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Short Communication

Obtainment of a highly concentrated pancreatic serine proteases extract from bovine pancreas by precipitation with polyacrylate

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ABSTRACT

Serine proteases have wide application in leather, food, meat and soap powder industries, among others. There are a lot of methodologies to obtain them in large quantities but most of them are expensive or contaminating. We then propose an economical and environmentally friendly method in which proteases are separated from a crude fresh bovine pancreas homogenate using precipitation with polyacrylate, a commercially available negatively charged and weak polyelectrolyte. The zymogens of the serine proteases were activated prior to precipitation by the addition of trypsin. The proteases were precipitated by adding polyacrylate at pH 4.50 to the pancreas homogenate. Under these conditions, serine proteases are positively charged and form non-soluble complexes with the negatively charged polyacrylate. The purity of proteases was increased 5-fold with a recovery of 33% under the best conditions tested. The volume of the final product was decreased to 5% of the feedstock, in order to concentrate the sample up to 20 times. The proposed method removed up to 96% of the contaminant proteins.

1. Introduction

Meat industries are very important in our country. Therefore, great amounts of meat waste are produced. One of these products is bovine pancreas: thousands of kilos of fresh pancreas are discarded daily, contributing to environmental pollution. However, there is an increasing commercial interest in the pancreas since it is very rich in enzymes such as proteases of wide application in numerous biotechnological processes [1–3]. Proteases are among the most important hydrolytic enzymes and have been studied extensively [4].

Due to their activity and stability at alkaline pH, serine proteases are of considerable interest and have applications in different types of industries [5]: pharmaceuticals, diagnostic reagents, leather, food, meat and soap powder industries, among others.

The three serine proteases present in bovine pancreas are trypsin (Tryp), chymotrypsin (ChTRP) and elastase. In order to use these enzymes it is necessary to isolate them and to preserve them properly to keep their functionality. There are a lot of methodologies to obtain these serine proteases in large quantities but most of them are expensive or generate byproducts that are not suitable to be discarded [6–10]. We have proposed different methods to isolate ChTRP [11–14], Tryp [15,16] and their zymogens [17,18]. However, it is also interesting to obtain a concentrated extract with protease activity (containing a mixture of the above mentioned) to be applied to many industries including the production of food products, leather, meat, soap powder, waste management and silver recovery where the different specificities are not needed.

Conventional multistep downstream processing of enzymes is often time consuming, labor intensive, requiring huge hold-up volumes to operate in batch or semibatch mode and usually accounts for up to 80% of the total process costs [6,18–20]. Some of the traditional methods suitable to be applied in scaling up are ion exchange adsorption [21] or chromatography [22]. However, chromatography is the single largest cost center in downstream processing and also the yield of a chromatography is usually low. Thus, alternatives to chromatography are an attractive option even if only a reduction in the extent of use of packed beds can be realized.

The introduction of expanded bed adsorption has allowed early process integration by protein sequestration from a crude feedstock, but reduces the overall system dynamic binding capacity for the target product due to drawback in maintaining an appropriate (close to plug-flow) hydrodynamic condition of the fluidized bed. The chromatographic resins exhibit several major limitations due to slow intra-particle diffusion within the porous beads which tend to limit the dynamic binding capacity of the resins to capture desired target product, the column design reduces throughput as a result of the increased pressure drop at higher flow rates and high material and operational cost.

As a consequence, the separation and purification schemes applied for the purification of enzymes require alternative

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operations to replace traditional packed or expanded-bed column resins. It is necessary to perform a biotechnological process consisting of as few steps as possible, to reduce the total mass and volume of the sample to obtain an extract rich in enzymes that can be freeze-dried [23,24].

Serine proteases have basic isoelectric pH values: 10.4 (Tryp), 8.7 (ChTRP) and 9.1 (elastase). This property opens up the possibility of using a purification technique which makes use of the positive charge of these enzymes in acid media. Charged proteins interact with polyelectrolytes (PE) to form soluble or non-soluble complexes, according to the experimental conditions of the medium. By changing these conditions, such as pH or ionic strength, the protein can be released, thus keeping its secondary and tertiary structure as well as its biological activity. Synthetic polyelectrolytes such as polyacrylate (PAA) have been used to precipitate proteins as an isolation method. PAA is not toxic; it is used in the pharmaceutical industry as a basis for preparing different edible products.

In this study, we proposed the precipitation of proteases with PAA from fresh pancreas as a generic, simple, low-cost, fast and scalable basic operation, which may be useful as first step in a purification method to concentrate the main serine proteases from its natural source, the bovine pancreas.

2. Materials and methods

2.1. Chemicals

Crystallized hemoglobin, polyacrylate sodium salt (35% w/v), benzyl-L-tyrosine ethyl ester (BTEE), crystallized Tryp and ChTRP were purchased from Sigma–Aldrich and used without previous purification. All others reagents were also analytical grade.

2.2. Bovine pancreas homogenate preparation

The pancreas from a recently killed bovine was removed, washed with isotonic saline solution, cut in small pieces, mixed with $CaCl_2$ solution in final concentration of 50 mM and homogenized for 5 min. The resulting homogenate was divided in aliquots and frozen at −30 °C.

2.3. Serine proteases activation from fresh homogenate pancreas

Since serine proteases are produced as zymogens in the pancreas (chymotrypsinogen, trypsinogen and proelastase), an activation step is required. The activation of zymogens was initiated by adding a small aliquot of Tryp (0.0001%, w/w) to the pancreas homogenate (medium: 45 mM $CaCl_2$, 90 mM Tris–HCl buffer, pH 8.20). The time required to complete the activation process was determined by measuring the protease activity at different intervals until a maximal value was reached.

2.4. Solubility diagram of polyacrylate–proteases mixtures

Turbidity (absorbance at 420 nm) of solutions of 0.5 g/L PAA and different final concentrations of mixtures of Tryp and ChTRP (1:1 weight ratio) was measured and plotted against pH. The pH variations were obtained by the addition of NaOH or HCl aliquots and leaving the system to equilibrate before measuring the turbidity. These titration curves were performed in order to estimate the pH at which the system composed by proteases and PAA becomes insoluble and to analyze how the different concentrations of proteases affect the soluble–insoluble pH range.

2.5. Turbidimetric titration curves of serine proteases with PAA

Solutions of different concentrations of Tryp and ChTRP (1:1 weight ratio) in 50 mM acetate–phosphate buffer were titrated at 25 °C with a 50 g/L PAA solution. The pH of all the solutions was properly adjusted to avoid changes during titration. The increase in the absorbance at 420 nm of the mixture is related to the PAA–protease insoluble complexes formation and was plotted vs. the total concentration of PAA in the tube. The amount of proteases both in the supernatant and in the redissolved precipitate was determined by measuring their proteolytic activity.

2.6. Determination of the ionic strength effect on serine protease complex solubility

Mixtures of proteases (0.5 mg/mL) and PAA (0.5 g/L) at three different pH values: 3.50, 4.00 and 4.50 were prepared. Each mixture was added with NaCl and the turbidity of the system was measured.

2.7. Determination of the effect of pH on the proteases precipitation effectiveness

PAA (0.5 g/L) was added to bovine pancreas homogenate at 3 different pH values: 3.50, 4.00 and 4.50. After a 1 h incubation, the decanted precipitate was separated from the supernatant and redissolved by addition of Tris–$CaCl_2$ buffer pH 8.20. The proteolytic activity was determined in the redissolved fraction. To calculate the percentage of recovery of proteases, the proteolytic activity in the bovine pancreas homogenate was also determined.

2.8. Determination of total protein concentration

Total protein concentration was determined using the bicinchoninic acid assay [25]. A fresh standard working reagent (SWR) was prepared mixing 100 vol of reagent A (Bicinchoninic acid solution purchased from Sigma–Aldrich) with 2 vol of reagent B (4% $CuSO_4$ solution prepared from $CuSO_4{\cdot}5H_2O$ purchased from Sigma–Aldrich). A volume of 50 μL of protein solution (maximum concentration of 1 mg/mL) was added to 1 mL of SWR. The tubes were incubated at 37 °C for 30 min. After leaving them to cool at room temperature, the absorbance was measured at 562 nm using a 1 cm of path length cell. The calibration curve was performed using dilutions of a standard BSA solution of 1 mg/mL.

2.9. Determination of protease activity

Protease enzymatic activity was estimated using hemoglobin as substrate. 250 μL of hemoglobin solution (50 g/L of hemoglobin in HCl pH 2.00) and 250 μL of 400 mM Tris–$CaCl_2$ buffer pH 8.20 were mixed in test tubes and added with an appropriate volume of the sample. After 30 min of incubation at 37 °C, 500 μL of 100 g/L TCA was added. The suspension was centrifuged at 2000*g* for 15 min. The supernatant was separated and its absorbance at 280 nm was measured to determine the free tyrosine residues.

ChTRP assay is based on the hydrolysis of BTEE [6]. The reaction rate was determined by hydrolysis of 0.6 mM of BTEE in 100 mM Tris–$CaCl_2$ buffer pH 8.20. The production of benzyl–tyrosine was measured by monitoring the increase in Abs at 256 nm every 2 s for 3 min. One ChTRP unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction and was calculated with the following equation: $U\ (\mu\text{mol/min}) = (\Delta A\ \ 256\ \text{nm/min}) \times 1000 \times 1/964$, where 964 is the benzyl-tyrosine molar extinction coefficient.

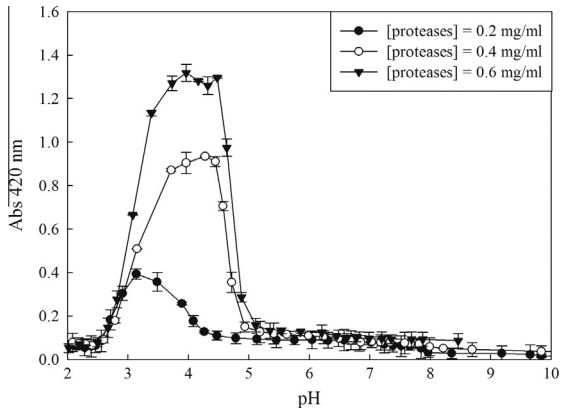

Fig. 1. Solubility diagrams of PAA–proteases. Medium: 50 mM sodium acetate – phosphate buffer. Temperature: 25 °C. PAA concentration: 0.5 g/L. Error bars correspond to the standard deviation of three independent determinations.

3. Results and discussion

3.1. Solubility diagram of polyacrylate–proteases mixtures

As a starting step to carry out this study, a mixture of the two main serine proteases present in fresh bovine pancreas (Tryp and ChTRP) was prepared. This step is useful to find out the experimental conditions needed to reach precipitation of the proteases. pH effect on the macroaggregates formation was assayed using different concentrations of a mixture of proteases with a fixed PAA concentration. The pH of the medium was varied by the addition of HCl or NaOH as shown in Fig. 1. In the pH range where the acid–base turbidimetric titration was assayed, these serine proteases have a net positive electrical charge. The formation of the insoluble protease–PAA complexes was dependent on the pH of the medium. The increase of pH above 3 induced an increase in the absorbance of the medium which is consistent with the formation of non-soluble protease–PAA aggregates. Below this pH, the PAA is protonated and the polyacrylic acid does not interact with the serine proteases to form non-soluble complexes. Around pH 5 the turbidity significantly decreased, that would suggest the solubilization of the complex. The maximum absorbance value was observed between pH 3 and 5 and that value was related to the concentration of the proteases in the media.

3.2. Turbidimetric titration curves of serine proteases with PAA

Fig. 2 shows a turbidimetric titration curve of the mixture of serine proteases with PAA. At different PAA concentrations the precipitate was separated and redissolved by changing the pH to 8.20. Then, the proteolytic activity was measured as shown in Fig. 2. This plot shows a typical titration curve of a protein interacting with a polyelectrolyte; two important characteristics were observed: at low PAA/protease ratios, absorbance increases with an increase in the PAA total concentration but at high PAA/protease ratios there is a plateau, indicating a saturation of the precipitation capability.

An increase in the proteolytic activity in the redissolved precipitate can be detected as the PAA concentration increases. The amount of precipitated proteases seems to be related to the turbidity of the media due to the PAA–proteases aggregation, as it was previously demonstrated [26]. On the other hand, a decrease in the serine protease activity in the supernatant is observed as turbidity increases. It should be noted that precipitation reaches the best performance when the PAA concentration is higher than 0.5 g/L.

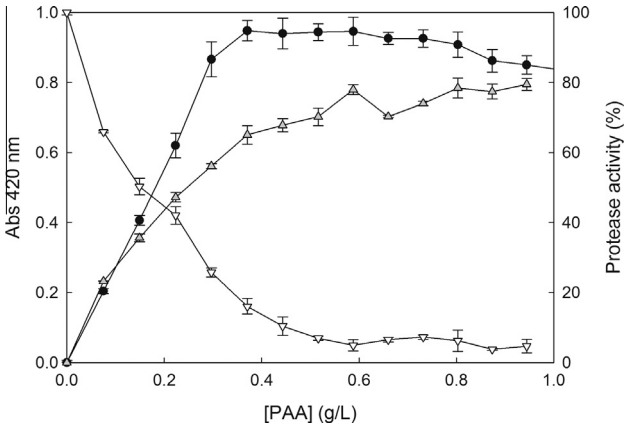

Fig. 2. Titration of serine proteases with PAA. Turbidity (●) is shown on the left *Y* axis, activity in the supernatant (▲) and activity in the redissolved precipitate (▼) are shown on the right *Y* axis. Serine proteases concentration: 0.5 mg/mL. Medium: 50 mM acetate buffer, pH 4.50. Temperature: 25 °C. Error bars correspond to the standard deviation of three independent determinations.

3.3. Ionic strength effect on protease–PAA complexes solubility

Fig. 3 shows the NaCl concentration effect on the solubility of proteases-PAA mixtures at different pH. Complexes solubility was dramatically increased by the salt presence at pH 4.00 and 4.50. The presence of NaCl 200 mM at pH 4.50 produces the complete solubilization of protease–PAA aggregates. However, at pH 4.00, complete solubilization only occurs from 300 mM NaCl. At pH 3.50, the increase in the salt concentration slightly affects the PAA–protease solubility. This may be due to the low electrical charge density of PAA at this pH value. This finding is consistent with an electrostatic mechanism of interaction between the polyelectrolyte and the proteases. This is the basis of the protein isolation method that allows precipitation using charged polymers, followed by the dissolution of the precipitate by the addition of a NaCl solution [27].

3.4. pH effect on the proteases precipitation effectiveness

In order to determine the best pH conditions to recover Tryp and ChTRP by means of precipitation with PAA, we assayed the precipitation of these proteases in different media. The PAA concentration chosen to precipitate the proteases was 0.5 g/L since

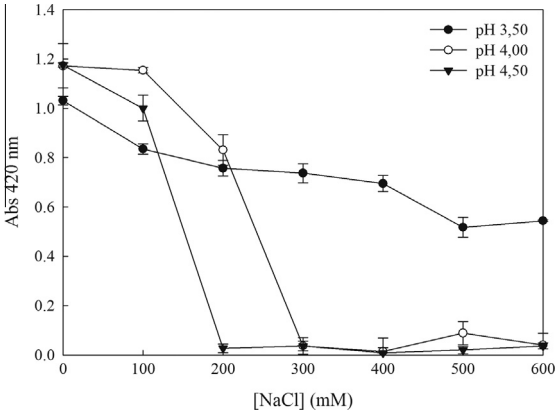

Fig. 3. Effect of the ionic strength on the solubility of the serine proteases (0.5 mg/mL) – PAA (0.5 g/L). Medium: 50 mM acetate buffer, pH 4.50. Temperature: 25 °C. Error bars correspond to the standard deviation of three independent determinations.

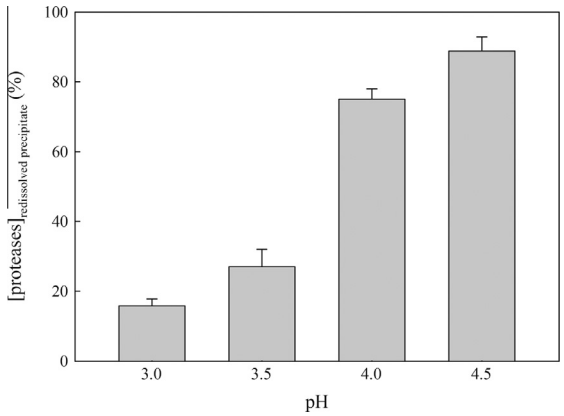

Fig. 4. Effect of the pH of precipitation on the recovery of the proteolytic activity from a 0.5 g/L PAA-precipitate of serine proteases. Error bars correspond to the standard deviation of four independent determinations.

this amount of PAA in the media was enough to assure the quantitative precipitation of the proteases, as shown in previous titrations. The pH range assayed was from 3.00 to 4.50 since it was determined to be the appropriate one to precipitate proteases through solubility diagrams (Fig. 1). Fig. 4 shows the recovery of the proteolytic activity depending on the pH of the precipitation. In all cases, the precipitate was redissolved in 100 mM Tris–HCl buffer, pH 8.20. The highest recovery of proteases was obtained by precipitating them at pH 4.50, but decreased as the acidity of the media increased. This may be due to the increasing PAA negative charge as the pH of the media increases, thus its interaction with positively charged proteases becomes more favorable.

3.5. Effect of PAA on the protease enzymatic activity

It is well documented that the presence of polymers in the media may affect the biological activity of many enzymes [28]. Therefore, the following step in the design of an isolation method using polyelectrolyte precipitation is to verify the applicability of the method. To achieve this goal, it is necessary to analyze whether there is any modification of the enzyme biological activity that is intended to be isolated in the presence of the polyelectrolyte. The enzymes were incubated during 1 h at pH 8.20 in a medium with different PAA concentrations and their biological activity was determined. The presence of PAA affects the proteolytic activity in different ways. Fig. 5 shows the effect of PAA on the biological activity of ChTRP. It can be seen that ChTRP lost around 30% of its activity in the presence of 0.7 g/L PAA; the decrease in the ChTRP activity is related to the PAA concentration, i.e., as the amount of PAA in the medium is higher, the loss of the activity increases. However, PAA has no significant effect on the Tryp activity ($p = 0.34$). This means that precipitation with PAA can be used as an isolation method of these proteases taking into account that there will be a loss in the recovery of ChTRP.

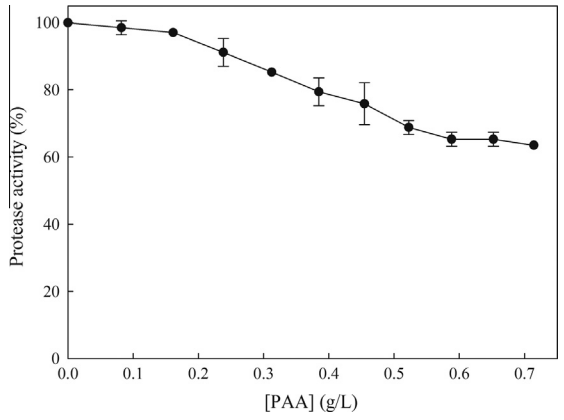

Fig. 5. PAA effect on the biological activity of chymotrypsin. Medium: 50 mM phosphate buffer, pH 8.00. Error bars correspond to the standard deviation of four independent determinations.

Table 1
Purification of serine proteases from bovine pancreas by means of precipitation with PAA. 0.5 g/L (extract 1) and PAA 2.5 g/L (extract 2).

	Homogenate	Extract 1	Extract 2
Volume (mL)	50	2.5	2.5
[Total protein] (mg/mL)	22 ± 3	128 ± 4	32 ± 1
Total protein amount (mg)	1100 ± 10	320 ± 1	81 ± 3
Protease activity (U)	6000 ± 200	3400 ± 100	1990 ± 20
[proteases] (mg/mL)	2.51 ± 0.08	28 ± 1	16.6 ± 0.2
Proteases amount (mg)	126 ± 4	70 ± 2	41.4 ± 0.4
Proteases purity (%)	11 ± 2	22 ± 1	51 ± 3
Proteases recovery (%)	100	56 ± 4	33 ± 1
Fold purification	1	1.9 ± 0.4	5.0 ± 1

3.6. Recovery of proteases from its natural source

Using the above determined conditions, the method was assayed for the recovery of proteases from a fresh bovine pancreas homogenate without previous treatment. It has been reported that the polyelectrolyte concentration to precipitate a pure enzyme in solution is very different from that needed that to precipitate the same enzyme in a complex mixture [12,14]. In the latter case, a much higher polyelectrolyte concentration is required due to the presence of other proteins having the same electrical charge as the target enzyme that consume polyelectrolyte. Taking this into account, the precipitation was assayed with different PAA concentrations ranging from 0.25 to 5 g/L. The precipitate obtained was redissolved, brought to pH 8.20 and the total protein concentration and proteolytic activity were determined. Different redissolution volumes were tested and in all the cases no difference was observed in the purification factor and recovery of proteases.

Table 1 shows the results obtained by redissolving the precipitate in a volume representing a 5% of the initial homogenate volume. This means that the obtained extract is highly concentrated; in addition, the method allows the removal of the contaminant proteins up to 96% and the purification of proteases up to 5 times.

The homogenate and both fractions corresponding to the redissolved precipitated (extracts 1 and 2) were analyzed by means of SDS–PAGE (included as supporting information). The purity increase of the proteases is checked in the gel. The main protease bands that can be observed in the gel in the lanes where the precipitate fractions were run are those corresponding to chymotrypsin and trypsin. This may be due to two reasons: (1) the precipitation conditions were determined using pure ChTRP and Tryp (not elastase) and (2) elastase level in pancreas is lower than the level of the other two proteases.

4. Conclusion

In this work, we used the capability of PAA to interact with positively charged enzymes like pancreatic serine proteases (Tryp, ChTRP and elastase) to form non-soluble macro-aggregates that can be redissolved by changing the pH. This purification strategy was used to recover proteases from bovine pancreas homogenate. Through a simple precipitation step by the addition of PAA and

after sedimentation of the enzyme–PAA aggregates, the solid can be separated and the proteases recovered by solubilization of the complex. One advantage of using PAA is the low concentration required to behave as a protein precipitant. In this case, a concentration of 0.5 g/L was enough to carry out the precipitation. A concentrated solution of the target proteases was obtained, reducing the sample volume up to 20 times. In addition, the protease–PAA precipitate (whose volume is less than 500 times the homogenate volume) may be stored for a long time before redissolution. This advantage reduces transport and storage costs. Although PAA induced a 30% loss in ChTRP enzymatic activity, the protease activity was increased around 10 times and up to 96% of the impurity proteins were eliminated. The effectiveness of this method lies in its simplicity, velocity, cost and environmental care.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2013.05.047.

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