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Interaction of lysozyme with negatively charged flexible chain polymers

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Abstract

The complex formation between the basic protein lysozyme and anionic polyelectrolytes: poly acrylic acid and poly vinyl sulfonic acid was studied by turbidimetric and isothermal calorimetric titrations. The thermodynamic stability of the protein in the presence of these polymers was also studied by differential scanning calorimetry. The lysozyme–polymer complex was insoluble at pH lower than 6, with a stoichiometric ratio (polymer per protein mol) of 0.025–0.060 for lysozyme–poly vinyl sulfonic acid and around 0.003–0.001 for the lysozyme–poly acrylic acid. NaCl 0.1 M inhibited the complex precipitation in agreement with the proposed coulombic mechanism of complex formation. Enthalpic and entropic changes associated to the complex formation showed highly negative values in accordance with a coulombic interaction mechanism. The protein tertiary structure and its thermodynamic stability were not affected by the presence of polyelectrolyte.

Keywords: Lysozyme; Poly vinyl sulfonate; Poly acrylic acid; Protein–polyelectrolyte complex

1. Introduction

Production of proteins is a prime biotechnological application and includes upstream and, often more expensive, downstream processing steps to obtain the final product in the desired purified form. Bioseparation steps for recovery of final product can account for 50–80% of overall production costs [1]. Most purification technologies employ precipitation of proteins as one of the initial operations aimed at concentrating the product stream for further downstream steps. Precipitation by salts, organic solvents and non-ionic polymers are well known and simple techniques for protein concentration [2,3]. Attempts are usually made to derive some degree of purification of target products in the precipitation step. The precipitation methods used, however, lack selectivity and thus limit their purification potential. Substantial advantage can be gained if the precipitation step is imparted with some degree of target specific selectivity.

There are several studies in which attention should be called to the formation of a flexible polymer chain (PCF)–protein complex [4,5]. In addition, with the intention of developing novel drug delivery systems, block and graft polymers with proteins have also been studied in terms of the formation of polymer–protein complexes, although the authors did not pay much attention to the role of the polymer moieties in the complex formation.

Complex formation by proteins with water-soluble synthetic polymers is interesting from two points of view [6]:

- First concerns the way in which globular proteins interact with flexible chain macromolecules through electrostatic, hydrogen bonding and hydrophobic interactions, an understanding of which could provide a better explanation of the mechanisms of macromolecular interaction available in nature.
- Second concerns the extent to which biochemical activity is maintained in the resulting complexes, the answer to which is central to the molecular design of composite protein–polymer systems, such as immobilized enzymes, as well as to the

Abbreviations: LYZ, lysozyme; PAA, poly acrylic acid; PVS, poly vinyl sulfonate; FPC, flexible polymer chain

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process design for protein separation using water-soluble polymers.

Protein precipitation by formation of insoluble-protein complex is a potential technique used in the isolation and purification of protein [7–9]. To make the use of PCF an attractive means of protein separation, two additional problems should be solved. The first problem is the protein recovery from the PCF medium and regeneration of the polymer. One of the potential solutions to this problem is the use of reversibly soluble systems based on a better understanding of factors those affecting phase state and stability of PCF–protein complex formed.

The second problem is the selectivity of the polymer–protein interaction. This problem could be addressed by specific interactions introduced by affinity ligands coupled to the polymer, so-called macroligands, so that a reversible biospecific water-soluble complex is formed with the desired target protein.

Precipitation methods have the advantage that low concentration of polymer is needed to precipitate the protein, but in some cases the protein loses part of its biological activity [10]; so, it is necessary to know the molecular mechanism by which PCF in aqueous solution interacts with proteins. We have used spectroscopic and calorimetric techniques to obtain information about the molecular mechanism of interaction between a basic model protein: lysozyme and two negatively charged polyelectrolytes: poly acrylic acid and poly vinyl sulfonate with the goal of applying this information in the polyelectrolyte–protein complex formation as tool for protein separation.

2. Materials and methods

2.1. Chemical

Egg white lysozyme (LYZ) was purchased from Sigma Chem. Co. (USA) and the polymers poly acrylic acid, sodium salt (PAA), 25% (w/w) sol. in water molecular average mass 240 kDa and poly vinyl sulfonic acid, sodium salt (PVS) 25% w/w. sol. in water, molecular average mass 170 kDa, were purchased from Aldrich and used without further purification. Buffers of different pH were prepared at concentration of 50 mM: phosphate buffer pH 5.5 and 7.0, and acetic acid/acetate buffer pH 3.1. They were adjusted with NaOH or HCl in each case.

2.2. Lysozyme turbidimetric titration curves with polymer

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration [11]. Buffer sodium phosphate solutions (10 mL) with a fixed protein concentration were titrated at 20 °C in a cubic 2.6 cm path-length glass cell with the polymer solution as the titrant. Unless otherwise noted, the concentrations of protein and polymer were 0.3 mg/mL and 0.25% w/w. (PVS) and 0.8 mg/mL and 0.42% w/w (PAA), 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 versus polymer/protein molar ratio. Therefore, polymer/protein complex with three different stoichiometry – selected from the plateau region of the cited above plot – are titrated with alkali and acid, the absorbance at 420 nm being plotted versus pH

2.3. Isothermal titration calorimetry (ITC)

Measurements were performed at 20 °C by using a VP-ITC titration calorimeter (MicroCal Inc., USA) [12]. The sample cell was loaded with 1.436 mL of lysozyme solution (90 μM) and the reference cell contained Milli-Q grade water. Titration was carried out using a 0.3 mL syringe filled with polyelectrolyte solutions. The experiments were performed by adding 30–100 aliquots of 3 μL of polymer solutions (0.63%, w/w) to the cell containing the protein solution. The heat associated in the interaction between protein and polymer was calculated by discounting the heat of dilution of protein and polymer, which was determined by the titration of a protein solution into buffer and the buffer into polymer 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 and the intrinsic molar enthalpy change for the binding, ΔH°, the binding stoichiometry, n, and the intrinsic binding constant, K_b, were thus obtained. The intrinsic molar free energy change, ΔG°, and the intrinsic molar entropy change, ΔS°, for the binding reaction were calculated by the fundamental thermodynamic equations:

$$\Delta G^\circ = -RT \ln K_b \quad (1)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (2)$$

2.4. Differential scanning calorimetry of LYZ experiments

Thermal desnaturation of proteins was monitored with a high sensitivity differential scanning calorimeter model VP-DSC from MicroCal Inc. [13]. Thermograms were obtained between 60 and 90 °C, at scan rate 30 °C/h. Protein was analyzed at concentration of 3 mg/mL and for both polymers concentration was 0.25% (w/w). All result were averages of, at least, three independent measurements. The calorimetric data were analyzed 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 (((H_{VH}). The unfolding constant K_u, (G° and (S° were calculated using the equations:

$$K_u(T) = \exp\left[-\frac{\Delta H_{cal}}{RT}\left(1 - \frac{T}{T_m}\right)\right] \quad (3)$$

$$\Delta G^\circ = -RT \ln K_u \quad (4)$$

$$\Delta S^\circ = -\left(\frac{\partial \Delta G^\circ}{\partial T}\right) \quad (5)$$

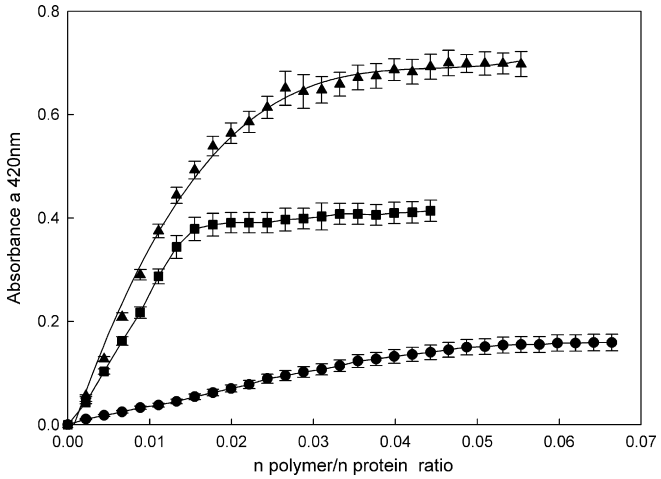

Fig. 1. Turbidimetric titration curves of LYZ (0.3 mg/mL) solution with PVS in a medium with 50 mM phosphate buffer. pH 5.5 (▲), 7.0 (●), and acetic acid/acetate buffer pH 3.1 (■). Temperature 20 °C.

3. Results

3.1. Lysozyme turbidimetric titration curves with the polymers

Figs. 1 and 2 show typical titration curves of LYZ with PVS and PAA, respectively, from which, two important characteristics were observed:

(i) at low polymer–protein ratio absorbance increases linearly with an increase in the polymer total concentration and,
(ii) when the polymer concentration increases, there is a plateau which depends on the medium pH.

The free LYZ concentration remaining in the titration system was measured by absorbance at 280 nm of the supernatant solutions from which the insoluble polymer–protein complex had been separated by centrifugation. The free LYZ concentration

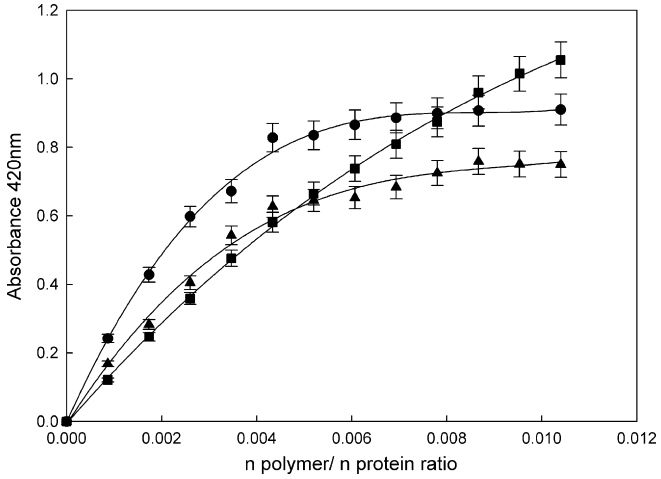

Fig. 2. Turbidimetric titration curves of LYZ (0.8 mg/mL) solution with PAA in a medium with 50 mM phosphate buffer pH 5.5 (▲), 7.0 (●), and acetic acid/acetate buffer pH 3.1 (■). Temperature 20 °C.

Table 1
Protein/polymer ratio obtained for turbidimetric titration curves of LYZ with polymers at different pH

System	Protein/polymer ratio
LYZ–PVS	
pH 3.1	66
pH 5.5	47
pH 7.0	23
LYZ–PAA	
pH 3.1	n.d.
pH 5.5	250
pH 7.0	285

in solution decreases linearly with increasing PVS concentration, it being negligible when the curve reached a plateau (data not shown) which suggests that all the protein in solution has been precipitated. The stoichiometric protein/polymer ratio which corresponds to the situation where all the protein has been precipitated as insoluble complex was 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. Table 1 shows the molar protein–polymer ratio which corresponds to the stoichiometry of the complex formation calculated from the titration curves for the different experiments. These values are important they because allow to calculate the minimal polymer amount needed to precipitate the protein, the data have been expressed as the number of LYZ molecules bound per polyelectrolyte molecule.

Lysozyme is a basic protein with 19 amino residues, an isoelectrical pH between 11.0 and 11.4 and a molecular mass of 14.3 kDa, therefore at the pHs where the turbidimetry titration were assayed the protein has a net positive electrical charge. Formation of LYZ–PVS complex was observed to be influenced by the medium pH; the increase of pH between 5.5 and 7.0 induced a dramatic decrease in the maximum absorbance values which suggested a minor amount of complex formation. However, at pH 3.1, a minor absorbance maximum value was observed than at pH 5.5, which can be assigned to the loss of the native structure of this protein by influence of the acid medium.

The LYZ titration curves by PAA were not significantly affected by the pH change. At pH 5.5 and 7.0 the curves reached a plateau, while at pH 3.1 this behaviour was not observed. The shape of this last curve showed an increase in the number of polymer molecules bound per protein molecule. This behaviour could be understood taking into account that at pH 3.1 an important fraction of the protein is in its unfolded form which exposes other basic groups to the attack of the negatively charged polymer.

Because the medium pH was critical to the insoluble protein complex formation, the turbidimetric titration was carried out at a constant polyelectrolyte–protein ratio while the pH of the medium was varied by acid or base addition. Fig. 3A and B show the absorbance dependence (at 420 nm) versus the pH change. LYZ–PVS and LYS–PAA complexes were soluble at basic pH values, from pH 6.0 to lower values a significant increase in the

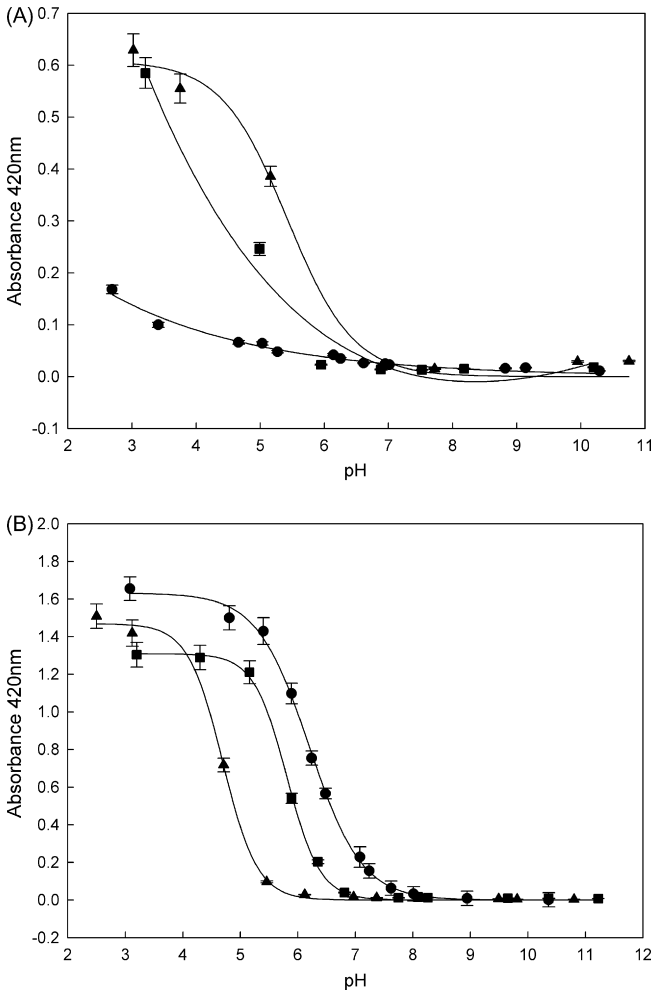

Fig. 3. (A) Dependence of the absorbance at 420 nm vs. pH at a constant protein–polymer ratio of LYZ–PVS:(●) 0.025, (■) 0.042, (▲) 0.057 μM/ μM. (B) Dependence of the absorbance at 420 nm vs. pH at a constant protein–polymer ratio of LYZ–PAA: (●) 0.0027, (■) 0.0065, (▲) 0.0010 μM/ μM. Temperature 20 °C.

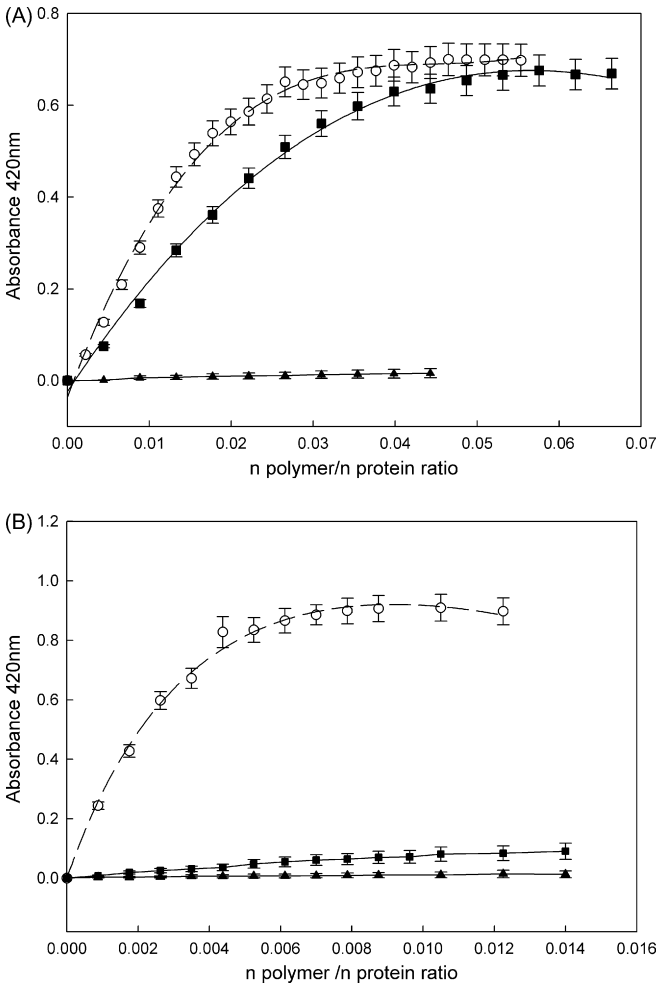

Fig. 4. NaCl concentration effect on the turbidity of (A) LYZ–PVS, pH 5.5, NaCl concentration: (◯) 0 M, (■) 0.05 M and (▲) 0.1 M. (B) LYZ–PAA, pH 7.0, NaCl concentration: (◯) 0 M, (■) 0.1 M and (▲) 0.5 M. Temperature 20 °C.

turbidity was observed corresponding to the insoluble complex formation. Similar behaviour was reported for the serum albumin titration with anionic polyelectrolyte. [11]. These curves determine the optimum pH interval in which the protein complex is in insoluble form.

Because the molecular mechanism of the LYZ–PVS and LYZ–PAA interaction is electrostatic in nature, the turbidimetric titrations (at pH 5.5 and 7.0) were performed in medium of different ionic strength such as shown in Fig. 4A and B. For the system LYZ–PVS and LYZ–PAA, a NaCl concentration of only 0.1 M is enough to avoid formation the insoluble protein–polymer complex. This finding may be interesting because it is the base of an isolation method of protein, allowing in a first step the precipitation by the polymer and then the precipitate may be dissolved by NaCl solution addition at low concentration. The low ratio polymer–protein values found suggested that one polymer molecule bound many protein molecules according to the model proposed by Dubin and coworkers [14] where one polymer molecule has the capability to interact with many proteins molecules.

3.2. Thermal stability of the LYZ in the absence and presence of the polymers

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. Because LYZ is one of the four proteins whose thermal denaturation is thermodynamically reversible, the equations for systems in thermodynamic equilibrium can be applied to obtain the thermodynamic functions (entropy and enthalpy of unfolding) directly from the thermograms, as described by Privalov [15].

A typical temperature function of the partial molar heat capacity of LYZ is shown in Fig. 5, the T_m is defined as the temperature at which a maximum change happens in the Cp value. No change in the shape and T_m of the thermogram was observed in the polymer presence. The T_m constant value is a proof that the protein retains its thermodynamic stability in the presence of both polymers. However the polymer presence induced a decrease in the area under the curves, in agreement with a diminution of the heat associated to the denaturation process. The unfolding entropic change showed not to be affected by the polymer presence, in accordance with the protein retaining

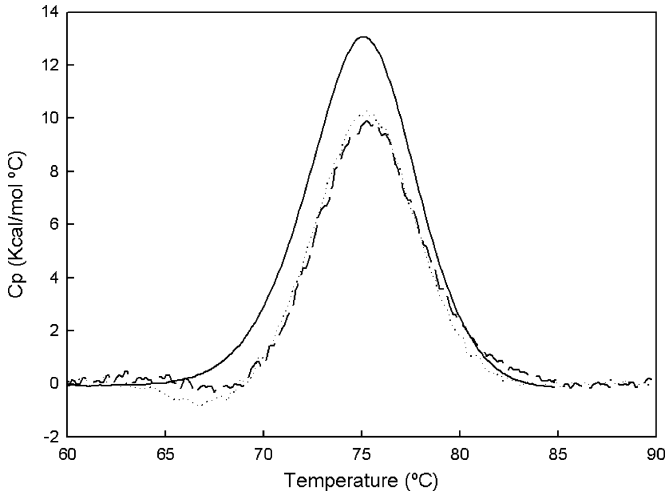

Fig. 5. DSC thermogram of the LYZ in the absence (—) and presence of PVS (- - -) and PAA (. . .). Heating rate 0.5° min^{-1}. LYZ (3 mg/mL), PVS and PAA (0.25%, w/w) pH 7.0.

its tertiary structure and no important conformational protein change is occurring. The ratio $\Delta H_{VH}/\Delta H_{cal}$ reached values greater than 1, suggesting that an intramolecular cooperation process is being carried out [16] and no aggregation of the LYZ molecules occurs. The evaluation of ΔH_{VH} gives an idea of the mechanism of the unfolding process. If a two state process is carried out under equilibrium condition, $\Delta H_{VH} = \Delta H_{cal}$. If $\Delta H_{VH} < \Delta H_{cal}$, it can be concluded that one or more intermediate states of significance in the overall process. If $\Delta H_{VH} > \Delta H_{cal}$ intramolecular cooperation is taking place which may require some degree of molecular association.

LYZ has only one domain with low molecular mass, its thermal unfolding has been described as reversible, however the capacity of LYZ to associate in aqueous solution is well known. The $\Delta H_{VH}/\Delta H_{cal}$ value greater than unity is an indication of the intermolecular cooperation presence during the thermal unfolding. The increase of this ratio, suggests more cooperative intramolecular process. 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 turbidymetric titration curves. In this way the intermolecular interaction between protein molecules is favoured.

The dependence of the Gibbs energy change of the unfolding process with the temperature was calculated from the Eqs. (3) and (4), a straight line was obtained (data not shown), the slope of this plot is the unfolding entropy (ΔS) as shown in Table 2. It can be seen that in the polymer presence unfolding entropy was not affected which suggests that the polymer–protein complex followed the same conformational state as the protein alone.

Table 2
Thermodynamic functions obtained for the thermal LYZ unfolding determined by DSC in the absence and presence of the studied polymers

	LYZ	LYZ–PVS	LYZ–PAA
ΔHcal (kcal/mol)	89.4 ± 0.3	72.0 ± 0.3	66.7 ± 0.4
ΔH_{VH} (kcal/mol)	139.0 ± 0.6	141 ± 0.8	151 ± 1.0
$\Delta H_{VH}/\Delta H_{cal}$	1.55 ± 0.05	1.96 ± 0.01	2.26 ± 0.03
T_m (°C)	75.01 ± 0.01	75.33 ± 0.02	75.2 ± 0.1
ΔS (e.u.)	399 ± 3	405 ± 4	405 ± 5

3.3. Isothermal calorimetric titration of LYZ with polymers

The complex formation between LYZ and polymers was investigated by isothermal titration calorimetry. The direct curves obtained showed negative heat associated with the titration, and the polyelectrolyte dilution into the buffer (in the absence of protein) was associated with a large heat release (data not shown), being the heat of dilution of the protein by the buffer negligible. The heat associated with the interaction polymer–protein (ΔH_i) was calculated by subtraction using the equation:

$$\Delta H_i = \Delta H_t - \Delta H_d \quad (6)$$

where ΔH_t is the polymer–protein titration heat and ΔH_d is the heat of dilution of the polymer in the buffer in the absence of the protein. Fig. 6 shows the calorimetric titration curve of LYZ with PVS in a 50 mM, pH 5.5 (PVS), medium buffer phosphate. By non-linear fitting of these data, 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 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 with the same affinity constant, K, for the ligand (lysozyme).

Turbidimetric titration and the ITC results yielded a stoichiometric ratio for LYZ–PAA ten times lower than that for the LYZ–PVS system. This finding is in agreement with the theory proposed by Dubin [17] for the molecular mechanism of protein–polyelectrolyte interaction by which one polymer molecule has capacity to bind to several protein molecules.

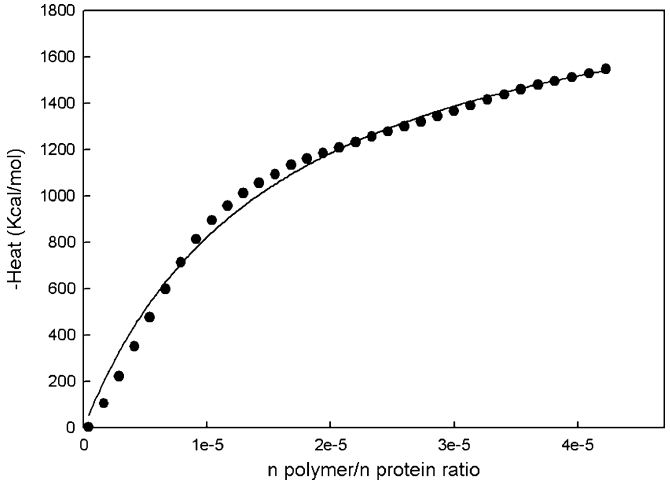

Fig. 6. Binding curve for the calorimetric titration of LYZ with PVS. Protein concentration 90 μM. Medium 50 mM sodium phosphate buffer, pH 5.5. Temperature 20 °C.

Table 3
Thermodynamic and binding parameters of the interaction LYZ–polymer from ITC experiments

System	LYZ–PVS	LYZ–PAA
n (protein/polyelectrolyte)	21.2 ± 0.2	294 ± 8
K (M^{-1})	$2.7\ 10^3$	$5.1\ 10^4$
ΔH° (kcal/mol)	−15.2	−10.0
ΔS° (e.u.)	−1103	−1033

The enthalpic change is expressed per mol of protein bound.

Negative and very high heats associated to the protein–polymer interaction were found, in the order of 320 kcal/mol for the LYZ–PVS and around 3000 kcal/mol for the LYZ–PAA complex formed, respectively. A better quantitative analysis of heat associated to the protein–polyelectrolyte complex formation can be made if the n values are expressed as protein mol per polymer mol in the same way as that in the turbidimetry titration. The values obtained are shown in Table 3. Values of 21.2 and 294 protein mol per polymer mol were found for LYZ–PVS and LYZ–PAA complex formation, respectively. The ΔH was normalized per mol of protein, therefore, heat values of 15.2 and 10.0 kcal/protein mol are yielded for both polymers, respectively. These heats of interaction are expected in the order of magnitude for interactions of electrostatic nature. The larger value observed for the LYS–PVS interaction suggests a stronger interaction of this polyelectrolyte with the protein. PAA has a pK_a value around 4.5 (which is the pK_a for carboxylic acids), and at pH 5.5, only 90% of the carboxylic groups will be dissociated in PAA, while PVS will be fully ionized which drives a greater interaction between the positively charged electrical groups of LYS and the negatively charged PVS.

The ΔS values were found negative as result of the formation of a more ordered structure by protein–polymer complex formation.

4. Discussion

Current methods of protein purification involve an extensive series of steps and processes that increase the cost of the final product. New techniques for large-scale protein separation are therefore of interest. One of these involves the addition of polyelectrolytes, leading to selective protein phase separation. Proteins interact strongly with both synthetic and natural polyelectrolytes. These interactions are modulated by variables such as pH and ionic strength, and may result in soluble complexes, insoluble complex or the formation of amorphous precipitates. This phase separation is a result of the electrostatic interactions between the protein and the polyelectrolyte which, at very low ionic strength, results in tight ion-pairing and the formation of amorphous precipitates. Several experimental studies have been reported for protein precipitation using polyethyleneimine. The most extensively studied proteins are lysozyme, ovalbumin and bovine serum albumin [14]. Oppositely charged polyelectrolytes such as carboxymethyl cellulose, poly acrylic acid and poly metha acrylic acid have been used to selectively precipitate proteins from an aqueous mixture on the basis of different affinities [2,7]. 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 [18].

Some authors [6,11,17] have postulated different theoretical models to describe the protein polyelectrolyte complex formation. However, these models are of difficult practical application to predict the behaviour of the system which makes it necessary to determine in a practical way the experimental variable values. These variables values reach the complex precipitation condition and then to dissolve it by salt addition. A better knowledge of the molecular mechanism of precipitation by which the complexes are formed allows to determine in an exact manner the interval values of the experimental variables. Different techniques have been used to study the complex formation, but the most used is the turbidimetry which is a simple methods. We have used ITC which gives the direct heat associated to the complex formation and compared the results from both. One parameter that is important to know is the stoichiometry ratio for polyelectrolyte–protein complex formation to cause complete protein precipitation. The values obtained from both techniques were in the order of 10^{-2}–10^{-3} polymer mol/protein mol which represent that 10–100 protein molecules are bound per polymer molecule. This finding is in agreement with previous theoretical model which supposes that one polyelectrolyte molecule has the capability to bound several protein molecules [11]. The mechanism occurred out between the electrical charged group of both which allow that one protein molecule is bound to the polymer by several charged group. Because the size of the polyelectrolyte molecule is ten times larger than the protein, the number of protein molecules bound per polymer molecule is high, decreasing as the size of the protein molecule was increased. The heat associated with the complex formation was extremely high; but when they are normalized, per protein molecule bound to the polymer, the heat associated yield was 10–15 kcal mol, which is a normal heat amount for a coulombic interaction between two charge groups in solution. These low interaction heats are in agreement with the low NaCl concentration needed to induces the dissolution of the insoluble complex (around 0.1 M). Other important parameters to know are the thermodynamic stability of the protein in the polymer presence. It is desirable that the protein retains its tertiary structure. DSC measurements demonstrated that the T_m of LYS was not modified by the polymer presence, only a decrease in the denaturalization heat was observed. [19]. Interaction between flexible polymer chain and protein has been used in the bioseparation methods: liquid–liquid partition in aqueous two phase system and protein precipitation by polyelectrolytes. The first technique has the disadvantage that requires high concentration of the polymer to produce the separation of the phases. In contrast, polyelectrolyte precipitation uses very low polymer concentration (0.1–1%) which makes this technique non-expensive. The solution has low viscosity and easy application in scale up having this methodology a significant advantage over the partition in aqueous two phase system.

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