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Polyethyleneimine phosphate and citrate systems act like pseudo polyampholytes as a starting method to isolate pepsin

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Abstract

The aqueous solution behaviour of polyethyleneimine (a cationic synthetic polymer) in the presence of anions (such as citrate and phosphate) was studied by means of turbidimetry. The variation of the absorbance at 420 nm of dilute mixture with pH, the polymer concentration and the ionic strength were examined. The mixture of polyethyleneinine citrate or polyethyleneinine phosphate behaves as a pseudo polyampholyte with an isoelectric point of 5.5 and 6.2 for phosphate and citrate respectively and a precipitation pH range between 3.5 and 8.0. Pepsin was completely precipitated with the polymer anion complex within this pH interval. Citrate showed a better precipitation effect than phosphate did. The precipitate was reversibly dissolved in NaCl (for concentrations higher than 0.2 M) and pepsin kept its biological activity. Studies of pepsin thermal stability (by differential scanning calorimetry) revealed that the polyethyleneimine presence increased the enzyme denaturation temperature. The circular dichroism spectrum of pepsin showed a non-significant loss of secondary and tertiary enzyme structure by the polyethyleneimine. However, the polymer presence increased the biological activity of pepsin.
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1. Introduction

Precipitation is one of the typical downstream processing of biological products, especially applied to the large scale recovery and purification of proteins in biotechnology. Among the different precipitation methods, polyelectrolyte precipitation has been widely investigated because of its low additive consumption, high protein recovery, and high degree of fractionation of target enzymes [1,2].

A number of studies have been carried out on the protein–polymer interaction and the effect of polymer molecular weight, as well as the nature of the protein. Due to the charged nature of proteins, electrostatic interactions play a particularly important role. Electrostatically driven associations between proteins and polymers have also been investigated regarding complex formation and phase separation, the characteristics of the aggregates formed in terms of size and composition, and the effect on these parameters have been studied by varying the protein–polymer electrostatic interaction through changing pH [3,4].

Some theoretical models about protein polyelectrolyte formation have been proposed for the internal structure of protein–polyelectrolyte complexes; however, it is desirable to know how the different experimental variables such as polyelectrolyte molecular mass, pH and ionic strength [5,6] influence the insoluble complex formation.

The mechanism of particle formation in polyelectrolyte precipitation has been early proposed [1,3,7]. The protein precipitate is produced through a two-stage process: the formation of protein–polyelectrolyte complexes and the com-

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plex aggregation between them to form an insoluble state [8].

In this work, we study the capacity of both the polyethyleneimine–phosphate and polyethyleneimine–citrate systems to form an insoluble complex which behaves as an ampholyte with the ability to interact with proteins having negative electrical charge. Pepsin was used as a model protein to achieve this goal.

2. Materials and methods

2.1. Chemicals

Pure liophylised pepsin (PEP), polyethyleneimine (PEI) (50% aqueous solution, Mn 60,000) were purchased from Sigma Chem. Co (USA). Stock solutions of PEI were prepared in water and the pH adjusted to a desired value using HCl.

2.2. Turbidimetric titration

The formation of polymer–anion complex was examined by means of turbidimetric titration [3,9]. Aqueous solutions (10 mL) with a fixed phosphate or citrate concentration were titrated at 20 °C with a PEI solution as titrant and the turbidity measured as absorbance at 420 nm. Solutions containing different PEI–phosphate or citrate ratio were also titrated with HCl and the turbidity measured.

2.3. Differential scanning calorimetry of the PEP

Thermal denaturalization of protein was monitored with a differential scanning calorimeter model VP-DSC from MicroCal Inc. Thermograms between 30 and 90 °C, at a scan rate of 30 °C h^{-1} were obtained. This protein was analysed at concentrations of 3 mg mL^{-1} and the polymer concentration was 5% (w/w). The results were averages of, at least, three independent measurements. The calorimetric data were analysed by using the software ORIGIN 7.0, MicroCal Inc. The parameters obtained from this analysis were: the temperature at which maximum heat exchange occurs (T_m), the area under the peak, which represents the enthalpy of transition for the reversible process ΔH_{cal} and the van't Hoff enthalpic change (ΔH_{VH}). The unfolding constants K_u, ΔG° and ΔS° were calculated using the following equations [10,11].

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

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

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

2.4. PEP enzymatic activity determination

It was measured according to the enzyme's capacity for milk clotting [12]. Skim milk powder was prepared by dissolving 10 g of a commercial skim milk powder in a solution of 100 ml of $CaCl_2$ 10 mM and stirring it for 30 min. The reconstituted milk was kept at 4 °C and used 1 h after preparation. Before use, the milk was equilibrated at 35 °C for 20 min. The clotting time was determined through a rotation movement until the first clot appeared. Control of the activity was performed adding PEP in a 50 mM pH 6.5 phosphate medium during 1 h and the activity of the increasing amounts of these solutions was measured through the slope of clotting time vs. 1/volume of added protein solution plot. The inverse of this slope is the U/mL of the solution used. As a reference plot, the same experiments were carried out incubating the same amount of PEP during 1 h and the slope of the same plot in order to find out the activity values of the unknown samples in all media used. In order to check the stability of the different PEP solutions, their activities were compared with the PEP calibration curve of 1.078 mg/mL. A PEP solution was prepared in 50 mM pH 6.5 phosphate buffer; using it later as a reference for PEP solutions in mg/mL by interpolation in the calibration curve.

2.5. Circular dichroism of the PEP solution in the presence of PEI

The CD spectra for PEP (90 μM) were obtained in the absence and presence of PEI (5%) in a Jasco spectropolarimeter, model J-815. The ellipticity values were obtained in mdeg directly from the instrument and five cycles were carried out to obtain the spectra.

3. Results

3.1. Turbidimetric titration of phosphate and citrate with PEI

Fig. 1 shows turbidimetric titration curves when phosphate (500 mM) or citrate (50 mM) was titrated by adding a PEI concentrated solution (1 mM). Both curves reached a plateau at a high polymer anion ratio, which suggests a complete precipita-

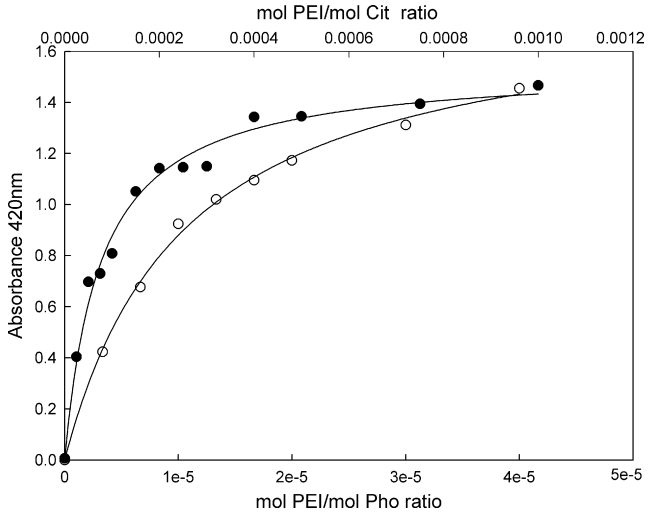

Fig. 1. Turbidimetric titration of phosphate (◯) and citrate (●) with PEI. pH medium 5.5 temperature 20 °C.

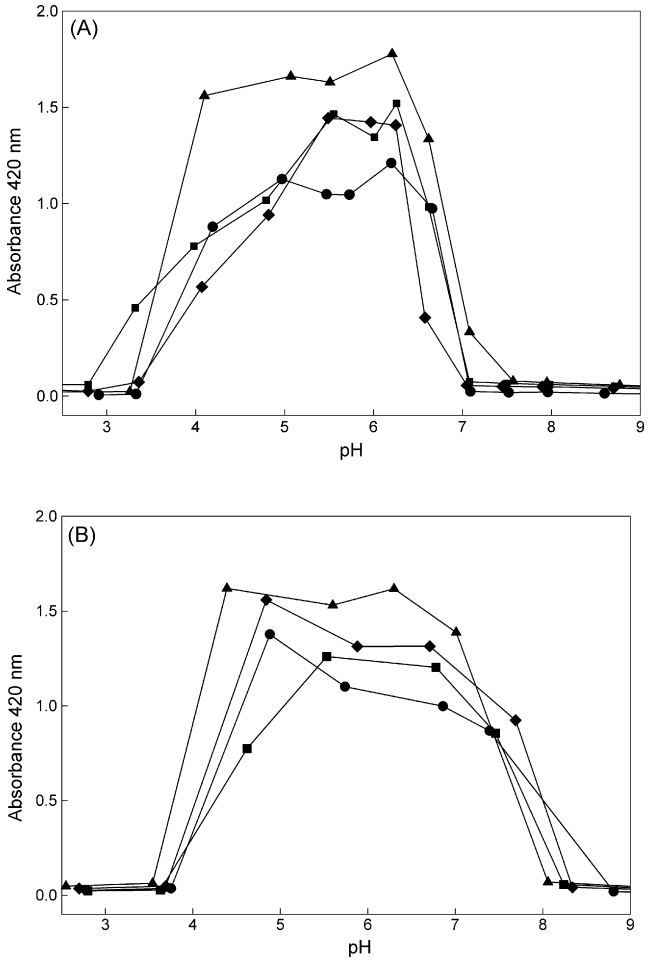

Fig. 2. (A) pH effect on the precipitation of the PEI–phosphate complexes. Temperature 20 °C. PEI/Pho molar ratios (●) 4×10^{-5}, (■) 7.7×10^{-5}, (▼) 1.13×10^{-4}, (▲) 1.5×10^{-4}. (B) pH effect on the precipitation of the PEI–citrate complexes. Temperature 20 °C. PEI/Cit molar ratios: (●) 0.003, (♦) 0.006, (■) 0.009, (▲) 0.012.

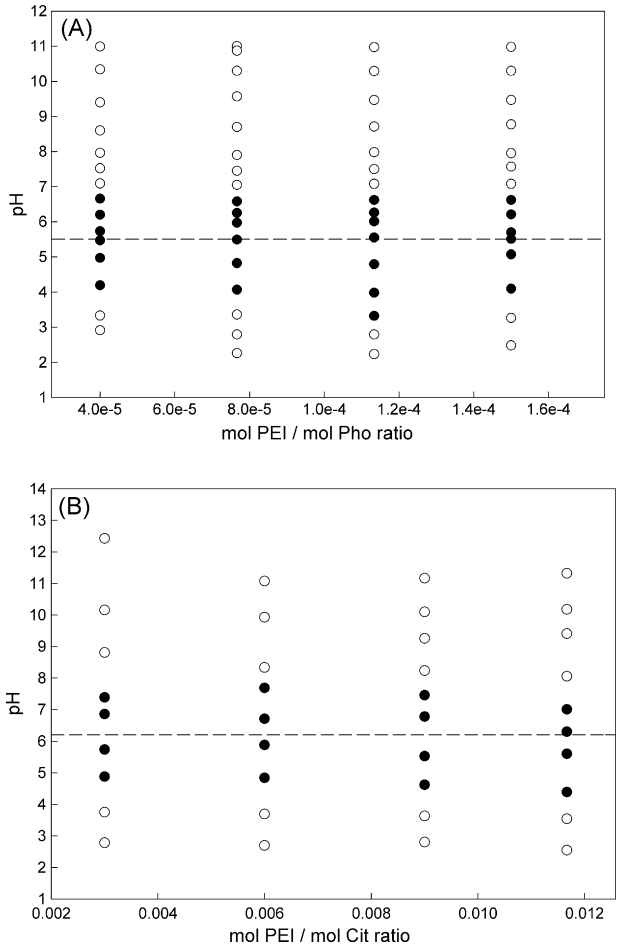

Fig. 3. (A) Phase diagram of PEI/Pho systems at different molar ratios of Fig. 2A. (B) Phase diagram of PEI/Cit systems at different molar ratios of Fig. 2B.

tion of the complex. It could be seen that the plateau was obtained at a polymer/anion ratio 10 times higher for citrate than for phosphate, suggesting that citrate has a greater precipitation capacity than phosphate.

The PEI–citrate system was dramatically affected by 0.5 for higher ionic strength; in this case, no formation of the insoluble complex was found while the PEI–phosphate system showed to be slightly affected by the NaCl increased concentration (data not shown). The inhibition of the precipitate formation in both systems was directly proportional to the salt concentration, in agreement with the presence of an important coulombic component in the insoluble complex formation [13,14].

Fig. 2A and B shows the pH variation effect on the insoluble complex formation obtained for different PEI/anion ratios. A pH interval can be observed (ΔpH) where the turbidity of the medium is increased, reaching a maximal value of about 5.5 of pH for phosphate and 6.0 for citrate, and decreasing with pH diminution. Each curve has a trapezoidal shape with a plateau which is the height of a trapezium. It depends on PEI concentration. The pH values corresponding to the edges of the trapezium are the critical pHs at which the transition from complete dissolution to precipitation occurs. The lower critical pHs of 4.0 for citrate and 3.5 for phosphate will be referred to as the acidic critical pH. The higher critical pH are 7.0 (phosphate) and 8.0 (citrate) and will be referred to as the basic critical pHs. Similar results have been previously reported [1].

The data shown in Fig. 2A and B can be used to make a phase diagram of the polymer behaviour in the pH variation range studied. The broken line is drawn at the polymer isoelectric point of 5.5 (phosphate) and 6.2 (citrate). The filled circles in Fig. 3A and B are drawn at the pH of non-zero absorbance in Fig. 2A and B, whereas the open circles indicate zero absorbance values of the solutions. The transitions from complete solubility to polymer precipitation occur at the same critical pHs independently of the polymer concentration. This arises from the acid and basic constant at critical pHs values observed in Fig. 3A and B. The acid and basis critical pHs are equally spaced from the polyanpholyte isoelectric point. Table 1 also shows the ΔpH values for the precipitate formation. Both media showed similar

Table 1

Medium	Polymer–anion ratio (mM/mM)	Isoelectric pH	ΔpH
Phosphate	1.33×10^{-5}	5.5	3.6
Citrate	1.5×10^{-4}	6.2	4

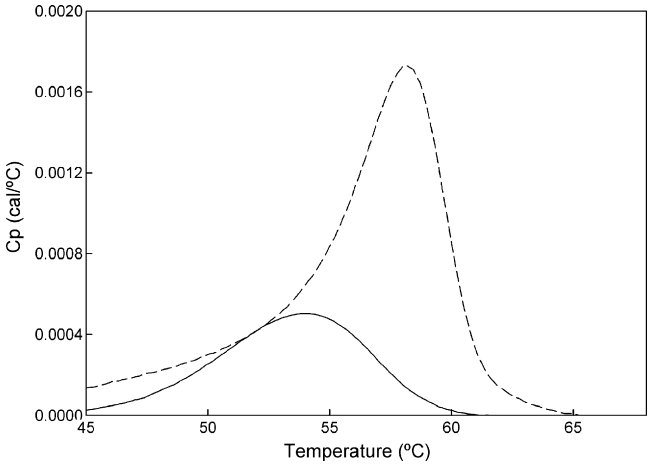

Fig. 4. Thermograms of PEP in the and absence (—) and in the presence (- - -) of PEI. Protein concentration: 90 μM, polymer concentration 900 μM Medium condition sodium phosphate buffer 50 mM, pH 5.5.

ΔpH values; however, the ΔpH for citrate was shown to be a unity shifted to the alkaline zone.

In the acid region (on the left of isoelectric pH), the complex has an excess of positive electrical charge and the same happens in the basic region, where there is an excess of negative charge.

3.2. Thermal stability of PEP in the absence and presence of the polymer

Differential scanning calorimetry is a useful tool for studying protein unfolding. Thermograms of excess specific heat capacity (C_p) are obtained as a function of temperature [10,11].

A typical temperature function of the partial molar heat capacity of PEP is shown in Fig. 4. The Tm is defined as the temperature at which a maximum occurs in the C_p value. A significant change in the shape and the Tm of the thermogram was observed by PEP due to the polymer presence. The T_m increased about 4 degree in agreement with an increase in the thermodynamical stability of PEP induced by the polymer presence which increased the area under the curve, due to an increase of the unfolding heat such as shown that in Table 2.

The ratio $\Delta H_{VH}/\Delta H_{cal}$ reaches a value higher than 1 for the protein alone, suggesting that an intramolecular cooperation process is being carried out [15] and no aggregation between the PEP 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 occur in the overall process. If $\Delta H_{VH} > \Delta H_{cal}$, intramolecular cooperation is taking place, which may require some degree of molecular association.

Table 2
Thermodynamic functions values obtained from the PEP thermogram

	Pepsin	Pepsin–PEI
T_m (°C)	53.64 ± 0.02	57.83 ± 0.02
ΔH (kcal/mol)	87.1 ± 0.74	172.0 ± 1.7
ΔH_{VH} (kcal/mol)	114.0 ± 1.2	178.0 ± 2.2
$\Delta H_{VH}/\Delta H$	1. 39	1.03
ΔS° (e.u.)	348 ± 26	536 ± 35

PEP and PEI concentrations were 87 and 83 μM, respectively.

The $\Delta H_{VH}/\Delta H_{cal}$ > than the unity is an evidence of the intermolecular cooperation presence during the thermal unfolding for the pepsin alone. The decrease of this ratio, suggests a minor cooperative intramolecular process. This finding is consistent with the proposed model of protein–polymer interaction, where one polymer molecule can bind several protein molecules. In this way, the intermolecular interaction between the pepsin molecules is disfavoured.

The dependence of the free energy change of the unfolding process on the temperature was calculated from Eqs. (3) and (4), and a straight line was obtained (data not shown), its slope being the unfolding entropy ΔS as shown in Eq. (5).

The unfolding entropic change was found to be increased by the polymer presence, in accordance with the formation of a complex structure. The increase of electrostatic bonds between the protein and polymer results in an increase in the disorder when the protein was unfolded by the action of the temperature.

3.3. PEP biological activity in the absence and presence of PEI

Fig. 5 shows the biological activity of PEP (determined as the clotting time of milk) in the absence and presence of polymer. It can be seen that at two concentrations of PEI, the clotting time is shorter than when the polymer is not present. Therefore, the PEI presence increases the biological activity of PEP. Fig. 6 shows the biological activity of PEP in the presence of PEI at a polymer/protein molar ratio of 83. It can be seen that the enzyme activity remains at a temperature of 20 °C for about 25 h. This high polymer–protein ratio was chosen to verify the polymer presence effect on pepsin, however, in the following experiments a lower ratio was assayed.

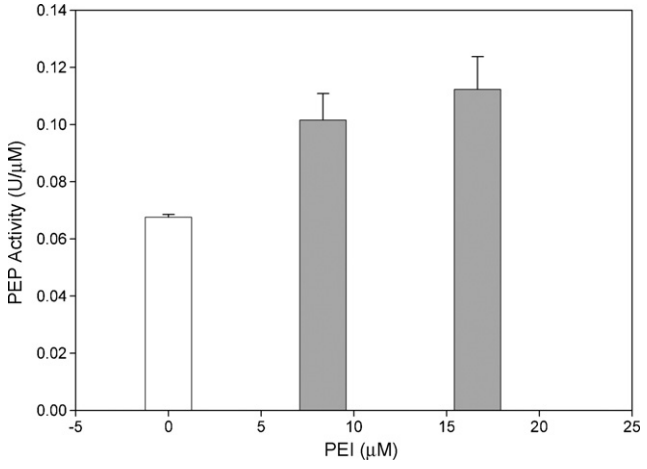

Fig. 5. Biological activity of PEP (0.1 μM) in the absent and the presence of two different concentrations of PEI using milk as substrate.

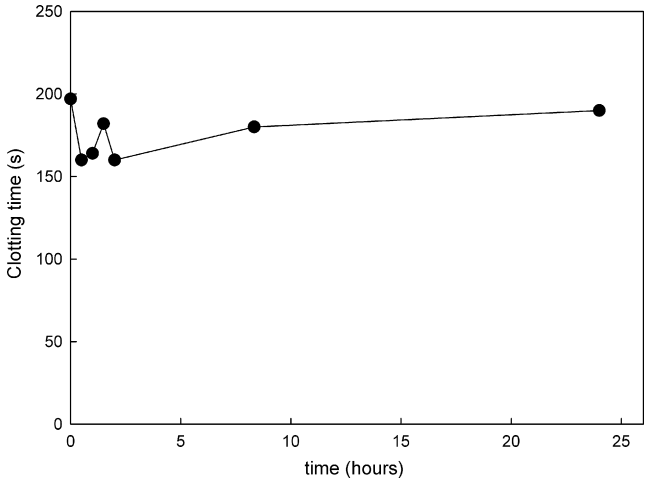

Fig. 6. Time dependence of the PEP activity measured using milk as substrate in the presence of PEI. PEP concentration 0.83 μM PEI concentration 0.1 μM. Temperature 20 °C.

3.4. PEI effect on the pepsin secondary structure

Circular dichroism (CD) is a useful tool to detect modifications in the secondary structure of a protein induced by temperature or by a cosolute presence such as a polyelectrolyte [16]. When a protein is isolated by liquid–liquid extraction using aqueous two-phase systems or by polyelectrolyte precipitation, both techniques use a flexible polymer chain due to the protein–polymer interaction. This should induce a modification in the tertiary or secondary protein structure or both. The final consequence is a loss of the thermodynamical protein stability and its biological activity. Therefore, it is necessary to determine its secondary structure in the absence and presence of the used polymer. Fig. 7 shows the CD spectrum of pepsin in the 200–300 nm spectral range, in the absence and presence of PEI. Since pepsin is a serin-protease, it has a poor CD signal in the 220 nm zone due to a low content of alpha helix. PEI presence did not induce a significant decrease in the CD signal in the 210–220 nm zone, which corresponds to the beta helix content, suggesting a stabilization of the protein.

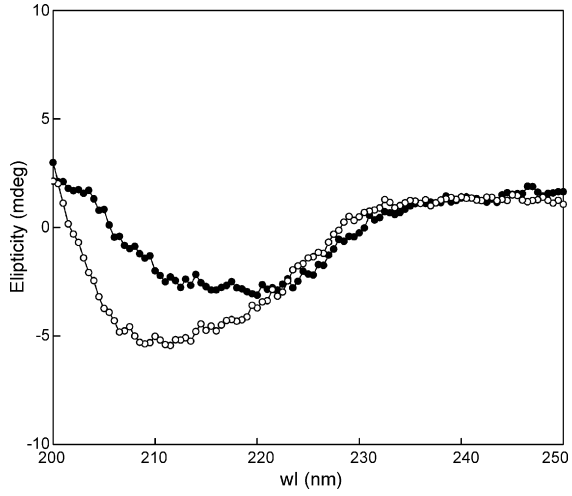

Fig. 7. Circular dichroism spectra of PEP (9 μM) in the absence (◯) and presence (●) of PEI (75 μM). Medium sodium phosphate buffer 50 mM, pH 5.5. Temperature 20 °C.

Table 3
Precipitation of PEP by PEI–salt complexes formation

Medium	% Pepsin recovery	PEI–anion ratio (mol/mol)
Phosphate	87.1	1/1000
Citrate	98.0	1/416

3.5. PEP precipitation by PEI–salt complex formation

A medium containing PEP (7.5 μM) and sodium phosphate (250 mM) or citrate (44.3 mM), at pH 5.5, was precipitated adding PEI. The precipitate formed was incubated at 20 °C for 30 min and centrifuged at 1000 G during 10 min. The precipitate was dissolved by the addition of NaCl 1.5 M. The protein concentrations were determined in the supernatant and in the precipitate dissolved by absorbance at 280 nm. Controls and a test curve were also carried out. Table 3 shows the PEP recovery for both systems. It can be considered that the recovery was complete (>98%) in the presence of citrate, while phosphate produced a recovery of 87%. At the pH where the PEP precipitation was assayed, the protein had a negative electrical charge because its isoelectric pH was near 1.5. At pH 5.5 the PEI–phosphate complex was near its isoelectic pH, therefore its electric net charge was expected to be zero or slightly positive, while PEI citrate complex at pH 5.5 had a positive net electrical charge. In this case, the complex interacted with the negatively charged PEP, which resulted in a better recovery of the enzyme.

4. Discussion

Polyampholytes are widely distributed in nature, where they are found as inorganic derivatives such as silver iodide salts and metal oxides such as aluminium. Polyampholytes have also been synthetically obtained as random linear polymers. Polyampholytes precipitate in dilute solution in a pH range. They have affinity for proteins and have been used for protein purification by anion exchange. Polyampholyte protein complex precipitation is driven by coulombic forces, which are highly dependent on protein and polyampholitic isoelectric pH values [5,17,18]. Precipitation begins at a critical pH where the attractive forces have just overcome electrostatic repulsion.

Classical polyampholytes have both anionic and cationic groups in their molecules; however, the aqueous solution of any polyelectrolyte may behave as a polyampholyte provided there is a small ion with multiple electrical charges (two or more) in the medium which interacts with the opposite charge of the polyelectrolyte to form a pseudo polyampholyte. Under these conditions, it is possible to find a pH interval where the pseudo complex behaves as an ampholyte.

Polyethyleneimine is a positively charged polyelectrolyte with a pK_a of 9.7, with the structural formula $(–CH_2–NH–CH_2–)_n$. In the presence of citrate or phosphate polyethyleneimine has been found to form an insoluble complex in the pH interval between 3.5 and 7 or 8, where it behaves as an

ampholyte. This behaviour has been used to precipitate acidic proteins such as pepsin. Both complexes do not show exactly the same ΔpH value of precipitation, because the PEI–anion stoichiometry is different. Phosphate and citrate have three pK_a with different values, which makes both acid basic parameters slightly different.

PEI does not produce any damage in the secondary and tertiary PEP structure; its biological activity does not vary within 24 h. This was not assayed for a longer time because our goal was to apply the precipitation effect of the PEI–anion complex to the isolation and purification of PEP, since only very few hours (one or two) are needed to precipitate and dissolve the insoluble complex. The precipitation of PEP (>98%) has shown a high yield in citrate medium, which allows for the possibility to use this method to isolate pepsin from its natural source. Experiments using gastric homogenate to isolate pepsin using this method are being carried out in our laboratory.

Our work attempts to prove that PEI–anion complex can be used as a method to isolate acid or basic proteins. This complex can act like a pseudo-ampholyte, which has an excess of positive or negative charge according to pH. According to our result, the PEI–anion complex, at the pH which has an excess of positive charge, causes the formation of a ternary complex with proteins which have a low isoelectric point.

The mechanism of formation of the complex can be interpreted in a first stage as the interaction of the high density positive electrical charge of the polymer with the polivalent anion (phosphate), which can precipitate themselves. The adjustment of pH can cause the complex to contain an excess of positive or negative charge, which then allows the interaction with the protein to precipitate jointly like a ternary complex.

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