt{\left(1 + \frac{1}{nkM_t} + \frac{X_t}{nM_t}\right)^2 - \frac{4X_t}{nM_t}}\right) \quad (1)$$

where V_0 is the active volume cell, X_t is the bulk concentration of ligand and M_t is the bulk concentration of the macromolecule in V_0 [19].

The mathematical model equation selected to fit the ITC data was derived from a model that assumes the polyelectrolyte molecule binding to several protein molecules, all with the same intensity; in other words, the polyelectrolyte was considered as a macromolecule having *n independent and equivalent sites*, all of which have the same affinity constant, K, for the ligand (trypsin) [20].

The heat associated with the interaction polymer–protein (ΔH_b) was calculated by subtraction using Eq. (2):

$$\Delta H_b = \Delta H_t - \Delta H_d - \Delta H_{dissol} \quad (2)$$

where ΔH_t is the total heat associated to each polymer addition, ΔH_d is the heat of dilution of the polymer in the buffer in the absence of the protein and ΔH_{dissol} is the heat of polymer

dissolution. The heat associated to the dilution of the protein in buffer was negligible. Then ΔH_i was plotted vs polymer/protein molar ratio and, by non-linear fitting of these calorimetric curve, the affinity constant (K) for the polymer binding to the protein and the number of polymer molecules (n) bound per protein molecule was calculated using the software provided by the instrument.

The intrinsic molar free energy change, ΔG°, and the intrinsic molar entropy change, ΔS°, for the binding reaction were calculated by the fundamental thermodynamic Eqs. (3) and (4):

$$\Delta G^\circ = -RT \ln K \quad (3)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (4)$$

2.7. Effect of ionic strength on complex formation

Solutions of EL100 6.23×10^{-3}% (w/w) were prepared with buffer citrate/Tris–HCl 100 mM pH 5.00 and different concentrations of NaCl (without salt; 0.50 M and 1.00 M) in order to obtained different ionic strength in each solution. These solutions were titrated at 25 °C with aliquots of 1 mM TRP and absorbance at 420 nm was measured in a 1 cm path-length cuvette for each aliquot. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The complex formation was followed through a plot of absorbance at 420 nm vs protein/polymer molar ratio [21].

Also, this analysis was performed in ITC experiments, working under the same conditions as in Section 2.6 but supplementing the medium with NaCl 1.00 M in order to ensure interfering the electrostatic interactions between the molecules of protein and polymer.

2.8. Differential scanning calorimetry of TRP experiments

Thermal denaturation of proteins was monitored with a high sensitivity differential scanning calorimeter model VP-DSC from MicroCal Inc. [22]. Thermograms were obtained between 25 and 85 °C, at scan rate 30 °C h^{-1} and at constant pressure of 28 psi. Protein was analysed at concentration of 0.3 mM in citrate buffer 50 mM pH 5.00 and EL100/TRP molar ratio was 0.092. All result were averages of, at least, three independent measurements. Buffer vs buffer baseline scans were determined and subtracted from transition scans prior to normalization and analysis of protein denaturation. Finally, the values of the excess heat capacity were obtained after subtraction of the baseline [23]. The calorimetric data were analysed by using the software ORIGIN 7.0, MicroCal Inc., following the methodology recommended by IUPAC. The parameters obtained from this analysis were: temperature at which maximum heat exchange occurs (T_m), the area under the peak, which represents the enthalpy of transition for reversible process (ΔH_{cal}) and the van't Hoff enthalpy (ΔH_{VH}). The evaluation of ΔH_{VH} gives an idea of the mechanism of the unfolding process [24]:

- $\Delta H_{VH} = \Delta H_{cal}$: *a two-state process* is carried out under equilibrium condition.
- $\Delta H_{VH} < \Delta H_{cal}$: one or more intermediate states of significance in the overall process.
- $\Delta H_{VH} > \Delta H_{cal}$: intramolecular cooperation is taking place which may require some degree of molecular association.

2.9. Trypsin precipitation with EL100

Solutions of EL100 and TRP at five different TRP/EL100 molar ratios (3.23; 5.53; 8.08; 16.18; 32.41) were prepared with buffer citrate/Tris–HCl 100 mM pH 5.00. The insoluble complexes formed were incubated for 10 min at 25 °C and centrifuged at 2292 × g for 10 min. The supernatants were separated and the precipitates were redissolved in citrate/Tris–HCl 100 mM pH 8.00 NaCl 1.00 M. The enzymatic activity and absorbance at 280 nm in the supernatants and in the redissolved precipitates were measured in order to determine the concentration of the enzyme in each fraction. Control curves were also carried out [25].

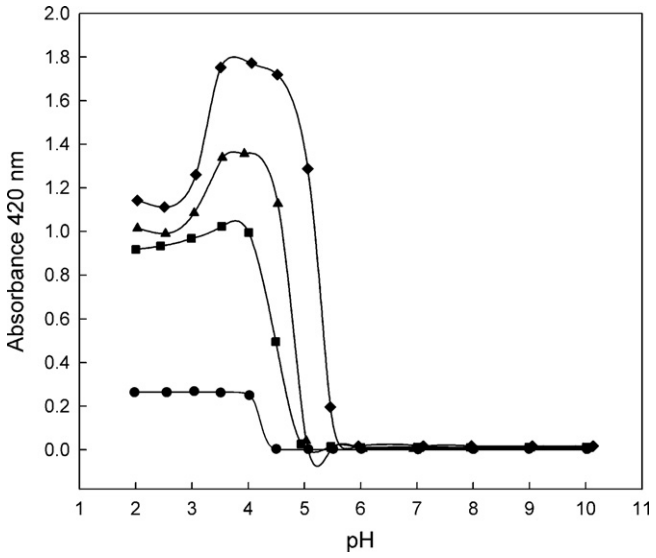

Fig. 1. Dependence of the absorbance at 420 nm vs the medium pH at three protein/polymer molar ratio of TRP/EL100: (●) without TRP, (■) 2.70:1, (▲) 5.34:1, (♦) 13.35:1. Temperature 25 °C.

3. Results and discussion

3.1. pH effects on the complex EL100–TRP formation

Fig. 1 shows the pH variation effect on the complex formation obtained in the presence of three different TRP/EL100 molar ratios. It can be seen that the polymer in the absent of TRP has a different insoluble range than in the presence of it, showing turbidity at pH lower than 4.00. This range shifts to higher pH as increasing TRP concentration. The presence of turbidity at pH 4.5–5.5 reveals the existence of complex formation between the polyelectrolyte and protein. It is consequence of strong electrostatic attraction between the protein and the polyelectrolyte, due to the opposite electrical charges of the two molecules at this pH range. The difference of absorbance 420 nm between pH 2–3.5 and pH 3.5–5 may be due to the lower positive charge of trypsin. Thus the structure of insoluble complex is different depending on pH.

3.2. EL100 turbidimetric titration curves with trypsin

Fig. 2 shows the TRP titration curves with EL100 obtained working at 5.00 in the presence and the absent of NaCl. The experimental data were fitted to a sigmoidal Weibull equation and as expected, all systems present a plateau zone indicating that EL100–TRP insoluble complex are saturated. The TRP/EL100 molar ratio e calculated from the intersection of a straight line which corresponds to the prolongation of the linear zone of the curve (at low polymer concentration) with a line which gives a plateau. These values allowed calculating the minimal polymer amount necessary to precipitate the maximum amount of protein. Molar ratios e in the experiment performed at pH 4.50 and 5.00 were 35 and 98 respectively.

Fig. 2 also shows the results obtained at different ionic strength. The values of turbidity yielded in the NaCl presence were smaller than the value obtained in absence of it; indicating that electrostatic forces are also involved in complex formation. This effect

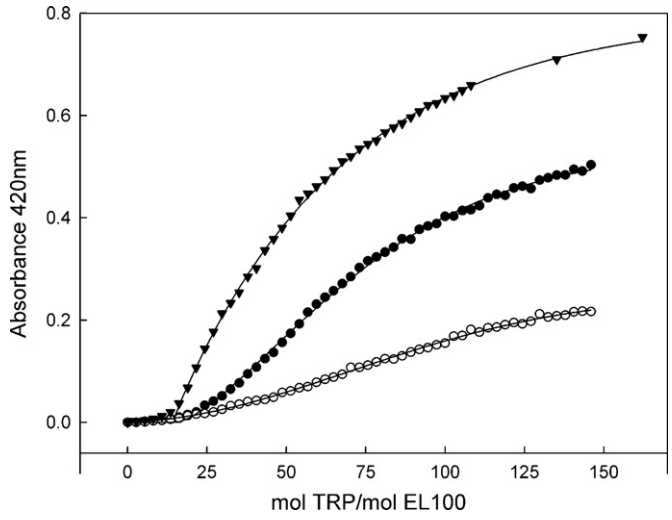

Fig. 2. Turbidimetric titration curves of EL100 (0.006%, w/w) solution with TRP (1 mM) in a medium in citrate/Tris–HCl 100 mM, pH 5.00. NaCl concentrations: (▼) 0 M NaCl, (●) 0.50 M and (◯) 1.00 M. Temperature 25 °C.

would indicate that the insoluble complex can be redissolved by increasing the ionic strength in the medium.

3.3. Kinetic assay of the complex EL100–TRP formation

The kinetic studies demonstrated that solutions of different TRP/EL100 molar ratio required less than 2 min of incubation to achieve the maximum quantity of complexes. In addition, as the concentration of polymer increased, the maximum turbidity value also increased. Fig. 3 shows the time required were independents of molar ratio.

3.4. Isothermal titration calorimetry of trypsin with EL100

This assay allowed to determine the thermodynamic parameters associated to the interaction between TRP and EL100 and the number of molecules of the protein interacting with one molecule of polymer.

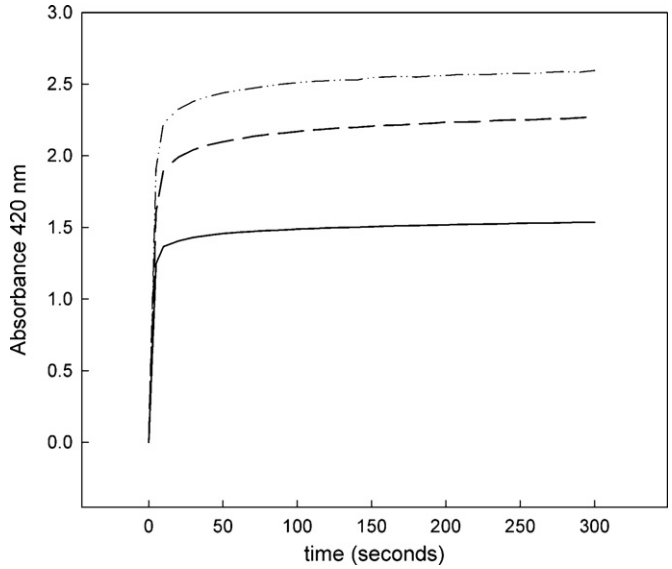

Fig. 3. Formation of complex TRP–EL100 through time at three protein/polymer molar ratio of TRP/EL100: (—) 32.41, (– –) 16.18, (– · ·) 8.08. Temperature 25 °C.

Table 1
Thermodynamics and binding parameters of the TRP–EL100 interaction from ITC experiments. Temperature 25 °C.

Binding parameter	Value
n (molar ratio) [mol TRP/mol EL100]	15.22 ± 0.05
K (affinity constant) [M^{-1}]	$9.8 \times 10^6 \pm 7 \times 10^5$
ΔH° (kcal/mol)	62.1 ± 0.3
ΔS° (e.u.)	240 ± 10
ΔG° (kcal/mol)	-9.59 ± 0.05

Fig. 4 shows the binding isotherm obtained when consecutive aliquots of EL100 were added to a solution of trypsin. The parameters calculated are summarized in Table 1. A value of 15 mol of protein per mol of polymer was found for the complex EL100–TRP formation. The high value of the affinity constant demonstrated that both molecules interact strongly with each other. The ΔH_b was normalized per mol of protein; therefore, heat value of 62.14 kcal/mol of protein is yielded. The positive value of ΔH_b indicates that the interaction between EL100 and TRP requires consuming of heat form de medium. The ΔS° value obtained was positive as a result of the increase of the disorder of the system due to release of structured water molecules.

EL100 is a charged polymer which also contains a hydrophobic framework in its linear chain. For such a complicated system it is not clear to what extent nonelectrostatic forces contribute to the observed complexation behavior. Besides, the value of ΔS° was positive indicating that the disorder of the system increased.

ITC experiment performed in presence of NaCl confirmed the results obtained by turbidimetry (data not shown). The values of heat measured during the experiment of titration are similar that obtained when studying the dilution of the polymer. This result is indicating that the TRP and the EL100 are not interacting when NaCl 1.00 M is added to the buffer.

Thermodynamic parameters were according to hydrophobic interactions between TRP and EL100. However, ITC and turbidimetric titrations experiments were altered in salt presence. It would demonstrate that the mechanism of interaction between these two molecules involves both hydrophobic and electrostatic interactions.

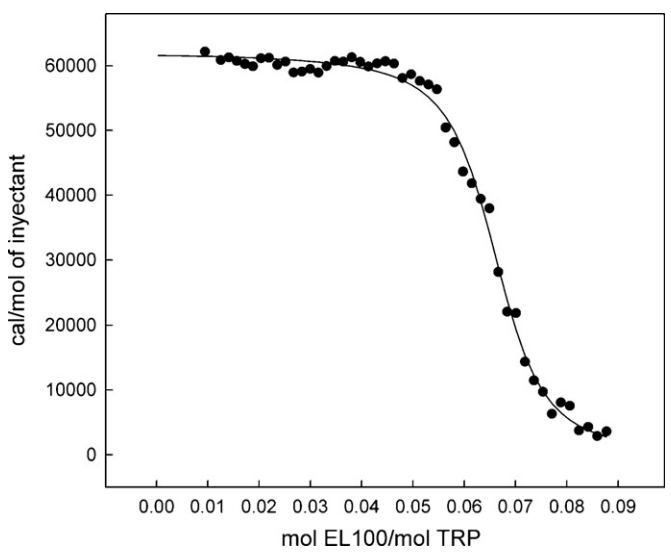

Fig. 4. Binding curve for the calorimetric titration of TRP with EL100 (0.175%, w/w). TRP concentration 0.30 mM. Medium citrate/Tris–HCl 100 mM, pH 5.00. Temperature 25 °C.

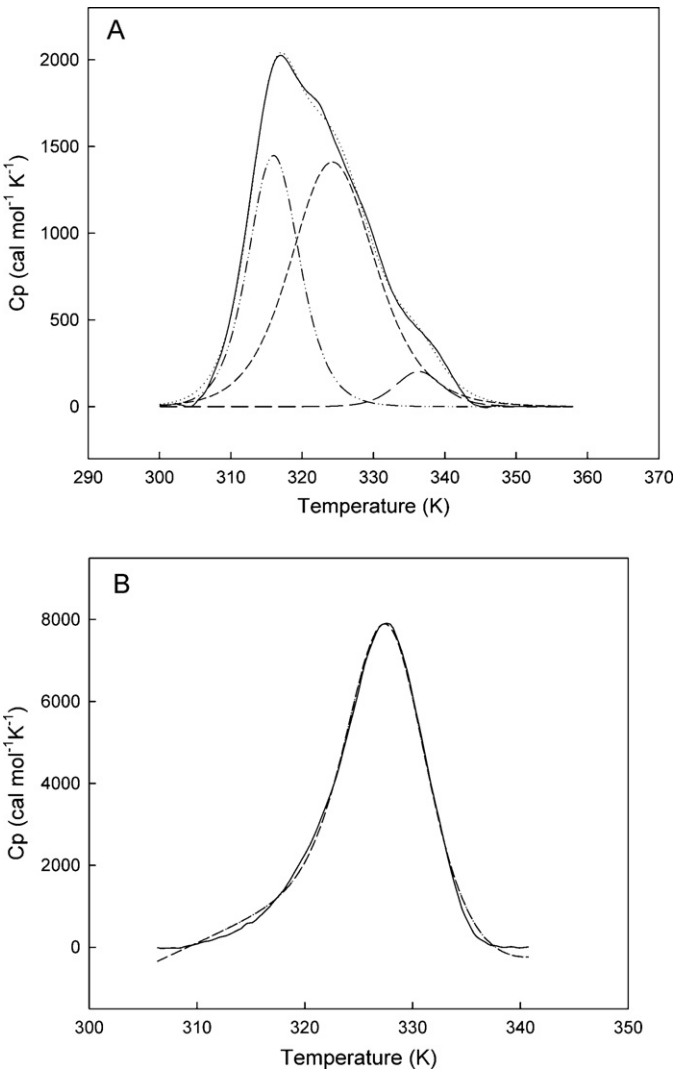

Fig. 5. (A) DSC thermogram of the TRP:(—) experimental data; (···) fit data; (··–) first transition; (- - -) second transition; (– –) third transition. Heating rate 0.5° min^{-1}. TRP (0.3 mM), citrate buffer pH 5.00. (B) DSC thermogram of the TRP in the presence of EL100: (—) experimental data; (– – –) fit data. Heating rate 0.5° min^{-1}. TRP (0.3 mM), EL100/TRP molar ratio 0.092, citrate buffer pH 5.00.

3.5. Thermal stability of the TRP in the absence and presence of the polymer

Differential scanning calorimetry is a useful tool for studying the protein unfolding in which values of excess specific heat capacity (*Cp*) are obtained as a function of temperature.

A typical temperature function of the partial molar heat capacity of TRP is shown in Fig. 5A. Trypsin is a small globular protein which has two domains with similar structures [26]. Its thermogram presents 3 transitions at pH 5.00. However, in the presence of polymer, it shows only one transition (Fig. 5B) with similar ΔH_{VH} and ΔH_{cal}. This behavior is compatible with a two-state model. Table 2 summarizes the DSC trypsin parameters obtained in the presence and the absent of EL100. The increase of T_m value obtained is a proof that the protein gains a major thermodynamic stability in the presence of the EL100. The polymer also induces an increase in area under the curve (ΔH_{cal}), according to a great interaction between EL100 and TRP.

The comparison of the two curves evidences two main differences. The first one is that in the TRP curve the T_m is about 48 °C, whereas for the TRP–EL100 complex the same parameter is about

Table 2
Thermodynamics functions obtained for the thermal TRP unfolding determined by DSC in the absence and presence of the EL100.

	T_m (K)	ΔH_{cal} (kcal/mol)	ΔH_{VH} (kcal/mol)
TRP	320.6 ± 0.1	38.7 ± 0.6	42.2 ± 0.8
	316.1 ± 0.1	13.9 ± 0.1	8.3 ± 3
TRP (transitions)	324.4 ± 0.3	22.2 ± 0.2	53.1 ± 3
	336.4 ± 0.3	1.8 ± 0.5	–
TRP–EL100	327.8 ± 0.1	82.0 ± 0.1	81.6 ± 0.1

55 °C. The shift of the T_m of TRP in the presence of the polymer means that the EL100 stabilizes the structure of the enzyme against thermal denaturation. The second difference is the change in the unfolding model: to *non-two-state model* for TRP and a *two-state model* for TRP–EL100.

This finding is in agreement with the proposed model of protein–polymer interaction, where one polymer molecule can bind several proteins molecules and with those obtained from the turbidimetric titration curves. In this way the intermolecular interaction between protein molecules is favoured.

3.6. Enzymatic activity in presence of EL100

Fig. 6 shows a plot of enzymatic activity vs TRP/EL100 molar ratio. The enzymatic activity was calculated from the slope of the straight line obtained plotting absorbance at 400 nm vs time. It can be seen that the biological activity of trypsin is practically the same either in the absence or presence of the polymer. This shows that the formation of the EL100–TRP complex does not significantly alter the structure of the catalytic site of the enzyme.

3.7. Trypsin precipitation by EL100–TRP insoluble complexes formation

Fig. 7 shows the results of % TRP in enzymatic activity and concentration (absorbance at 280 nm) at different molar ratios after precipitation. It can be seen that the percentage of precipitated protein is larger than in the supernatant indicating that the polyelectrolyte is an efficient precipitant of this protein (around 76%). On the other hand, Fig. 7 was presented in the form to verify mass and enzymatic activity balances.

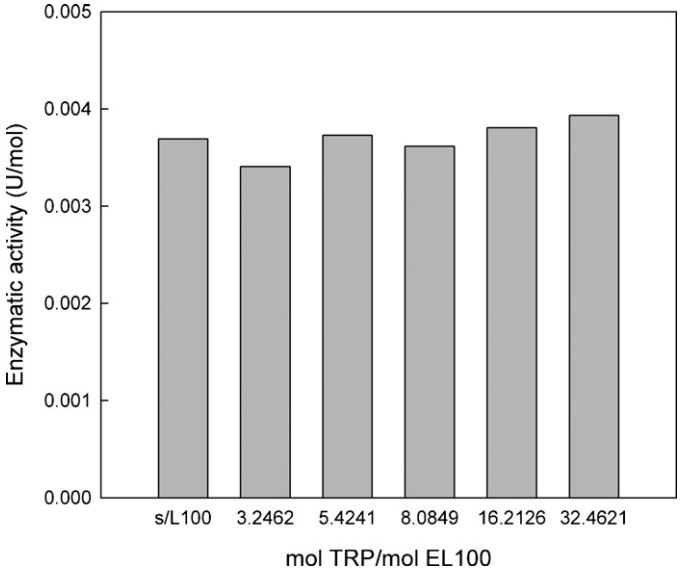

Fig. 6. Catalytic activity of TRP (0.11 mM) at different protein/polymer molar ratio of TRP/EL100. Medium citrate/Tris–HCl 100 mM, pH 5.00. Temperature 25 °C.

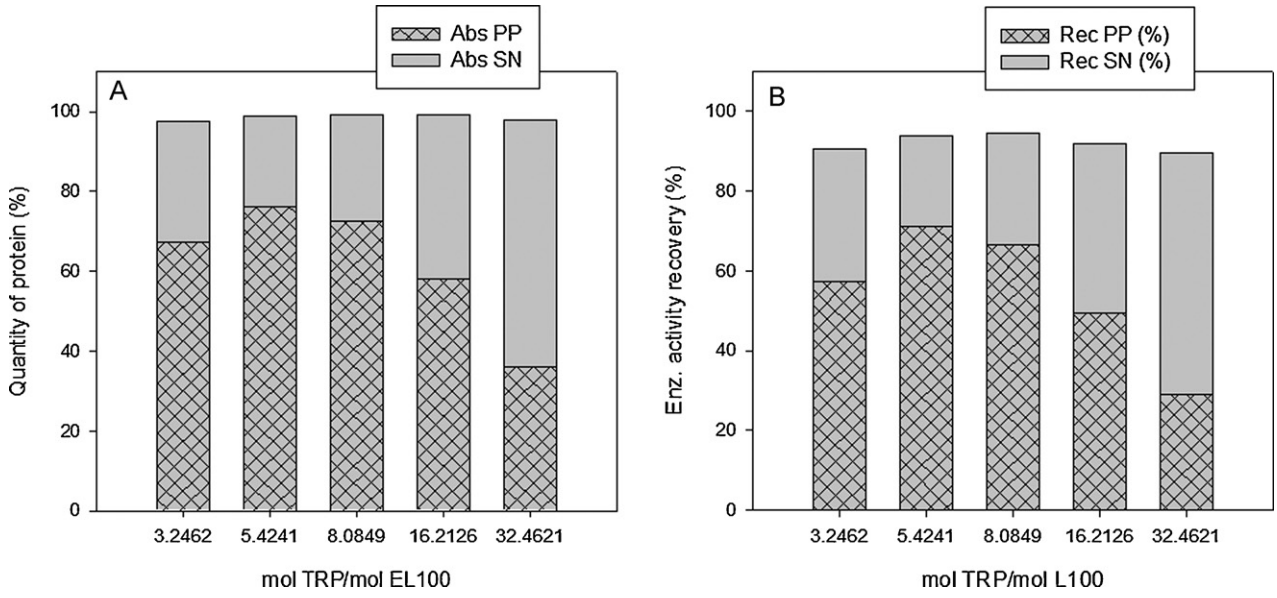

Fig. 7. Precipitation of TRP in (A) % quantity of TRP and (B) enzymatic activity recovery. Redissolved precipitated medium: citrate/Tris–HCl 100 mM, pH 8.00, NaCl 1.00 M. Temperature 25 °C.

Results obtained were similar measuring absorbance at 280 nm or enzymatic activity. It demonstrates that both techniques are useful for determining the amount of TRP in the different fractions.

4. Conclusion

Precipitation plays an important role in the down-stream processes in biotechnology. Methods based in the formation of insoluble protein–polyelectrolytes complexes not only provide an effective technique for purification and concentration of a protein in a dilute solution, but also allow immobilizing and stabilizing enzymes in order to produce bioreactors. A variety of charged precipitating agents such as carboxymethyl cellulose, poly acrylic acid and poly vinyl sulfonic acid have been used to selectively precipitate proteins with high industrial value from an aqueous mixture on the basis of different affinities [27,28]. Although protein precipitation and colloid flocculation with polyelectrolytes have been studied for many years, few theoretical studies have been directed toward understanding the mechanism of precipitation [29].

Experimental conditions of complex formation were identified by turbidimetric assays, but in determining the molar protein/polymer ratio was found a very different value determined by turbidimetric methods in comparison to the ITC. This shows that the former is not an appropriate methodology to estimate how many enzyme molecules bind per molecule of polymer.

DSC measurements showed that the T_m and denaturalization heat of trypsin increased in the polymer presence and the complex unfolded according to a *two-state model*. ΔH° and ΔS° binding parameters obtained by ITC were positives agree with hydrophobic interaction between trypsin and polymer. However, ionic strength of 1.0 M modified the insoluble complex formation. We propose a mechanism of interaction between Eudragit® L100 and trypsin molecules that involves both hydrophobic and electrostatic interactions. A high percentage of active trypsin was precipitated (approximately 76% of the total mass of protein).

The calorimetric techniques (ITC and DSC), turbidimetry and enzymatic activity studies provide useful quantitative information about the interaction of trypsin and Eudragit® L100 in aqueous solution. These findings may be useful in different protocols as a protein isolation strategy, immobilization or in purification of a target protein.

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