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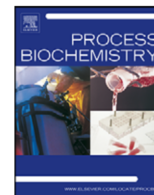

Aqueous two-phase extraction and polyelectrolyte precipitation combination: A simple and economically technologies for pepsin isolation from bovine abomasum homogenate

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ABSTRACT

The combination of two bioseparation techniques, partition in aqueous two-phase systems and polyelectrolyte precipitation of the target enzyme from the phase where it is present, was assayed to purify pepsin from bovine abomasum homogenate. Pepsin was partitioned in favor of the polyethyleneglycol-rich phase in an aqueous two-phase system of polyethyleneglycol 600 and 1450-sodium phosphate; however, a great amount of impure proteins were present. Chitosan (a cationic natural polyelectrolyte) was added to precipitate this acid enzyme as a form of insoluble complex. The addition of this second step increased the purity of the enzyme significantly while the yield was not significantly decreased. The combination of both partition in polyethyleneglycol 1450-phosphate system and chitosan precipitation produced a pepsin recovery of 48.5% with a purification factor of 9.0. The biological activity of the recovered enzyme remained unaltered.

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1. Introduction

A downstream process usually accounts for 50–80% of the total production costs of enzymes. Conventional methods used for protein purification are usually expensive because they involve several steps of unit operation, high cost of the reactants and they are difficult to scale up. Hence, in recent years there has been an ongoing interest in biotechnology for the development of innovative separation and purification methods that are both economically viable and gentle enough to preserve biological activity of proteins. Two methods have been considered to be very useful to scale up in the purification process of macromolecules: the partition in aqueous two-phase systems [1–5] and the protein–polyelectrolyte complex formation [6–10].

Aqueous two-phase systems (ATPSs) have been widely used as a method of separation and purification of biomolecules. These systems are particularly useful for partitioning of large biomolecules or even organelles. ATPSs are produced by combining two chemically different water-soluble polymers, or a water-soluble polymer (polyethylene glycol) and salt (potassium phosphate or citrate), added to water, resulting in the formation of two immiscible water-rich phases. Due to higher water content, the aqueous two-phase systems have several advantages compared to the commonly used separation and purification techniques and provide a secure separation and purification technique for biomolecules [1–5].

The non-soluble protein–polyelectrolyte complex formation is a common approach to obtain enzymes and other macromolecules. This technique offers the possibility of concentrating and purifying the target macromolecule at a low cost. Polyelectrolyte precipitation uses a poly-charged macromolecule of opposite electrical charge to the target macromolecule, forming a soluble protein–polyelectrolyte complex under desired experimental conditions; these complexes interact among each other, producing insoluble macro-aggregates [11]. This is a suitable method for protein isolation because very low polyelectrolyte concentrations are used (up to 0.1%, w/v). This method sometimes offers a high selectivity and the insoluble complex can be re-dissolved by a pH change or by adding a salt [6,8]. In a previous paper, we have studied the pepsin partitioning in ATPS of PEG–phosphate and found that this enzyme has a high affinity for the PEG-rich phase [12]. The high affinity of PEG for pepsin makes the polymer bound to the enzyme very difficult to eliminate by a simple dialysis method.

Chitosan, a polysaccharide comprising units of glucosamine and N-acetylglucosamine, is positively charged at acidic pH values and can interact with negatively charged proteins [13,14]. Chitosan solubility in aqueous media follows rules similar to those that apply to the solubility of proteins, depending on variables such as

Abbreviations: PEP, pepsin; Chi, chitosan; PEG600 and PEG1450, polyethyleneglycol of average molecular mass 600 and 1450.

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pH, temperature and ionic strength of the dissolving medium. This polymer exhibits a pH-sensitive behavior as a weak polybase due to the large quantities of amino groups on its chain, and it dissolves easily at low pH while it is insoluble at higher pH ranges.

In this paper, continuing a previous work [12], we combined the partition in ATPS and enzyme precipitation with chitosan to isolate pepsin, an acidic aspartic protease, from bovine abomasum. This combination increased the purification factor compared to each extraction process separately. To obtain this goal, we precipitated the pepsin partitioned in the PEG-rich phase by adding this cationic natural polyelectrolyte.

There are some reports [15,16] in which different polyelectrolytes, including chitosan, have been included in the PEG phase, leading to a combined process of partition and affinity precipitation. In our case, we do not have an affinity ligand which binds specifically to pepsin. Instead of that, our aim is to eliminate the PEG from the PEG-rich phase bound to PEP by the chitosan–PEP precipitation.

2. Materials and methods

2.1. Chemical

Crystaline pepsin (PEP) (Cat.: P6887, lot.:074K77165, 3300 units/mg protein), polyethyleneglycol (PEG) of average molecular mass 600 and 1450, chitosan (Chi) – minimum 75% desacetylation grade given by the manufacturer – were purchased from Sigma and used without further purification. Chi was dissolved in acetic acid 0.1 M at a concentration of 2%.

2.2. Abomasum homogenate preparation and activation

The abomasum was removed from a recently killed bovine, washed with isotonical saline solution (sodium phosphate 50 mM pH 7.0–NaCl 100 mM) and cut in small pieces. 100 g of gastric mucosa was mixed with 300 mL of sodium phosphate buffer 50 mM, pH 7.0 and homogenized for 5 min in a Minipimmer homogenizer. The resulting homogenate was filtered, divided in 35 ten-gram aliquots and frozen at −30 °C. Before using it, total protein concentration and enzymatic activity were assayed.

The pepsinogen activation was carried out at 8 °C by slow addition of HCl 0.3N to the homogenate solution in order to diminish the pH until a value of 2.5 was reached. The mixture was allowed to rest for 30 min. Then, a concentrated NaOH solution was added until a pH 6.4 was reached [17].

2.3. Determination of PEP activity

The pepsin assay is based on the hydrolysis of haemoglobin [18]. The reaction rate was determined by measuring the increases in the absorbance at 280 nm, at 37 °C, resulting from the hydrolysis of 400 μL of haemoglobin 2.5% in HCl pH 2 by adding 100 μL of sample. The reaction was stopped 30 min later by adding 500 μL of trichloroacetic acid (TCA) 10% (w/v) and the supernatant was separated by centrifugation for 5 min at 16,500 rpm. There is no international agreement about units obtained by haemoglobin digestion method. This work used an arbitrary system whereby the amount of enzyme that causes an increase in the absorbance of the haemoglobin filtrate of 1 at 280 nm in 1 min is taken as 1 unit.

The specific activity (SA) of PEP was defined as:

$$\mathrm{SA} = \frac{\text{protease activity}}{\text{protein concentration}}\,(\text{units/mg protein}) \tag{1}$$

2.4. Determination of total protein concentration

It is based on the turbidimetric response of protein with TCA 50% (w/v). The procedure consists of mixing equal volume (1 mL) of sample solution (standard or unknown) with a solution of TCA 50% (w/v) and measuring the absorbance at 420 nm after 20 min [19].

2.5. Preparation of the aqueous two-phase system and partition of PEP from the homogenate

Two ATPS were prepared according to the binodal partition diagram [12]: (A) PEG1450, 1.86 g; 30% (w/w) potassium phosphate solution, 5.06 g; water, 1.08 g and 2 g of activated abomasum homogenate. (B) PEG600, 1.83 g; 30% (w/w) potassium phosphate solution, 5.80 g; water, 0.37 g and 2 g of activated abomasum homogenate. Low-speed centrifugation (5 min at 1700 rpm) was used after gentle mixing of the system components to speed up phase separation. Both phases were separated and PEP activity and total proteins were determined in the PEG-rich phase.

2.6. Determination of the partition coefficient (K)

Once the thermodynamic equilibrium was reached, both phases were separated and the total protein and enzymatic activity was determined in each phase. The partition coefficient was defined as:

$$K = \frac{[P]_{\mathrm{top}}}{[P]_{\mathrm{bottom}}} \tag{2}$$

where $[P]_{\mathrm{top}}$ and $[P]_{\mathrm{bottom}}$ are equilibrium concentrations of partitioned protein in the PEG and phosphate-rich phases, respectively. In the protein concentration range assayed, a plot of $[P]_{\mathrm{top}}$ vs. $[P]_{\mathrm{bottom}}$ showed a linear behavior, K being its slope.

2.7. Pepsin turbidimetric titration curves with chitosan

The formation of the insoluble polymer–protein complex was followed by turbidimetric titration. Sodium phosphate 50 mM buffer solutions (10 mL) with a fixed protein concentration were titrated at 25 °C in a cuvette with the polymer solution as the titrant. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the pH 6.5. The solution absorbance at 420 nm was used to follow the protein–polyelectrolyte complex formation and plotted vs. the total concentration of the polymer in the tube. These plots were fitted with the following hyperbolic equation, which was empirically chosen:

$$\tau = \frac{\tau_{\max}[\mathrm{Chi}]}{[\mathrm{Chi}]_{0.5} + [\mathrm{Chi}]} \tag{3}$$

where $\tau_{\max}$ is the maximum absorbance of the solution and $[\mathrm{Chi}]_{0.5}$ is the Chi concentration at half the maximum turbidity. Solution absorbances were measured using a Jasco 520 spectrophotometer with a thermostatized cell of 1 cm of path length.

2.8. PEP precipitation from the activated bovine abomasum homogenate

Aliquots with a mass of 10 g of activated homogenate were stirred and small aliquots of Chi concentrated solution were added. The system was allowed to rest for 30 min and the precipitate obtained was separated by centrifugation (4 min at 500 × g) and then dissolved by addition of 4 mL of acetate buffer, pH 3.8. The PEP activity and the total protein concentration were determined in the supernatant in the re-dissolved precipitate and in the homogenate. Systems with different Chi solution volume and constant mass of homogenate were assayed. The recovery of PEP (with respect to the initial total activity in the same mass of homogenate) and the purification factor were calculated.

The PEP activity recovery was defined as:

$$\text{yield}\,(\%) = \frac{A_{\mathrm{f}}}{A_{\mathrm{i}}} \times 100 \tag{4}$$

where A_{f} is the total protease activity after a purification step (partition or precipitation) and A_{i} is the initial biological activity in the abomasum homogenate.

The purification factor was defined as:

$$\mathrm{PF} = \frac{\mathrm{SA_f}}{\mathrm{SA_i}} \tag{5}$$

where $\mathrm{SA_i}$ is the specific activity in the abomasum homogenate and $\mathrm{SA_f}$ the specific activity after a step of purification (partition or precipitation).

3. Results

3.1. Solubility curves of Chi vs. the medium pH in the presence of PEP

Titration curves were made in order to estimate the pK_{a} of polymer amino groups and analyze how the presence of pepsin affects the polymer acid–base state. The Chi was dissolved in 0.1 M HAc; therefore, the chitosan acid–base titration curve only showed the titration of acetic acid and did not allow us to study the polymer acid–base behavior. For the same reason, the chitosan and the PEP-chitosan acid–base titration curves could be superposed (data not shown). This might indicate that the protein does not modify the polymer acid–base state in this way.

It is well known that the solubility of Chi is highly dependent on the pH of the medium because the protonation state of the Chi amine groups induces the repulsion between them. Solubility curves of Chi solution were obtained to characterize the Chi used in this work and expressed as medium absorbance as shown in Fig. 1. The medium pH variation was obtained by adding NaOH or HCl aliquots, allowing the system to equilibrate and then measuring the pH of the medium. The curve was also obtained in the presence

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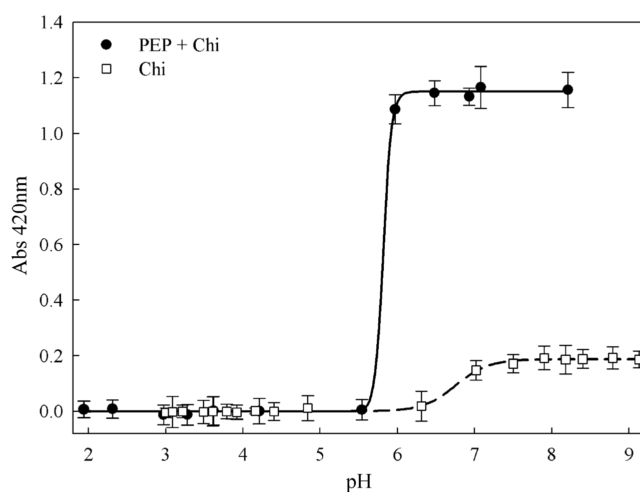

Fig. 1. Absorbance at 420 nm of Chi solution vs. the medium pH. The medium pH was varied adding increasing amounts of NaOH. Chi concentration: 0.1% (w/v). PEP concentration: 0.25 mg/mL. Temperature: 25 °C. Medium: sodium phosphate mM.

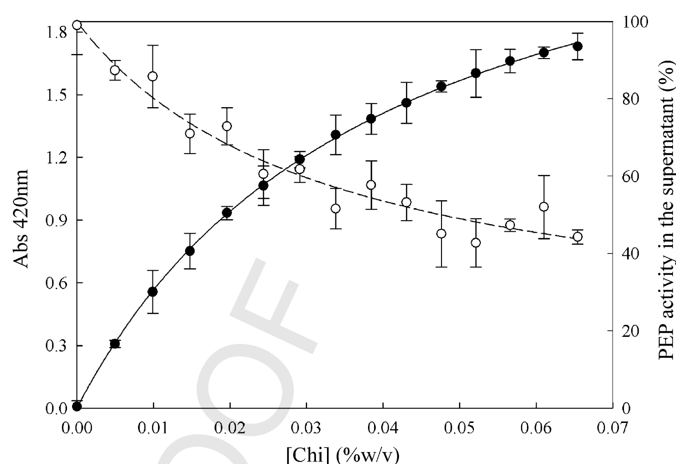

Fig. 2. Turbidimetric titration of PEP (0.25 mg/mL) with increasing Chi concentration (●), medium buffer phosphate 50 mM, pH 6.5, and temperature 25 °C. PEP activity in the supernatant (○) expressed as percentage relative to the initial activity (in the absence of Chi).

of protein to test any possible protein effect on the solubility curve of the polyelectrolyte. A typical sigmoidal titration curve was obtained. It can be seen that the titration curve of Chi in the presence of protein does not overlap that in the absence of PEP. This finding suggests that the presence of protein modifies the solubility behavior of Chi in a significant way. This may be due to the interaction between the Chi amine groups with the negatively charged groups of the protein and could be regarded as a proof of how strong the Chi–protein interaction is. The pH of semi-precipitation value for Chi was 6.90 but in the presence of PEP, the complexes precipitate after pH 5.82. This finding allows us to select the optimum pH of the medium to precipitate PEP (between 5.82 and 6.90). It has been postulated [10] and confirmed from our finding that the interaction of chitosan with proteins is mainly electrostatic. However, hydrophobic forces may always be involved, by participating in the chitosan aggregation and precipitation process at high pH.

3.2. Turbidimetric titration of protein with Chi

To assay the capacity of Chi to interact with the protein, a pH value of 6.5 was selected to carry out the Chi–PEP complex formation. PEP has an isoelectrical pH around 1.5, therefore, under this pH value, PEP has a net negative electrical charge value around 23. Fig. 2 shows the absorbance dependence at 420 nm when PEP is titrated with increasing concentrations of Chi at a constant concentration. Curves with a hyperbolic shape were observed. The non-precipitated protein concentration in the supernatant was shown to decrease in the same way as the precipitate was formed, which reflects the disappearance of the enzyme in the supernatant. However, at high Chi concentrations, the protein precipitation was not complete. The supernatant protein concentration curves vs. Chi concentration show a plateau that indicates that there is remaining PEP (around 40%).

From the non-linear fitting of the turbidimetric titration curve, the protein–polymer ratio which corresponds to the stoichiometry of insoluble complex formation was calculated. As it was pointed out by other reports [11], this protein–polyelectrolyte ratio corresponds to the final state of the complex in its non-soluble form. It is well demonstrated that the formation of this complex is produced by two steps [6]: first, the formation of a primary protein–polymer complex which is soluble; and second, the interaction of soluble complex particles among them to form high molecular and non-soluble aggregates which are determined by turbidimetric titration. The above stoichiometry ratio corresponds to the formation of this last state. Therefore, this value is a mean value of the PEP/Chi mass ratio in the precipitate.

The protein–polyelectrolyte ratio values estimated at the plateau of the turbidimetric curves are important because they allow us to calculate the minimal polymer amount to precipitate the protein. A high protein–Chi ratio was obtained with a value of (1.26 ± 0.02)g of PEP per polymer mass (g), which is in agreement with the results reported for the precipitation of other proteins by polyelectrolytes [20]. The mass of Chi was expressed in g because this polyelectrolyte is a natural product and a polydisperse polymer. Also, the stoichiometry ratio was calculated by measuring the PEP concentration in the supernatant and the PEP bound to Chi was obtained by calculating the difference between the initial aliquot and the supernatant concentration. The value of stoichiometry ratio was (1.5 ± 0.5)g PEP/g Chi which was similar to that estimated from the turbidimetric curve.

The total Chi concentration in equilibrium necessary to precipitate the protein was in the order of 0.05–0.07% (p/v), which is a very low concentration value compared to that used in other systems. Using little amounts of polymers implies an economical advantage. Another one is the low viscosity of the medium and the lack of interference for the subsequent use of the protein. This is important because of the potential application of the method in scaling up. Therefore, this technique appears to be an excellent method to precipitate proteins due to the fact that it is non-expensive and uses limited amounts of polyelectrolyte. This is important to design scaling up methods to precipitate proteins by a polyelectrolyte because the target proteins are present in high volumes of solution, so small masses of this polyelectrolyte are necessary to precipitate the desired enzyme.

Complex precipitation is driven by a balance between the attractive and repulsive forces among the particles or chains. Van der Waals and hydrophobic interactions are attractive non-pH-dependent forces, which favor precipitation. Electrostatic repulsive forces, which oppose precipitation, are pH-dependent and are present due to the fact that the complex has a net charge. The electrostatic attraction among the complex sectors with opposite charge should be added to the attractive forces. Therefore, precipitation begins when the attractive forces just overcome the electrostatic repulsion. The results shown in Fig. 3 agree with a net electrostatic mechanism proposed for this complex formation; the increase in the NaCl concentration at level around 0.6 M inhibited the PEP–Chi complex formation completely.

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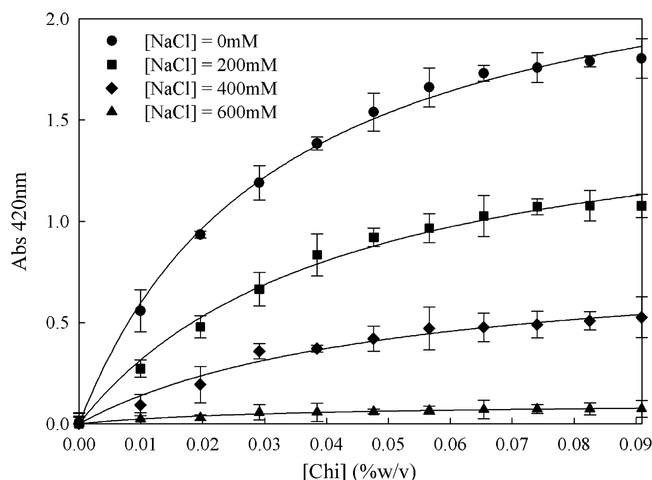

Fig. 3. Salt effect on the turbidimetric titration curve of PEP (0.25 mg/mL) with increasing Chi concentration. Medium buffer phosphate 50 mM, pH 6.5, and temperature 25 °C.

3.3. Chi effect on the enzymatic PEP activity

PEP was precipitated at pH 6.5 by Chi addition, the precipitate was dissolved at pH 3.8 and the enzymatic activity of PEP was determined. A control was carried out by the same amount of PEP. Neither a difference in the PEP activity in the presence of Chi (up to 0.1%) nor a change after 12 h was observed (data not shown).

3.4. PEP precipitation from the activated bovine abomasum homogenate

Fig. 4 shows the recovery of PEP and the purification factor vs. the Chi concentration used in the precipitation. It can be seen that, at increasing Chi total concentration, the PEP activity recovery increases up to a constant value around 60%, while the purification factor diminishes, which suggests that a greater Chi concentration induces the precipitation of other proteins.

3.5. Partition of PEP from the activated freshly bovine abomasum homogenate in ATPS

Table 1 shows the pepsin recovery and the purification factor obtained from these ATPSs. The partition of bovine abomasum

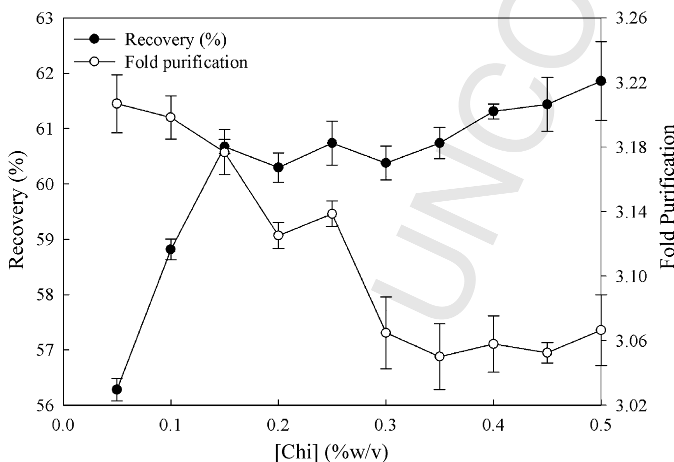

Fig. 4. Recovery of the PEP activity and purification factor from the solubilization of the PEP–Chi precipitated at different initial concentration of precipitant agent (Chi). Medium buffer phosphate 50 mM, pH 6.5, and temperature 25 °C.

Table 1
Recovery and purification factor obtained from partition in ATPSs and Chi precipitation of an abomasum homogenate.

Purification technique	Recovery (%)	Purification factor
Partition in ATPS (PEG600)	42 ± 3	2.0 ± 0.1
Partition in ATPS (PEG1450)	55 ± 2	4.0 ± 0.1
Chi precipitation (0.35%)	60 ± 4	3.0 ± 0.1
Partition in ATPS (PEG600) + Chi precipitation (0.35%)	40.1 ± 0.4	5.1 ± 0.2
Partition in ATPS (PEG1450) + Chi precipitation (0.17%)	48.5 ± 0.9	9.0 ± 0.6

homogenate in ATPS yielded 42–55% of PEP activity for systems in PEG600 and 1450, respectively; however, PEG1450 showed a better purification factor.

3.6. PEP precipitation from the top phase of ATPS

The pH of the top phases of the above ATPSs (PEG-rich phase) was changed to 6.5. Small aliquots of Chi concentrated solution were added to them. The systems were allowed to rest for 30 min and the precipitates obtained were separated by centrifugation (4 min at 500 × g) and then dissolved by addition of 4 mL of acetate buffer, pH 3.8. The PEP activity and the total protein concentration were determined in the supernatant and in the re-dissolved precipitate, and the purification factor and recovery were calculated as shown in Table 1. It can be seen that the PEP % recovery value did not change with the precipitation with respect to the partition in ATPS; however, a significant increase in the purification factor value was observed. The system composed by PEG600 showed a purification factor of 2, while this value increased to 5 with the precipitation. In the case of the ATPS formed by PEG1450 the purification factor increased from 4 to 9.

4. Discussion

The first step to apply the ATPS method in the isolation of an enzyme is to assay the pure enzyme in different biphasic systems. The enzyme recovery obtained is very high (between 80 and 95%). However, when the same method is applied to the purification of this enzyme in a complex mixture (i.e. an animal or a plant tissue), the recovery observed is lower. Rito-Palomares [21] explained this recovery loss due to the presence of other macromolecules (proteins and nucleic acids) which lead to the macromolecule–macromolecule interaction inducing the target enzyme precipitation at the interphase. One disadvantage of the use of the ATPS in the purification is that when the target enzyme has high affinity for the PEG-rich phase (due to the high interaction between the enzyme and PEG) the PEG bound to the macromolecule is very difficult to eliminate. In a previous paper [22] we studied the partition of PEP in ATPS of PEG–phosphate, and we found an anomalous high affinity of this aspartic protease for the PEG-rich phase, so it is important to find a method to solve the problem of the elimination of PEG from the PEP solution.

Nowadays, the interest in Chi has increased to design downstream processing methods in the biotechnological separations, due to the fact that Chi is non-expensive and has mild properties for the macromolecules.

Our finding suggests that the presence of a protein significantly modifies the solubility of Chi in an aqueous medium according to a coulombic mechanism of interaction, being a proof of the interaction between the amino groups of Chi and groups with high negative density of change in the protein (carboxylic groups). The final consequence is a significative modification in the Chi solubility with the pH.

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Some authors [23,24] have postulated different theoretical models to describe the protein–polyelectrolyte complex formation. However, from these models it is difficult to predict the behavior of the system, which makes it necessary to determine the experimental values of variables in a practical way.

Polyelectrolyte precipitation method uses very low polymer concentration, in our case a maximal Chi total concentration of 0.07% in the medium was necessary to precipitate the maximum amount of PEP, however, when this method was applied to an abomasum homogenate, around 5-fold Chi concentration was necessary to obtain the maximal precipitation of PEP from the homogenate. In this case, part of Chi reacts with the DNA present in the homogenate forming insoluble complexes which precipitate with the PEP–Chi complex.

The use of Chi precipitation for enzymes already partitioned in top phase of ATPS increased the purification factor compared to each extraction technique separately and it is useful to eliminate PEG from PEP solution. The results obtained in this work are important since we got a greater purification factor and recovery yield of PEP than other authors [25,26].

PEP preserves its biological activity after the ATPS partition and the Chi precipitation, even when the enzyme was in the presence of polyelectrolyte for some time in the solution. These findings show that a combination of both extraction techniques: ATPS partition and Chi precipitation is a potential framework to be applied in the isolation of different enzymes or other macromolecules from a natural source. The use of a non-toxic and natural polymer such as chitosan adds another favorable quality to this methodology.

However, it is necessary to develop experimental measurements to know how the experimental variables of the medium have an influence on the complex formation and dissolution. Some of the factors which can affect the protein–polyelectrolyte interaction are: the polymer and protein charge densities [6,7,24] (both related with the pH, the isoelectrical pH of the protein and the pK_a of the polymer), the ionic strength [8], the presence of salts which modifies the structure of the ordered water [11], temperature, the molecular weight of the polymer [9], the surface hydrophobicity of the protein and the presence of different ions which specifically affect the solubility of the polymer [27].

In this paper, we have used bovine pepsin as a model protein to assay the combination of the partition–precipitation methods due to its biotechnological importance. However, as it can be seen from the present results, the methodology is not specific for pepsin and can be applied to other macromolecules with low isoelectric pH.

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