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A new platform for chymotrypsin isolation from fresh bovine pancreas using an environmentally friendly polyelectrolyte: Alginate

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ABSTRACT

The separation of chymotrypsin from a crude filtrate of fresh bovine pancreas homogenate was carried out using precipitation with a commercially available negatively charged natural weak polyelectrolyte: sodium alginate. The zymogen form of the enzyme was activated by the addition of trypsin at pH 8.2 in the absence of Ca^{++}, then, the enzyme was precipitated by sodium alginate addition at pH 5.00. The non-soluble complex was separated by simple centrifugation and re-dissolved by a pH change to 8.20. The recovery of chymotrypsin biological activity was 36.1 % of the initial activity in the pancreas homogenate with a 3.2 fold increase in its specific activity. The volume of the final product decreased to 6.25 % of the initial feedstock, concentrating the sample up to 16 times.

KEYWORDS

Alginate;
Chymotrypsin;
Polyelectrolyte;
Enzyme-polyelectrolyte complex.

INTRODUCTION

Downstream processing is a critical limiting factor in the commercial development of biotechnology because bioseparation step can account for 50-80 % of the overall production cost[16]. The development of methods for the separation and purification of biological macromolecules has been an important requirement for much advancement in biotechnology. Most purification technologies employ precipitation of proteins as one of the initial operations aimed at concentrating the product stream for further downstream steps.

Proteins interact with polyelectrolytes to form soluble or non-soluble complexes according to the experimental conditions of the medium[9, 13, 17, 8]. By changing these conditions, such as pH or ionic strength, the protein can be released, keeping its secondary and tertiary structure as well as its biological activity. Synthetic polyelectrolytes such as polyacrylate derivates[18, 12], polyvinylsulfonate[4], Eudragit[6] as well as natural ones like chitosan[1] have been used to precipitate and concentrate proteins as an isolation method.

Chymotrypsin (Chy) is a serine protease widely used in food and pharmaceutical industry; it has a molecular weight of 25.7 kDa with an isoelectric point of 9.1 and optimum activity at pH 8.2[23]. A great amount of this enzyme is required for different industrial purposes, which makes it necessary to de-

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velop scaling-up methodologies to obtain it.

Currently, the main source of serine proteases is fresh pancreas because these enzymes have shown a low level of expression in plants and microorganisms. In the area where our laboratory is located, meat industries are very important and great amounts of meat waste are produced. One of these products is bovine pancreas, which is very rich in enzymes such as different types of proteases, amylases and lipases of wide application in numerous biotechnological processes. Chy was early isolated by the Kunitz´s[15] method, which is easy to carry out at laboratory scale in order to obtain small amounts of these enzymes. However, the scaling up of the method is expensive because it requires high amounts of salt and it also has many incompatibilities due to the necessity of recycling the salt which cannot be discarded in the environment due to the high toxicity of ammonium cation. In fact, Chy and related proteases are obtained by different protocols involving ammonium sulfate precipitation, adsorption and chromatographic techniques based on: affinity, ionic exchange, gel filtration and hydrophobic principles[5, 10, 7]. The need for a new protocol is based on economic and environmental issues. High amounts of salts are known to be contaminant and chromatography is not an economically feasible technique to be used as an early unit operation of downstream processing.

Alginate (Alg) is a linear polymer based on two monomeric units, β -D-mannuronic acid and α -L-guluronic acid with carboxilic group substituents, wich have a pKa around of 3.7[11]. Alg is refined from brown seaweeds of cold seas. Since Alg is non-toxic it is used as food additive, especially in the production of gel-like foods and as a pharmaceutical incipient.

In a previous paper[4] we described the molecular mechanism of interaction between polyvinil sulfonate, a strong anionic polyelectrolyte (molecular mass: 220 kDa) and Chy, determining the medium variable values at which the insoluble protein–polyelectrolyte formation is optimal. However, the use of synthetic polyelectrolytes may have negative effects on the final product due to their toxicity, their use not being allowed by the codes.

There are not many reports about Alg - Chy non-soluble complex formation, because the presence of Ca^{++}, which is necessary to activate the chymotrypsinogen to Chy, induces the formation of a gel between alginate and Ca^{++}[19, 11]. In this paper, we developed a chymotrypsinogen activation method in the absence of Ca^{++} in order to precipitate Chy using Alg from a fresh bovine pancreas homogenate at low ionic strength.

EXPERIMENTAL

Chemicals

α chymotrypsin from bovine pancreas (Chy), Trypsin from porcine pancreas, Alginate sodium salt, N-benzoyl-l-tyrosine ethyl ester (BTEE) and Bicinchoninic Acid Kit for Protein Determination were purchased from Sigma-Aldrich and used without further purification. Buffers of different pH were prepared: 50 mM phosphate buffer, pH 7.0; 50 mM acetic acid/acetate buffer, pH 4.0, 4.5 and 5.0 and 200 mM Tris-HCl buffer, pH 8.2. The pH was adjusted with NaOH or HCl in each case.

Methodology

Bovine pancreas homogenate preparation

The pancreas was removed from a recently killed bovine, washed with isotonical saline solution, cut in small pieces, mixed with 200 mM Tris-HCl buffer, pH 8.2 and homogenized for 5 min. with a Minipimer homogenizer. The resulting homogenate was divided in aliquots and frozen at -30 °C.

Chymotrypsin activation from fresh homogenate pancreas

Since Chy is produced as chymotrypsinogen in the pancreas, a previous activation step was required. The zymogen activation was initiated by adding a small aliquot of Tryp (0.0025% w/w) to the pancreas homogenate. The time required to complete the activation process was determined by measuring Chy activity at different intervals until a maximum value was reached. Due to the high ionic strength of the medium buffer used, the homogenate was dialyzed to lower salt concentration before the precipitation.

Solubility diagram of Alg-Chy complex

Turbidity (absorbance at 420 nm) of solutions of 0.25 mg/ml of Chy with 0.002% w/v Alg was measured and plotted against pH. The medium pH variations were obtained by adding NaOH or HCl aliquots and letting the system equilibrate before measuring turbidity. Complex formation was followed in the absence and presence of different ionic strengths adding NaCl to the medium. These titration curves were made in order to estimate the pH range where the polymer-protein complex is either soluble or insoluble.

Chy turbidimetric titration curves with Alg

The formation of the insoluble Alg–Chy complex was monitored by means of turbidimetric titration. A fixed Chy concentration (0.5 mg/ml) in 10 mM acid acetic/acetate buffer was titrated at 25 ºC in a glass cell with Alg solution as the titrant. To avoid changes in pH during titration, both Chy and Alg solutions were adjusted to the same pH value. The absorbance of solution at 420 nm was used to follow Chy–Alg complex formation and plotted vs. the total Alg concentration in the tube. The results were fitted with a 4-parameter sigmoidal function in order to determine the value of the Alg minimum concentration required to precipitate Chy. This parameter was calculated as the intersection of the tangent at the inflection point with the plateau of the plot. The [Chy]/[Alg] ratio can be calculated as the rate between Chy total concentration and the [Alg] calculated[8]. Absorbance solutions were measured using a Jasco 520 spectrophotometer with constant agitation in a thermostatized cell of 1 cm of path length.

Determination of Chy activity

Chy assay is based on the hydrolysis of BTEE[10]. The reaction rate was determined by measuring the absorbance increase at 256 nm, at 25 ºC, which results from the hydrolysis of the substrate at 0.6 mM concentration in 200 mM buffer Tris–HCl pH 8.2-200mM $CaCl_2$.

Determination of total protein concentration

It was carried out using the bicinchoninic assay[21]. A fresh standard working reagent (SWR) was prepared by mixing 98 vol of reagent A (Bicinchoninic acid solution) with 2 vol. of reagent B ($CuSO_4$ solution 4%w/v). A volume of 50 µL of protein solution (maximum concentration of 1mg/mL) was added to 1 mL of SWR. The tubes were incubated a 37ºC for 30 min. After letting them cool down at room temperature, absorbance was measured at 562 nm using a 1 cm path length cell. The calibration curve was performed using dilutions of a standard solution of BSA 1 mg/mL.

Evaluation of the performance of the purification process by SDS-PAGE

Aliquots of activated pancreas homogenate and re-dissolved precipitate were analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) using a vertical system. The running time was about 120 min and the constant intensity was 25 mA for the resolving gel. Proteins were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Influence of pH on Alg – Chy complex formation

Chy is a basic protein with an isoelectrical pH of 9.1. Therefore, at pHs between 3 to 9, where the turbidimetry titration was assayed, the protein has a net positive electrical charge. Alg possesses carboxyl groups which are dissociated at pH above 3.6, this polyelectrolyte being soluble[11].

Figure 1 shows the pH variation effect on the complex solubility obtained at constant Chy / Alg mass ratio. Blank titration curves of Alg without Chy and the enzyme without Alg were made in the same pH range, and no absorbance changes were observed (data not shown). The decrease of pH below 7 induced an increase in the maximum absorbance values, which suggested a major amount of complex formation. The curve reached a maximum absorbance at pH 5.0. The optimum pH interval where the polymer–protein complex is non-soluble can be determined from this curve. Figure 1 also shows the effect of increasing NaCl concentration on Alg- Chy complex solubility. The presence of salt increases the solubility of the complex, as it is observed by

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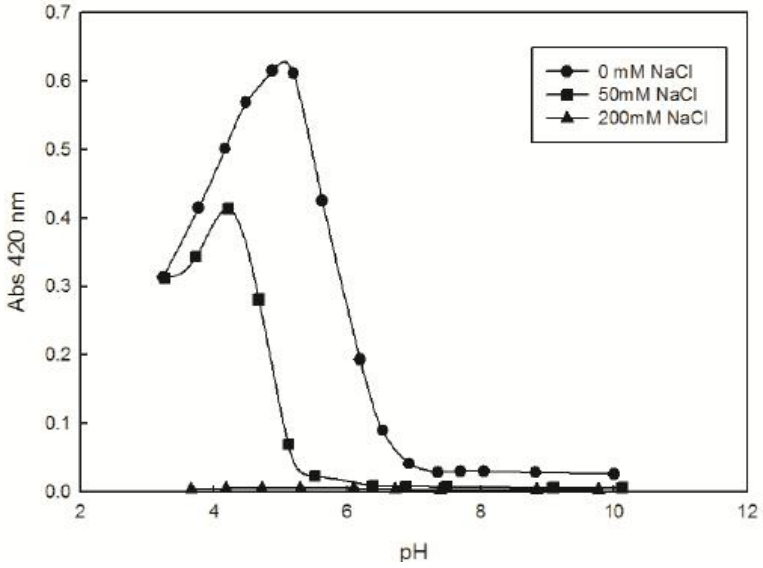

Figure 1 : Turbidimetry acid–base titration of a mixture of Alg–Chy. Alg concentration 0,002 % w/v; Chy concentration 0,5 mg/ml. Medium: 10 mM sodium acetate–phosphate buffer. Temperature: 25°C

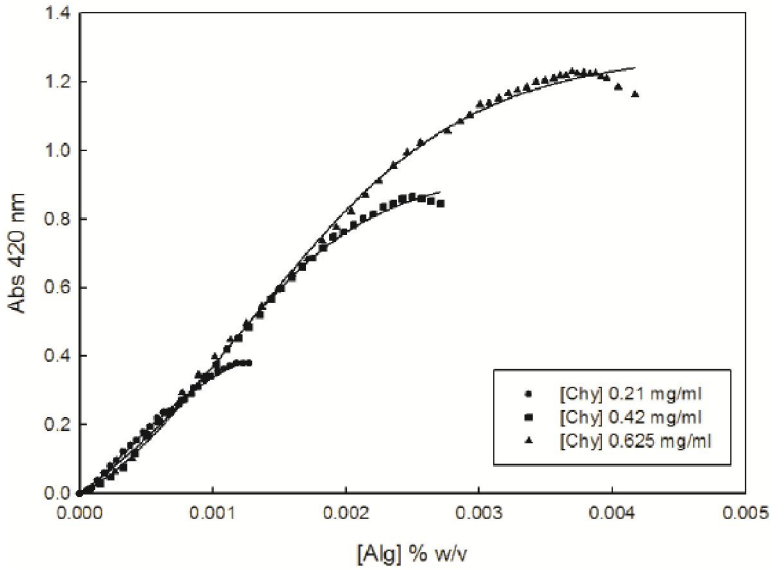

Figure 2 : Titration of Chy with increasing concentration of Alg. Medium: 10 mM acetate buffer, pH 5.00. Temperature 20°C

the decrease of turbidity. The presence of NaCl 200 mM produces the complete solubilization of the complexes. This is consistent with the fact that coulombic interactions drive the association between a polyelectrolyte and a protein of opposite charge[8].

Titration of Chy with Alg

Figure 2 shows absorbance dependence at 420 nm when Chy, at different concentrations, is titrated with Alg in pH 5.0 media. This pH was selected because it is the maximum non-solubility of the complex (see Figure1). The titration curves show a plateau at high Alg concentrations.

From these curves, the stoichiometric Chy/Alg ratio can be calculated, which corresponds to the point where most Chy has been precipitated as an insoluble complex. These values are important because they allow us to calculate the minimum Alg amount needed to precipitate Chy. From the non-linear fitting of the data, the Chy/Alg ratio was obtained at pH 5.00. The values obtained are shown in TABLE 1. The amount of Alg necessary to precipitate Chy is

Extremely small when compared with the val-

ues obtained by other authors for the same polymer as well as with the values obtained for other polyelectrolytes used as precipitating agents[9, 20] or the classical protein precipitants such as inorganic cations and anions[15]. Moreover, it can be seen that the ratio Chy/Alg does not depend on the total concentration of enzyme. This is important due to the potential applications of the method in scaling up. In many cases, the target proteins are present in high volumes of solution, but even so, a small mass of this polyelectrolyte is necessary to precipitate the enzyme.

Alg effect on the Chy activity

Previous reports have demonstrated that polyelectrolytes influence enzymatic activity because they induce a modification of the secondary and tertiary protein structure[9, 14]. Chy at constant concentration (0.5 mg/ml) was incubated for 30 min in media of increasing Alg concentrations at the pH of maximun enzyme activity (8.2). Under these conditions, the Chy–Alg complex remains in the soluble form. The enzymatic activity of Chy was determined with respect to a reference medium in the absence of Alg. The polyelectrolyte does not affect the enzymatic activity in any of the conditions assayed (data not shown).

Zymogen activation from the bovine pancreas homogenate

Chy is present in pancreas in a biological inactive form, chymotrypsinogen, so that an activation step is required. The traditional method for the activation of the pancreatic enzyme precursors is carried out at pH 8.2 in a Tris-HCl medium 200 mM in the presence of Ca^{++} and with the addition of a small amount of trypsin[6]. Under these conditions, precursors are active in less than 5 hours. In this particular case, the presence of Ca^{2+} make the process difficult due to the ability of Alg to form gels in the presence of this cation. Thus, activation was carried out in the absence of Ca^{2+}, keeping all the other variables constants. We found that the activation process has a good yield in the absence of Ca^{2+} and goes to completion in 270 min (data not shown).

Chy precipitation from the activated homogenate of bovine pancreas

Two experiments were carried out for Chy precipitation with Alg. First, a control experiment was performed using a pure Chy solution, adding increasing concentrations of Alg at pH 5.00, and then measuring enzyme activity in the supernatant and in the precipitate before its re-dissolution by change of the medium pH to 8.2 Figure 3 (insert) shows that an

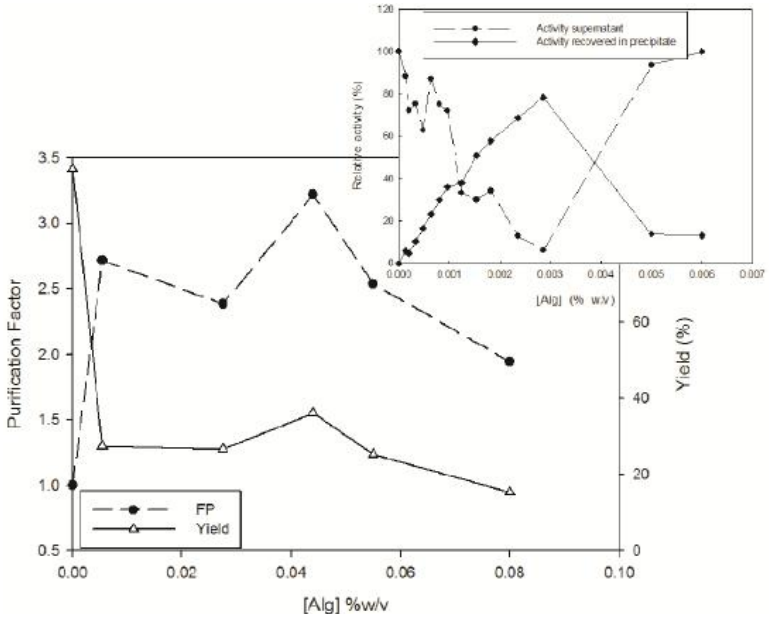

Figure 3 : Chy recovery activity in the precipitate and purification factor at increasing Alg concentration, *Insert:* Recovery of pure Chy activity in the precipitate and in the supernatant at increasing Alg concentration, Enzyme activity was measured at pH 8.2. Medium: 10 mM acetate buffer pH 5.00. temperature: 25°C

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increase in Alg total concentration favors Chy recovery, reaching a maximum yield of 80% around Alg concentration of 3. 10^{-3} %. At higher Alg concentrations, complex formation decreased as a result of its solubilization caused by the increase in the medium ionic strength. Therefore, there is a critical concentration value of Alg, where higher values induce a decrease in the amount of complex obtained. The second experiment was performed with the activated fresh pancreas homogenate. It has been reported that the polyelectrolyte concentration needed to precipitate a pure enzyme in solution is very different from that needed to precipitate the same enzyme in a complex mixture. In the latter case, a much higher polyelectrolyte concentration is required due to the presence of other proteins with the same electrical charge as the target enzyme that consumes polyelectrolyte. Taking this into account, aliquots of 1 ml of activated homogenate at pH 5.0 were titrated with increasing concentrations of Alg by adding small aliquots of Alg concentrated solution at pH 5.00. The precipitate obtained was separated by centrifugation (10 min at 5000 g) and then dissolved by addition of 200 mM Tris–HCl buffer, pH 8.2. Chy activity and the total protein concentration were determined in the supernatant and the re-dissolved precipitate. The recovery of Chy (with respect to the initial total activity in the same mass of homogenate) and the purification factor were calculated. Figure 3 shows that the best purification of the enzyme is reached at an Alg concentration eight times higher than that required to precipitate the pure enzyme, this difference may be explained by the interaction of the Alg with other positively electrical charged proteins and also cell membranes. Higher Alg concentrations induce a decrease in the yield and purification, consistent with the results obtained with pure Chy.

To verify the purity of Chy in the final solution, a SDS-PAGE of the different fractions was made, as shown in Figure 4. The lane corresponding to the re-dissolved precipitate shows a predominant band corresponding to Chy.

Finally, the effect of the final volume where the non-soluble complex was dissolved was also assayed. In this experiment, a constant volume of homogenate (4ml) was treated with the same concentration of Alg (0.005% W/V). The precipitate was separated and re-dissolved in different final volumes of 200 mM buffer Tris-HCl 8.2. The enzymatic activity recovered was constant in the different conditions, i.e., achieving a 16 times decrease in the volume of the sample.

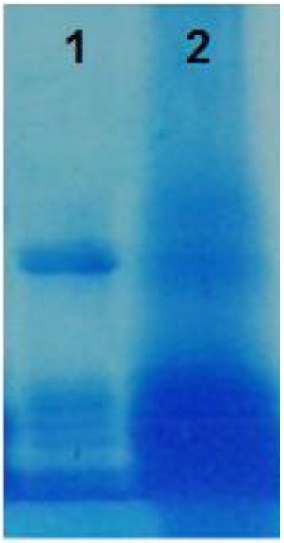

Figure 4 : SDS-polyacrylamide (13%) gel electrophoresis (Coomassie blue staining) of the proteins present in the re-dissolved precipitate (first lane) and in the activated pancreas homogenate (second lane)

CONCLUSIONS

In this work, Alg was found to be effective in precipitating Chy from fresh bovine pancreas homogenate. Precipitation using this polysaccharide is more advantageous compared to other synthetic polyelectrolytes previously used with the same aim[6, 12] that can be toxic or not allowed by the alimentary codex. Our finding showed that this polymer can precipitate around 80% of Chy when the protein is pure in an aqueous solution. Higher Alg concentrations produce the dissolution of the non-soluble complex by an increase in the ionic strength of the medium, resulting in a decrease in enzyme recovery. Fresh pancreas homogenate has a significant amount of cellular debris and other positively electrically charged proteins, so a higher Alg concentration was necessary to induce complex formation, which, in turn, led to a decrease in Chy recovery to 36.1%.

Also, this finding may be due to a displacement equilibrium effect, where the Alg concentration is no enough to totally displace the reaction to the complex formation. However, the method can be performed in only one step, ideally effective in terms of cost and processing time, with a purification factor of 3.2 and yield of 37%. On the other hand, this polyelectrolyte does not modify the biological activity of the enzyme at the concentration used to form the complex. Also, this methodology allowed concentrating Chy activity by reduction of the final volume where the precipitate is dissolved.

We are proposing an economical and environmentally-friendly method to obtain a Chy concentrated and clarified extract, from its natural source. This extract can be further purified, if necessary, according to its final application, by different techniques, including classical chromatography. The most remarkable advantages of this protocol is that we are reducing the volume and clarifying the sample, thus reducing the operation and reagent costs of the following steps in the process.

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