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Physicochemical study of the formation of complexes between pancreatic proteases and polyanions

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ABSTRACT

The formation of insoluble complexes between proteins and oppositely charged polyelectrolytes was assessed. Two pancreatic enzymes: trypsin and chymotrypsin, and two anionic synthetic polyelectrolytes: polyacrylate and polyvinylsulfonate, were used for the study at the pH range between 3.00 and 5.00. Two different titration curve shapes, representing two insoluble complexes formation mechanisms, were found. The turbidity of enzyme–polyelectrolyte mixtures is related to the increase either in the size or in the quantity of the insoluble complexes. Ionic strength destabilized insoluble complex formation. Finally, the kinetics of the process of insoluble complex formation at different conditions was studied.

1. Introduction

Synthetic and natural polyelectrolytes (PE) are widely used for different purposes in various fields of pure and applied science, like protein immobilization [1,2], purification [3,4] and targeted transport of drugs [5,6]. Research on the interaction between PE and proteins is important to predict the effect of PE on proteins (stabilization or denaturation) [7,8]. It also allows the choice of PE that selectively interact with proteins [9], in order to purify them by means of precipitation or adsorption [10,11], among others. Studies on the mechanism of complexation as well as on the molecular characteristics of the resulting complexes would be of particular interest [12,13].

At first, a number of non-covalent forces between polymers and proteins can contribute to their interaction and the formation of complexes. The interaction between proteins and hydrophilic polymers involves hydrogen bonds and the availability of acceptor and donor groups depends on the pH [14]. The predominance of electrostatic interactions between PE and proteins is widely accepted; however, there are some points of discussion in this regard [15].

The binding of polyanions and polycations to proteins below and above their isoelectric points (p*I*), respectively, has been reported [16,17], which serves as evidence of other driving forces in the interaction and complex formation. In addition, the total absence of hydrophobic interactions in protein–PE complexes is difficult to sustain [18].

In this work two synthetic anionic PE were used: polyacrylate (PAA), which contains carboxylic groups (with a pK_a between 4 and 4.5 [19,20]) and the more acid polyvinylsulfonate (PVS), which contains sulphonic groups (pK_a < 1) [21]. These polyacids are ionized in aqueous solution when the pH is higher than their pK_a. Such ionization caused the expansion of their chains due to charge repulsion. The ionization grade of a polyelectrolyte determines the volume it occupies: the higher the ionization grade, the more expanded the chain and the higher its volume [22,23].

The enzymes studied here were chymotrypsin (ChTRP) and trypsin (Tryp), two alkaline serine proteases (p*I* 8.7 and 10.4 respectively [4]). These enzymes have similar aminoacid composition; however, according to Horn and Heuck [21], they not only expose different number of charged aminoacid residues but also, the charged aminoacids are differently distributed on the surface [21,24,25].

Because of their wide application in leather, food, meat and soap powder industries, many methods have been developed to obtain the above mentioned enzymes from their natural source in large quantities. Precipitation with PE is one of them [3,4] and the characterization of protein–PE interaction is an essential primary step to carry it out.

Abbreviations: PE, polyelectrolytes; PAA, polyacrylate; PVS, polyvinilsulfonate; Tryp, trypsin; ChTRP, chymotrypsin.

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The aim of this work is to study, from a physicochemical point of view, the formation of soluble and insoluble complexes between ChTRP and Tryp with PAA and PVS.

2. Materials and methods

2.1. Chemicals

Polyacrylate sodium salt (PAA) (35% w/v), polyvinylsulfonate sodium salt (PVS) (25% w/v), crystallized trypsin (Tryp) and chymotrypsin (ChTRP) were purchased from Sigma Chem. Co.

2.2. Phase boundaries of protease–polyelectrolyte systems

Turbidity (absorbance at 420 nm) of enzyme–PE mixtures at constant concentration ratio was measured and plotted as function of pH to obtain solubility diagrams. The pH variations of the medium were obtained by adding HCl aliquots to the alkaline protein–PE mixture and allowing the system to equilibrate before measuring the turbidity. Coacervation is observed when the formation of insoluble enzyme–PE complexes occurs and is characterized by the appearance of an interface. Veis and Aranyi suggested the formation of soluble complexes prior to coacervation [26]. Even, Dubin et al. had demonstrated, using different techniques, the existence of such soluble complexes for protein–PE systems [27,28]. These "primary" soluble complexes were on the same order of size as the free PE, ranging from 40 to 200 nm [29,30], depending on the polymer, its concentration and the conditions of the medium: pH and ionic strength. Their formation is initiated at a specific pH called pH_c [31,32]. The value of pH_c preceded the pH of visual phase separation, designated as pH_Φ.

The ionic strength dependence of both pH_c, and pH_Φ, can be viewed as phase boundaries. The absorbance at 420 nm of protein–PE mixtures with different NaCl concentration was measured at several pH values. Phase boundaries were constructed considering the pH_c and pH_Φ obtained from the solubility diagrams [33].

2.3. Turbidimetric titration curves of serine proteases with polyelectrolytes

Solutions of different concentrations of each pancreatic protease were titrated at 25 °C using each PE as titrant. Protein and PE solutions were prepared in 50 mM acetate-phosphate buffer and the pH of each solution was properly adjusted in order to avoid changes during titration. Different pH values included in the pH range of non-soluble complex formation were assayed. The absorbance at 420 nm of the enzyme–PE solution was plotted vs. the final PE concentration added.

2.4. Study of the size and compactness of the insoluble enzyme–polyelectrolyte complexes

Changes in size and compactness of the insoluble enzyme–PE aggregates were assessed using the dependence of turbidity (τ) on the wavelength (λ). τ was measured as the absorbance in the (400–600) nm range, where there is no absortion of protein chromophoric groups.

The parameter β is related to the size, shape, and compactness of the particles in a suspension. β can be calculated from the slope of the $\log \tau$ vs. $\log \lambda$ plots in such λ range, applying the following equation [34]:

$$\beta = 4.2 + \frac{\partial \log \tau}{\partial \log \lambda} \quad (1)$$

in which τ was measured after each aliquot addition of a PE solution to an enzyme solution using a diode array spectrophotometer Spekol 1200. The spectrophotometer cuvette has 1 cm of path length and the temperature was maintained by water circulation.

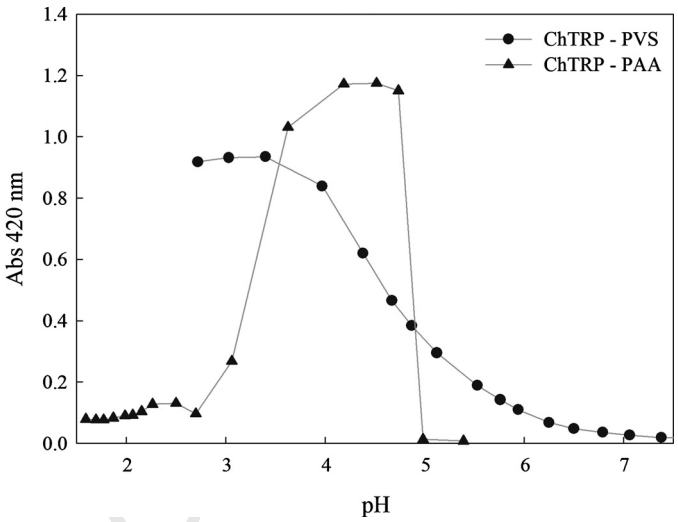

Fig. 1. Solubility diagrams of ChTRP–PVS and ChTRP–PAA mixtures. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C. ChTRP: 0.5 mg/mL. Polyelectrolyte concentration: 0.1 g/L.

2.5. Kinetics of insoluble enzyme–polyelectrolyte complex formation

The kinetics of the process of insoluble complex formation depends on the conditions of the medium such as pH, ionic strength, and concentration of each reactant: PE and protein. The system requires a specific time (which depends on each enzyme–PE pair) to reach the maximum turbidity value (τ_{max}). Enzyme solutions of different concentrations were prepared at different precipitation pH values. Then, a fixed PE concentration was added to each solution and the turbidity was measured over time (t). Data were fitted to the following first order exponential equation:

$$\tau = \tau_{max}[1 - \exp(-kt)] \quad (2)$$

being k the first order kinetic constant of insoluble complex formation.

3. Results and discussion

3.1. Phase boundaries of protease–polyelectrolyte systems

Turbidimetric titration of each enzyme–PE system was carried out in the presence and absence of different NaCl concentrations. Fig. 1 shows the solubility diagrams of ChTRP with each PE, without NaCl salt. The turbidity dependence on the pH of the Tryp–PE systems showed the same profile than those in Fig. 1, i.e., the shape of the solubility diagrams depends on the PE but not on the enzyme. Insoluble enzyme–PVS complexes were formed below pH 5, when the turbidity of the solution increase. The pH value of the insoluble–soluble transition can be explained taking into account that at this pH there is a significantly increase in the superficial charge of both proteins. According to Horn and Heuck [21] Tryp has ≈7 superficial electrical charges at pH 7, ≈13 at pH 5 and ≈20 at pH 3, whereas ChTRP has ≈3 superficial electrical charges at pH 7, ≈7 at pH 5 and ≈21 at pH 3. The aminoacid residues that may be mostly contributing to this change in the superficial electrical charge are histidine (His) residues ($pK_a \approx 6$) and glutamate residues ($pK_a \approx 4.5$). As both enzymes contain His in their primary structure (ChTRP has 2 and Tryp 3) [21], proteins gain more positive charges at pH 5 due to their protonation. Moreover, the

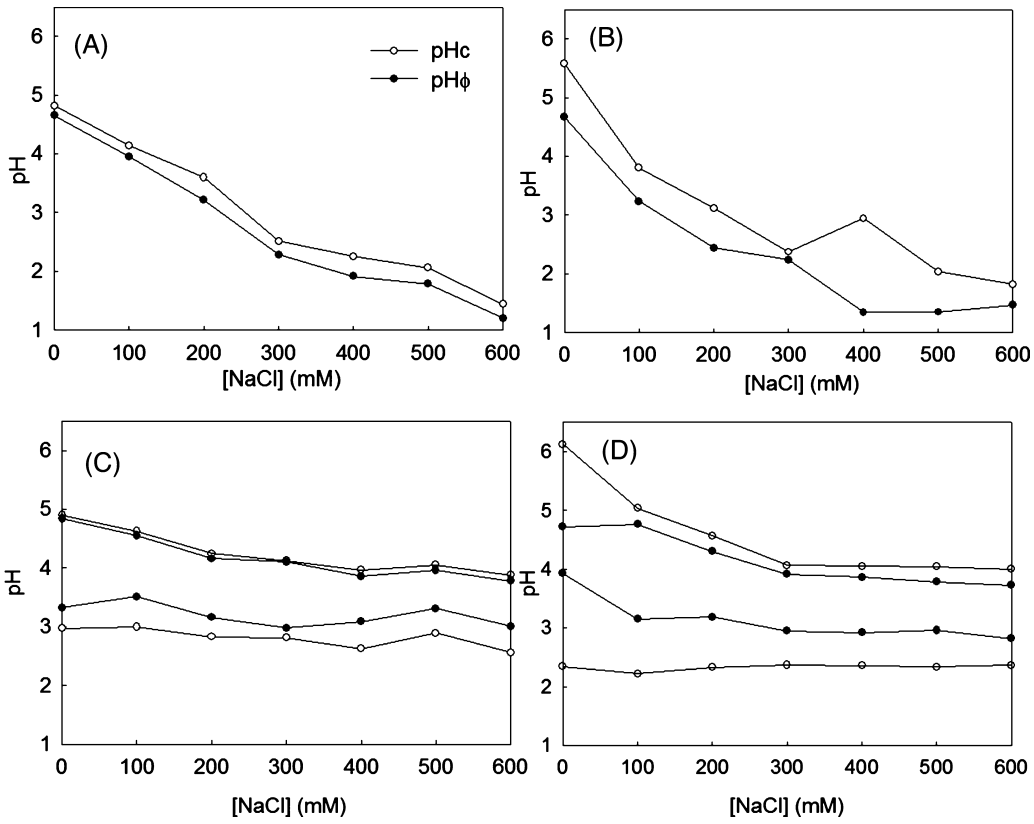

Fig. 2. Phase boundaries of A) ChTRP–PVS, (B) Tryp–PVS, (C) ChTRP–PAA and (D) Tryp–PAA systems. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C. Enzyme concentration: 0.5 mg/mL. PE concentration: 0.1 g/L. (●) pH_Φ; (○) pH_c.

protonation of the Glu residues (ChTRP has 5 while Tryp 4) produces an additional increase in the positive charge of the proteins. Below pH 5.0, the increase in the positive electrical charge of the proteins may favor the interaction with the PE, thus allowing the formation of the insoluble aggregates.

Insoluble enzyme–PAA complexes were formed between pH 3 and 5. This pH range of precipitation can be explained taking into account not only the charges of the enzymes, as was explained for the enzyme–PVS interaction, but also the different acidity of the PE. PAA is less acid than PVS, losing its negative charge close to pH 3. Under this pH value, PAA interacts weakly with the serine proteases (or does not interact at all), resulting in a decrease in the turbidity measured. The stronger acidity of PVS maintains this macromolecule charged along the pH range assayed.

pH_c and pH_Φ were graphically determined from the solubility diagrams. pH_c was determined as the intersection point between a straight line tangent to the inflexion point and the baseline. pH_Φ was determined as the pH at which half of the maximum turbidity was measured.

Since solubility diagrams of enzyme–PAA systems have two soluble–insoluble transitions, one at acid pH and the other at a higher value of pH, two values of pH_c and two values of pH_Φ were determined, one for each transition. Phase boundaries arise when these pH values are plotted against NaCl concentration. Fig. 2 shows the phase boundaries for (A) ChTRP–PVS, (B) Tryp–PVS, (C) ChTRP–PAA and (D) Tryp–PAA systems. White circles represent the transition to soluble complex (pH_c), while black ones represent the transition to coacervate (pH_Φ). In ChTRP/Tryp–PVS phase boundaries the insoluble enzyme–PVS complexes are formed below pH_Φ and the soluble ones are formed between pH_Φ and pH_c. In contrast, coacervation in the systems containing PAA occurs between both pH_Φ. Soluble enzyme–PAA complexes were formed between pH_Φ and pH_c. Below the lowest pH_c, PAA loses its charge and becomes insoluble, while the proteins are released to the solution.

NaCl decreased insoluble complex formation: the maximum turbidity of the solutions was lower and the pH range of coacervation was narrower as the NaCl concentration increased. For enzyme–PAA systems, the decrease of the pH range of coacervation was smaller at acid pH, which can be due to two issues: (1) net positive charge of the proteins is higher at acid pH, due to the protonation of the acid aminoacid residues; therefore, the electrostatic interaction is stronger and thus, higher ionic strength is required to dissociate the complex at low pH. (2) The PAA dissociation at a pH close to its pK_a is favored by an increase of NaCl concentration due to the screening of the charges of the protons and the carboxylate groups. For enzyme–PVS systems the solubility diagrams shifted to more acid pH values. This may also be due to the higher net positive charge of the proteins at lower pH values. It is remarkable that enzyme–PVS interaction has a more electrostatic character than that of the enzyme–PAA. It can be noted that PVS phase boundary slopes ($\partial pH/\partial[NaCl]$) are more negative than those obtain with PAA, i.e., NaCl affects more the enzyme–PVS interaction than the enzyme–PAA interaction.

Comparing the transitions of both proteases, the pH ranges corresponding to ChTRP–PE transitions were narrower than those corresponding to Tryp–PE transitions. The difference may be due to two reasons: (1) the presence of ChTRP oligomers [35] that make the aggregation process more cooperative or (2) the sharply increase in the superficial positive charge of ChTRP and their distribution on the surface (more sectored in ChTRP and more scattered in Tryp).

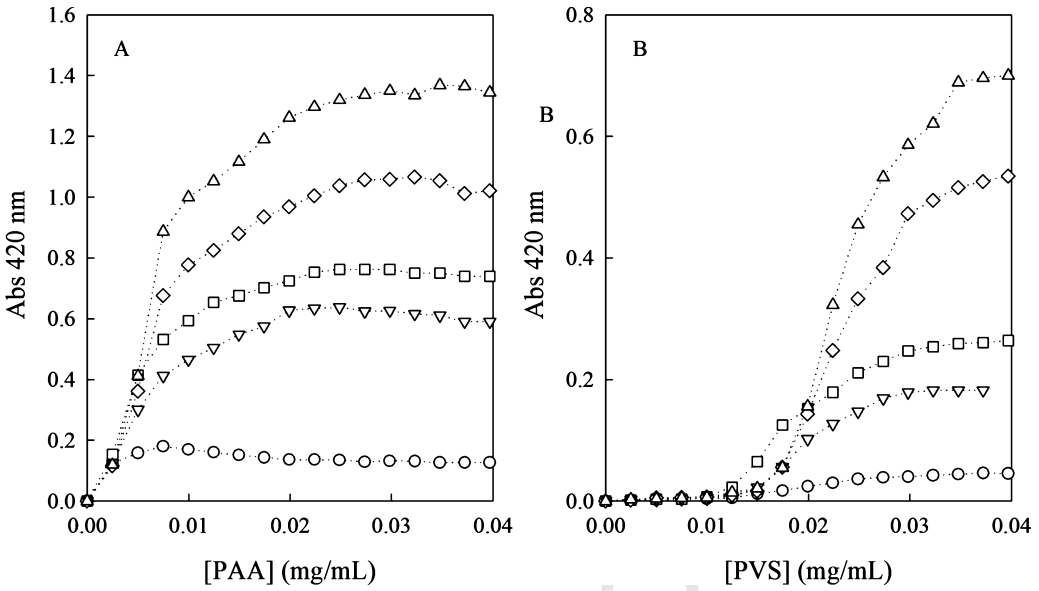

Fig. 3. Titration of ChTRP using PAA (A) and PVS (B) as titrant. Medium: 25 mM acetate-phosphate buffer pH 4.50. Temperature: 25 °C. ChTRP concentration: (○) 0.125 mM; (▽) 0.250 mM; (□) 0.375 mM; (◊) 0.500 mM and (△) 0.625 mM.

3.2. Protease titration with polyelectrolytes

3.2.1. Formation of the insoluble enzyme–polyelectrolyte complexes

Titrations of each protease using PAA and PVS as titrants were carried out. Three different pH values: 2.50, 3.50 and 4.50 were assayed with PVS, whereas PAA titrations were carried out at pH 3.50 and 4.50 because 2.50 is outside the pH range of the formation of the insoluble enzyme–PAA complexes. The enzyme concentrations assayed were: 0.125, 0.250, 0.375, 0.500 and 0.625 mg/mL.

Fig. 3 shows the titration curves of ChTRP using PAA (A) and PVS (B) as titrants at pH 4.50. Turbidity of the mixtures increased with the addition of titrant, verifying the formation of the insoluble enzyme–PE complexes. The increase in measured turbidity with the addition of PE may be due to an increase in either the number of insoluble complexes formed or in their size. There was a specific PE concentration at which the solution's turbidity reached a maximum, this means that under those assay conditions as many aggregates as possible were formed, i.e., higher PE concentration will not increase the amount of insoluble complexes. This maximum turbidity value depended on the medium pH and increased with higher enzyme concentrations in the medium.

A significant difference was found between the shapes of the titration curves observed in Fig. 3, suggesting there are two aggregation mechanisms:

In Fig. 3(A) the experimental data were fitted to a hyperbolic function, meaning that the formation of insoluble ChTRP–PAA complexes took place as the concentration of PAA in the medium increases. At low PE concentrations, the measured turbidity is proportional to the PE concentration until a plateau was reached. This behavior was also observed in Tryp–PAA and Tryp–PVS systems. This might indicate that insoluble ChTRP–PAA, Tryp–PAA and Tryp–PVS complexes were formed with the first additions of PE and then their size or number increased as higher the PE concentration.

In Fig. 3(B) the experimental data were fitted to a sigmoid function. At low PVS concentrations the turbidity of the solution did not increase, which would indicate that insoluble complexes were not being formed. This behavior was observed until a given concentration of PVS was reached in the medium; for higher concentrations of PE, the turbidity increased with the addition of PVS until a plateau. The insoluble complex formation process seems to be cooperative in this case, probably due to the aggregation of different soluble complexes when PVS concentration exceeds a specific value. The formation of ChTRP–PVS soluble complexes was previously studied [30].

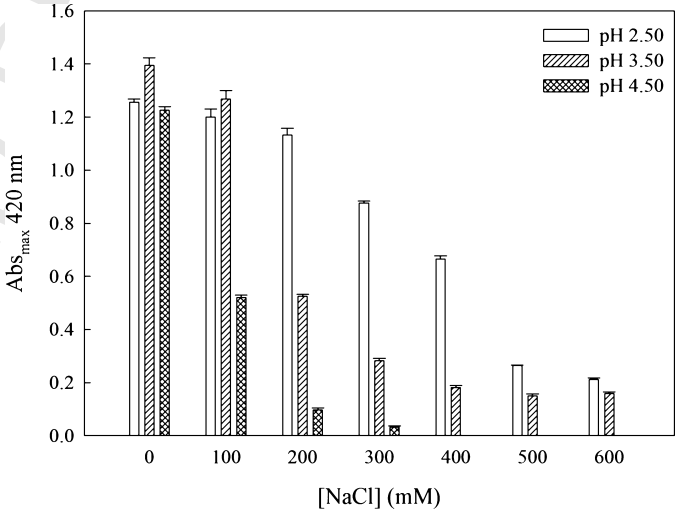

Fig. 4. Ionic strength effect on the maximum turbidity value reached in the turbidimetric titrations of Tryp with PVS as titrant at all the pH values assayed. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C. Tryp concentration: 0.5 mg/mL.

3.2.2. Ionic strength effect on serine proteases complex solubility

Turbidimetric titrations of proteases using the PE as titrants were also carried out in the presence of different NaCl concentrations. In all cases, the maximum measured turbidity decreased as the ionic strength in the medium increased. This can be due to a decrease either in the amount or in the size of the insoluble complexes formed. Fig. 4 shows the NaCl effect on the maximum value of absorbance at 420 nm of Tryp–PVS systems at different pH values. The presence of NaCl 300 mM at pH 4.50 produced the complete solubilization of Tryp–PVS aggregates. At the other two pH values assayed, the increase in salt concentration favored the solubility of Tryp–PVS aggregates, causing a decrease in the maximum

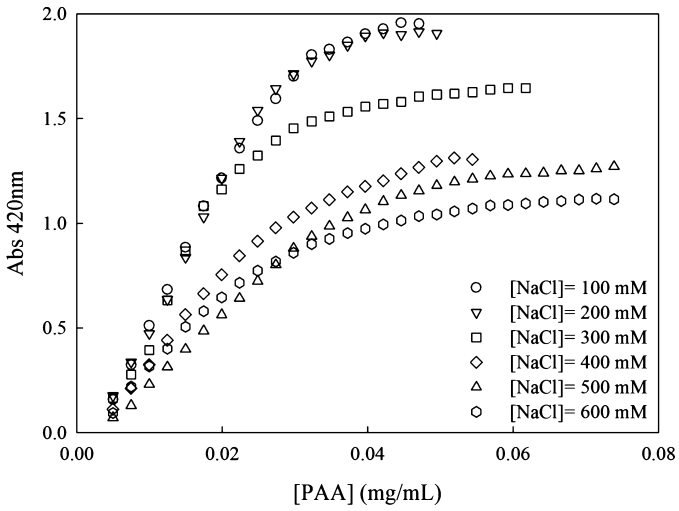

Fig. 5. Dependence of turbidity and β with PE concentration. Titration of Tryp using PAA as titrant. Medium: 25 mM acetate-phosphate buffer pH 3.50. Temperature: 25 °C. Tryp concentration: 0.5 mg/mL.

turbidity measured. However, a concentration of NaCl of 600 mM was not enough to completely solubilize the insoluble aggregates. This finding is consistent with an electrostatic mechanism of interaction between PVS and Tryp: the enzyme losses net positive charge as the pH of the medium increases making the enzyme–PE interaction weaker. The same trend was observed in all enzyme–PE systems.

However, the ionic strength effect on the enzyme–polyelectrolyte interaction at pH 3.50 was different for each PE. When PVS was used as titrant, the maximum turbidity of the solution gradually decreased as the salt concentration in the medium increased (see above). Instead, when PAA was used, the maximum turbidity decreased up to a given NaCl concentration and remained practically constant as shown in Fig. 5. This can be due to the fact that enzyme–PVS interaction is more electrostatic than the interaction enzyme–PAA at this pH. At pH 3.50 PAA loses most of its negative charge; thus, in enzyme–PAA systems, such interactions as hydrogen bond or hydrophobic effect which are not affected by NaCl concentration, begin to gain importance.

3.3. Study of the size and compactness of insoluble enzyme–polyelectrolyte complexes

The parameter β was determined under the same conditions as turbidimetric titrations. This parameter is directly related to the size and inversely related to the compactness of insoluble enzyme–polyelectrolyte complexes. Fig. 6(A) shows the results obtained in the titration of Tryp with PAA as titrant at pH 3.50. At low PAA concentrations, as the turbidity of the medium increased, the β parameter decreased. However, at higher PAA concentrations, both the turbidity of the medium and the β parameter increased as the PE is added. This indicates that at low PAA concentrations, the mean size of the insoluble complexes decreased up to a given PAA concentration at which the Tryp–PAA complex size increased, thus forming larger insoluble complexes. This same tendency was observed in all the titrations with PAA as titrant in all the conditions assayed. These results could be explained by the fact that the size of the first insoluble complexes formed decreases as the proteins are distributed among the new PAA molecules added to the solution. Thus, at this first stage the increase in the turbidity of the medium might be due to the increase in the quantity of insoluble complexes and not to the increase in their size. Finally, the insoluble complexes formed would aggregate forming bigger macroaggregates of bigger size.

Fig. 6(B) shows the results obtained in the Tryp titration using PVS as titrant at pH 4.50. In this case, β and turbidity values are correlated, i.e., they increased as PVS was added to the medium. This behavior was observed in all the titrations in which PVS was used as titrant in all the conditions assayed. In these cases, the increase in the turbidity as the PVS concentration increased might be due to the increase in the size of the insoluble complex.

When comparing maximum β values obtained, it was noticed that insoluble Tryp–PE complexes are less compact than those made up of ChTRP. This can be explained by Manning's theory [22,23], which predicts that the conformation of a PE depends on how charged its chain is: the more charged the PE chain, the more expanded the conformation. Thus, due to the fact that ChTRP dimerizes at acid pH [35], it can neutralize more charges in the PE chains, being the insoluble complexes with ChTRP more compact than those made up of Tryp. Moreover, despite both pancreatic enzymes have similar aminoacid composition, ChTRP has more aminoacids with acid pK_a values (Asp and Glu) [21] gaining more

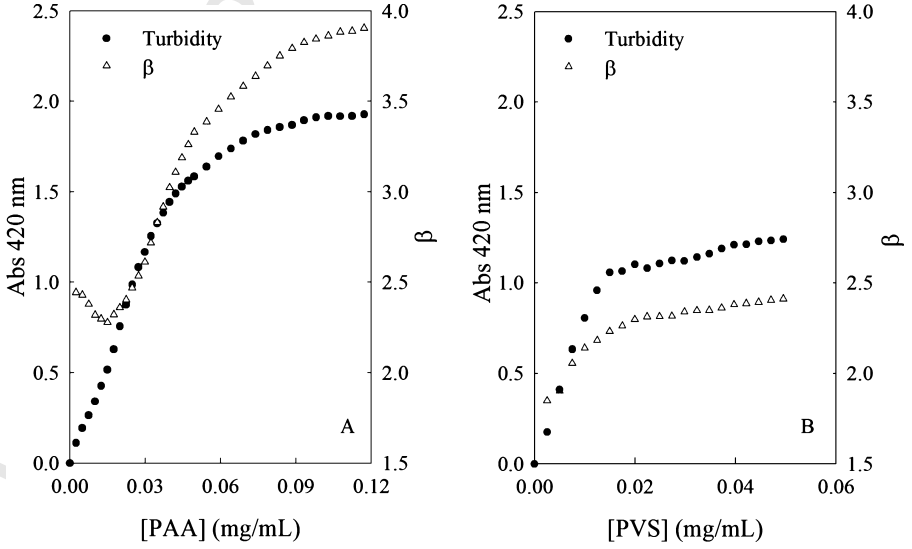

Fig. 6. Titration of Tryp with (A) PAA as titrant at pH 3.50 and (B) PVS as titrant at pH 4.50. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C. Tryp concentration: 0.5 mg/mL.

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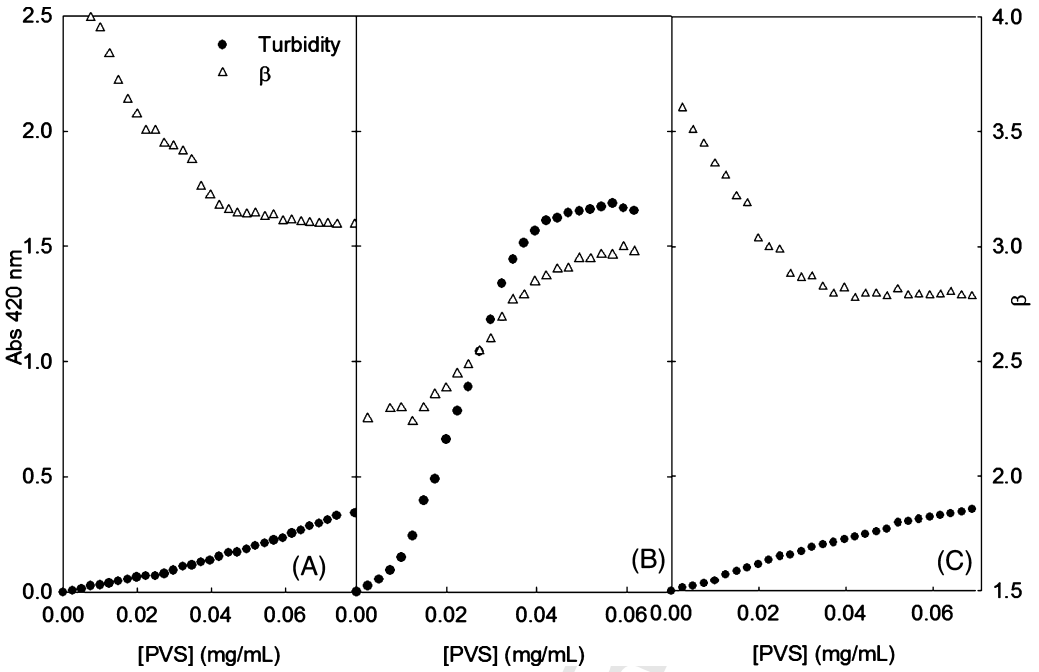

Fig. 7. Dependence of turbidity and β with PE concentration. Titration of ChTRP with PVS as titrant. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C. ChTRP concentration: 0.5 mg/mL. (A) pH of the medium: 2.50. NaCl concentration: 0.5 M. (B) pH of the medium: 3.50. NaCl concentration: 0.1 M (C) pH of the medium: 3.50. NaCl concentration: 0.2 M.

positive charge than Tryp at acid pH. This contributes also to the neutralization of more negative charges in the PE generating more compact insoluble ChTRP–PE complexes.

3.3.1. Ionic strength effect on compactness

At pH 3.50, in the enzyme titrations in which PAA was used as titrant, the behavior of β parameter as the PE concentration increased was the same in the absence and presence of different NaCl concentrations. However, at pH 4.50, the tendency changed as the NaCl concentration increased. In Tryp titration, when the NaCl concentration was 200 mM or higher, the graph of β parameter in function of polyelectrolyte concentration was fitted to a hyperbolic function, i.e., the increase in the turbidity of the solution was related to the increase in the size of the insoluble Tryp–PAA complexes. In the titration of ChTRP with PAA, when the NaCl concentration was 300 mM or higher, β parameter decreased as the PAA concentration increased.

During Tryp titration with PVS as titrant, the graph of β parameter in function of polyelectrolyte concentration fitted to a hyperbolic function in the presence of low NaCl concentration (up to 400 mM). However, at higher ionic strength (from 500 mM), β parameter decreased as the PVS concentration increased. This happens at pH 2.50 and 3.50, whereas at pH 4.50 the decrease on β parameter occurs at concentrations of NaCl equal to or higher than 200 mM.

In ChTRP titration using PVS as titrant at pH 2.50, β parameter decreased with the increase in PE concentration up to a point at which β parameter remained constant. This happens at all NaCl concentrations assayed. The titration of ChTRP using PVS as titrant at pH 2.50 and with a NaCl concentration of 500 mM is shown in Fig. 7(A). At pH 3.50 and 4.50 up to a NaCl concentration of 100 mM, β parameter in function of PE concentration was fitted to a sigmoidal function. At higher salt concentrations, β parameter decreased as PE concentration increased until a constant value was reached. As an example, the results of the titration of ChTRP using PVS as titrant at pH 3.50 in the presence of two different NaCl concentrations are shown in Fig. 7(B) and (C).

Table 1
First order kinetic constants (10^{-4} s^{-1}) of the polyelectrolyte–enzyme aggregation process at different pH. Polyelectrolyte concentration: 0.0375 mg/mL. Medium: 25 mM acetate-phosphate buffer. Temperature: 25 °C.

	PAA		PVS		
pH	3.50	4.50	2.50	3.50	4.50
ChTRP	150 ± 8	177 ± 2	130 ± 3	217 ± 2	217 ± 2
Tryp	344 ± 6	61 ± 1	97 ± 2	120 ± 2	111 ± 2

In the titrations with a given NaCl concentration in which β parameter decreased with the addition of PE, it is thought that salt would be interfering in the interaction between small insoluble complexes to form larger ones. As the enzyme and PE charges were screened by the salt ions, they interact weaker than in the absence of NaCl.

3.4. Kinetics of insoluble enzyme–polyelectrolyte complexes formation

The data of absorbance at 420 nm against time were fitted to a first order exponential function at all the assayed conditions. The protein concentration did not have a significant effect on the first order kinetic constant of insoluble complex formation. Table 1 shows the results of the kinetic study at the different pH assayed. It was noticed that the kinetics of precipitation of ChTRP was faster at pH 4.50. The average time ($t_{0.5}$) of insoluble ChTRP–PAA complex formation was 39.2 s and the $t_{0.5}$ for ChTRP–PVS complexes was 32 s. In contrast, the kinetics of precipitation of Tryp was faster at pH 3.50, probably because of the higher net positive charge of the enzyme at this pH. The $t_{0.5}$ of insoluble Tryp–PAA complexes formation was 20.2 s and the $t_{0.5}$ for Tryp–PVS complexes was 58 s.

4. Conclusions

In this work, we characterized the interaction between ChTRP and Tryp with PAA and PVS. The pH range of precipitation was determined for each enzyme–polyelectrolyte system. In

enzyme–PAA systems, there are two limits for insoluble complex formation. On the other hand, enzyme–PVS systems have only one phase transition, a pH value under which insoluble complexes are formed. This difference can be explained by the fact that PAA is less acid than PVS. Therefore, it does not have the same charge density in the pH range assayed, PAA loses charge at acid pH, interacting weakly with enzymes and giving place to soluble complexes. The effect of NaCl on the precipitation pH was also studied and enzyme–PVS interaction showed a more electrostatic character than enzyme–PAA one.

Turbidity of enzyme–polyelectrolyte systems was related either to the size of the insoluble complexes formed or to the number of complexes present in the solution.

Two mechanisms for insoluble enzyme–polyelectrolyte complex formation were proposed, depending on the system: (1) the insoluble ChTRP–PVS complex formation occurs in a cooperative way by the interaction of soluble ChTRP–PVS complexes; (2) the formation of the other insoluble complexes (ChTRP–PAA, Tryp–PVS and Tryp–PAA) follows a different mechanism. In the pH range of precipitation, all the PE in the solution take part of the insoluble complexes and the sequential addition of PE increases either the number or the size of the insoluble aggregates.

Acknowledgments

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