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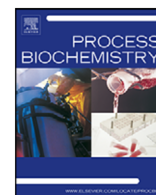

Short communication

Precipitation with poly acrylic acid as a trypsin bioseparation strategy

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ABSTRACT

Precipitation of enzymes with reversible soluble–insoluble polymers is a simple approach which can be easily scaled up. This work reports investigations aiming at verifying the existence of specific interactions and complex formation between porcine trypsin and poly acrylic acids using spectroscopy techniques. The trypsin–polymer complex was insoluble at pH lower than 5, with a stoichiometric ratio polymer mol per protein mol of 1:148. It took only a minute for the insoluble complex to form and it was redissolved modifying the pH of the medium. The enzymatic activity of trypsin was maintained even in the presence of the polymer and after precipitation poly acrylic acid presence protect the enzyme from itself degradation. The conditions of complex formation were studied using pure proteins that could be applied on porcine pancreas homogenates as an isolation strategy of trypsin.

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1. Introduction

The interaction between proteins and natural and synthetic polymers has been extensively studied, in particular for the modulation of living processes, immobilization or stabilization of enzymes, modification of substrate affinity, changing properties of food products, and for the development of many pharmaceutical applications [1–3].

Precipitation finds a place in most protein purification protocols and has traditionally been applied as a simple and rapid technique for protein concentration at the beginning of downstream processing [4].

A wide variety of synthetic and natural polyelectrolytes can interact with globular proteins to form stable protein–polyelectrolyte complexes (PPC) that result in the formation of soluble or insoluble complexes. The insoluble complex can be easily separated by centrifugation or simple decantation [5,6].

Precipitation as a product concentration step offers several advantages in that it is easy to scale up, uses simple equipment and can be based on a large variety of alternative precipitants [7,8]. When PPC is specifically formed with one of the proteins in the crude extract followed by a phase separation, the process could be used as a convenient strategy for the isolation and purification of the target protein [9].

Trypsin is a serin protease found in the digestive system. It is used for numerous biotechnological processes. It is produced in the pancreas as an inactive zymogen, trypsinogen [10].

We have used spectroscopic techniques to obtain information about the molecular mechanism of interaction between trypsin and a negatively charged polyelectrolyte (poly acrylic acid) with the aim of applying this information to the polyelectrolyte–protein complex formation as a tool for protein separation from porcine pancreas.

2. Materials and methods

2.1. Chemical

Trypsin (TRP) from porcine pancreas and α-*N*-benzoyl-DL-Arginine-*p*-nitroaniline (BAPNA) was purchased from Sigma Chem. Co. (USA) and poly acrylic acid, sodium salt (PAA), 25 w/w% sol. in water molecular average mass 240 kDa, were purchased from Aldrich and used without further purification. Phosphate buffer solutions of different pH were prepared at concentration of 50 mM. They were adjusted with NaOH or HCl in each case.

2.2. Turbidimetric titration curves vs. pH

Three different molar ratios PAA/TRP were selected. They were titrated with alkali and acid, the absorbance at 420 nm was plotted vs. pH. These phase diagrams show the pH range where the polymer–protein complex is soluble or insoluble.

2.3. Tripsin turbidimetric titration curves with polymers at different ionic strengths

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration [11,12]. Buffer sodium phosphate solutions (pH 3.00) with a fixed protein concentration were titrated at 20 °C in a cubic 1 cm path-length glass cell with the polymer solution as the titrant (0.20 w/w%). 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

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at 420 nm vs. polymer/protein molar ratio in the absence and presence of different ionic strengths adding NaCl to phosphate buffer.

The stoichiometric protein/polymer ratio 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. This corresponds to the situation where the protein has been precipitated as an insoluble complex with an minimal amount of PAA.

2.4. Enzyme assays

Trypsin activity was determined using the substrate α-*N*-benzoyl-DL-Arginine-*p*-nitroaniline (BAPNA) applying a method modified from Gildberg and Overbo [13]. BAPNA was used in the assay at a final concentration of 0.85 mM in 50 mM buffer phosphate pH 7.0. The reaction was followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10,500 M^{-1} cm^{-1}) for 5 min. The activities were calculated from the slope of initial linear portion of the absorbance vs. time curve.

The enzyme assays were performed at a constant temperature of 20 °C in the presence and absence of PAA. Three different PAA/TRP molar ratios were chosen from the plateau of titration curves: 0, 1:132, 1:106 and 1:90.

In order to evaluate the activity of the enzyme in the presence of the polymer, TRP was incubated in PAA and the activity was measured for 24 h. The molar ratio selected was 1:132.

2.5. TRP precipitation with PAA

A solution of PAA and TRP at a ratio of 1:132 mol of polymer per mol of protein in buffer Pi 50 mm of pH 3.0 was prepared. The precipitate formed was incubated for 30 min at 20 °C and centrifuged at 3500 rpm for 10 min. The supernatant of the precipitate was separated. Then, the precipitate was redissolved in two different ways: by addition of phosphate buffer with NaCl 1 M and by addition of phosphate buffer solution of pH 7.00 [14]. The enzymatic activity in the supernatant and in the redissolved precipitate was measured.

3. Results

3.1. pH effect on the complex formation

Trypsin 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 [10]. Therefore, at the pHs where the turbidimetry titration was assayed, the protein has a net positive electrical charge. The formation of a TRP–PAA complex was observed to be influenced by the pH medium. Fig. 1 shows the pH variation effect on the insoluble complex formation obtained for different PAA–TRP molar ratios. The increase of pH above 5 induced a dramatic decrease in the maximum absorbance values, which suggested a minor amount of complex formation [15]. From these curves, the optimum pH interval in which the polymer–protein complex is insoluble was determined.

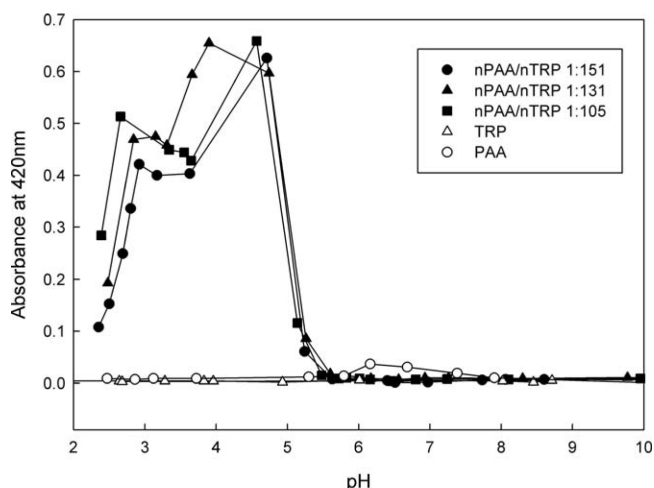

Fig. 1. Phase diagrams: dependence of the absorbance at 420 nm vs. the medium pH at a constant protein/polymer molar ratio of TRP–PAA. Temperature 20 °C.

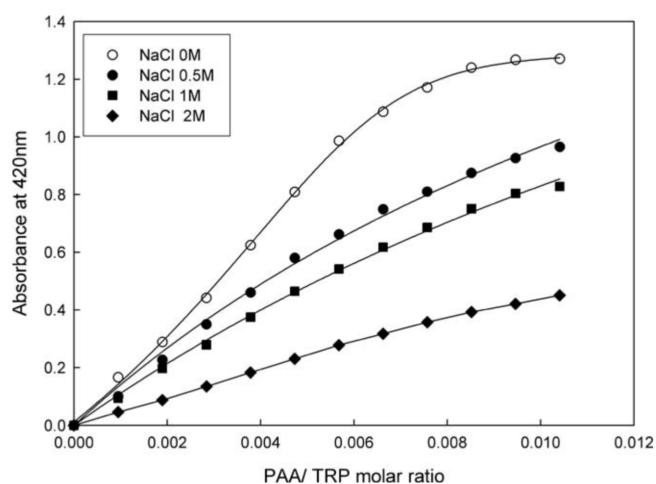

Fig. 2. Salt effect on the turbidimetric titration curves of TRP (70 μM) with PAA, pH 3.00. Temperature 20 °C.

3.2. Ionic strength effect on complex formation

Fig. 2 shows the trypsin turbidimetric titration curves with PAA at different ionic strengths. The insoluble complex formation was dramatically affected by ionic strength (0.5 M or higher) and it was directly proportional to the salt concentration, consistent with the presence of an important coulombic component in the insoluble complex formation [6].

This finding may be interesting because it is the basis of the protein isolation method which allows precipitation using charged polymers, followed by the dissolution of the precipitate by the addition of a NaCl solution at low concentration. However, in this case, NaCl 2 M was not enough to inhibit the complex precipitation. For this reason, the complex was dissolved by a change in pH.

3.3. Time of TRP–PAA complex formation

The time needed to form the complex was evaluated measuring the time required to obtain the maximal absorbance of the highest molar ratio of polymer protein: 1:132. The experiment shows that only 1 min is needed for the complex to form (data not shown).

3.4. TRP biological activity in the absence and presence of PAA

Table 1 shows the biological activity of TRP in the absence and presence of different concentrations of polymer. The polymer–protein ratios assayed were 1:132, 1:106 and 1:90. These three molar ratios correspond to an excess of PAA. We chose them to verify the effect of the polymer on the enzymatic activity. Although the presence of the polymer decreases the enzymatic activity when the protein is bound to the polymer, the results demonstrate that the activity is maintained even in the presence of excessive amount of polymer.

These results show that at molar ratio PAA:TRP 1:132, the enzyme activity is practically the same either in the absence or

Table 1
Enzymatic activity of TRP at three different molar ratio PAA:TRP.

PAA/TRP molar ratio	Enzymatic activity TRP (UI)	% enzymatic activity
0 (TRP alone)	$2.80 \times 10^{-3} \pm 1.5 \times 10^{-4}$	100
1:132	$2.75 \times 10^{-3} \pm 1.4 \times 10^{-4}$	98.2
1:106	$1.63 \times 10^{-3} \pm 8.0 \times 10^{-5}$	58.2
1:90	$1.62 \times 10^{-3} \pm 8.0 \times 10^{-5}$	57.8

% activity calculated regarding TRP in phosphate buffer pH 7.00, 50 mM.

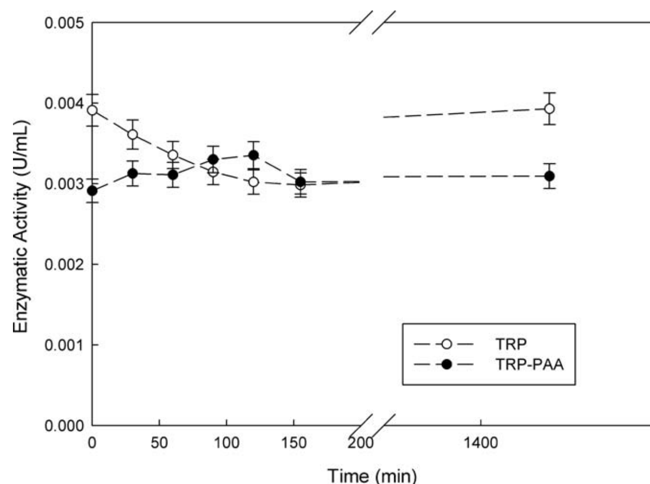

Fig. 3. Biological activity of TRP (25 μM) in the absent and the presence of PAA, molar ratio PAA/TRP 1:132. Temperature 20 °C.

presence of the polymer. This shows that the formation of the PAA–TRP complex does not significantly alter the catalytic ability of the enzyme when there is excess of polymer.

3.5. TRP biological activity through time, in the absence and presence of PAA

Fig. 3 shows the biological activity of TRP in the presence of PAA at a polymer/protein ratio of 1:132. Although the precipitation protein–polymer ratio is 148:1, we work in excess of polymer to make sure that all the protein is precipitated. We choose the ratio 132:1 because the protein maintains the 98% of its activity, even in presence of PAA.

The enzyme activity can be seen to remain at a temperature of 20 °C for about 24 h. This high polymer–protein ratio was chosen to verify the polymer presence effect on trypsin through time. For this reason, in the following experiments the same ratio was assayed.

3.6. TRP precipitation by PAA–protein insoluble complex formation

A medium containing TRP (25 μM) in 2 mL sodium phosphate buffer, 50 mM pH 3.00, was precipitated adding 65 μL PAA (0.20 w/w%). The precipitate formed was incubated at 20 °C for 5 min and centrifuged at 3000 rpm for 10 min. The precipitate was dissolved in pH 7.00 phosphate buffer. The protein concentration was determined in the supernatant and in the dissolved precipitate by trypsin activity. Control and test curves were also carried out. Table 2 shows the TRP recovery after precipitation. The recovery of the enzymatic activity of TRP was considered to be around 49% in the redissolved precipitate and only 3.28% in the supernatant.

4. Discussion

We have carried out an experimental study on protein titration using a polyelectrolyte. Our experimental findings agree with other authors who have shown that the complex formation is strongly dependent on the pH and the presence of salts [16]. Although protein precipitation with polyelectrolytes has been studied for many years, few theoretical studies have been directed toward understanding the mechanism of precipitation [17].

Oppositely charged polyelectrolytes have been used to selectively precipitate proteins from an aqueous mixture on the basis of different affinities [18]. This study showed that TRP could interact with poly acrylic acid and form either soluble or insoluble complexes depending on the solution pH, ionic strength, etc. Insoluble complexes were formed at pH values where the protein and polymer had opposite electrical charges (pH 2.5–5) because of a strong electrostatic attraction between the two biopolymers.

In turbidity assays, it was shown that 148 protein molecules are bound to a polymer molecule. The low ratio polymer–protein values found suggested that one polymer molecule is bound to many protein molecules according to the model proposed by Kokufuta and co-workers [19], where one polymer molecule has the capability to interact with many protein molecules.

The presence of high ionic strength of 2 M could not redissolve the complex completely. For this reason, the precipitate was redissolved by adding phosphate buffer pH 7.00.

Fig. 3 shows that the activity in the presence of PAA is lower than in its absence; however the variation of the activity through time is lower in the presence of the polymer. For this reason, PAA protects the enzyme from itself degradation and make it more stable. It can be demonstrated that similar polyelectrolytes give stability to the protein through time [20].

Table 2 shows that the enzymatic activity changes in presence of the polymer and its determination is more affected in the redissolved precipitate where there is a high concentration of polymer with regard to supernatant. Activity percentages were calculated with respect to a control of TRP in phosphate buffer. As these media cannot be reproduced to make a calibration curve, it is not possible to carry out activity balances. However, the determination of the TRP activity in both fractions demonstrates a high tendency of it to precipitation.

Redissolved precipitate contains both the protein and the polymer. The removal of the later requires an experimental procedure such as molecular exclusion chromatography, ultrafiltration, etc. However, removal of the polymer depends on the subsequent application of the protein. Trypsin is involved, for example, in the process of leather softening. In this case, there is no need to remove the polymer.

This method could be suitable for the precipitation of the protein from its natural source. Unlike other traditional protein isolation methods, it has the advantage of concentrating the sample.

Table 2
Enzymatic activity recovery after precipitation of TRP with PAA.

PAA/TRP molar ratio	Enzymatic activity TRP (UI)	% activity
Buffer Pi, pH 7.0	$1.8 \times 10^{-3} \pm 1 \times 10^{-4}$	100
Supernatant	$5.9 \times 10^{-5} \pm 2 \times 10^{-6}$	3.3
Redissolved precipitate	$8.8 \times 10^{-4} \pm 1 \times 10^{-5}$	48.8

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