G Model
PRBI-9673; No. of Pages 5

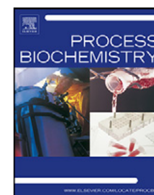
ARTICLE IN PRESS

Process Biochemistry xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Short communication

Precipitation of chymotrypsin from fresh bovine pancreas using ι-carrageenan

Nadia Woitovich Valetti, Julia Lombardi, Valeria Boeris, Guillermo Picó*

Laboratorio de Fisicoquímica Aplicada a Bioseparación, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 570, S2002RLK Rosario, Argentina

ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form
12 September 2012
Accepted 26 September 2012
Available online xxx

Keywords:
Carrageenan
Chymotrypsin
Polyelectrolyte

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 strong polyelectrolyte: ι-carrageenan. The zymogen form of the enzyme was activated by addition of trypsin (0.0001%, w/w), then, the enzyme was precipitated by polyelectrolyte addition at pH 4.50. 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 60% of the initial activity in the homogenate with 3-fold increase in its specific activity. The volume of the final product decreased to 10% of the feedstock, concentrating the sample up to 10 times.

© 2012 Published by Elsevier Ltd.

1. Introduction

Chymotrypsin (ChTRP) is a protease widely used in food and pharmaceutical industry; it has a single polypeptidic chain of 245 amino acid residues and a molecular weight of 25.7 kDa [1–3]. ChTRP is one of the proteolytic enzymes of vertebrates pancreas juice with an isoelectric point of 9.1 and optimum activity at pH 8.2 [4]. A great amount of this enzyme is required for different industrial purposes, which makes it necessary to develop scaling-up methodologies to obtain it.

Nowadays, chymotrypsin and related proteases are obtained by different protocols involving ammonium sulfate precipitation [5], adsorption [6] and chromatographic techniques based on: affinity [7,8], ionic exchange [5,9,10], gel filtration [5,11] and hydrophobic [6,10] principles. The need of a new protocol is based on economic and environmental issues [12]. High amounts of salts are known to be contaminants and chromatography is not a suitable technique to be used as an early unit operation in a downstream process from the economic point of view.

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.

Bioseparation steps for the recovery of the final product can account for 50–80% of overall production costs. Most purification technologies use precipitation of proteins as one of the initial operations aimed at concentrating the product for further downstream steps. Attempts are usually made to derive some degree of purification of target products in the precipitation step [13].

Proteins interact with polyelectrolytes to form soluble or non-soluble complexes according to the experimental conditions of the medium [14,15]. 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 [16,17], polyvinylsulfonate [18], Eudragit [19,20] as well as natural ones like chitosan [21,22] have been used to precipitate proteins as an isolation method. We have previously used different polyelectrolytes to isolate enzymes from its natural source [17–19,22,23].

Carrageenan (Carr) is a generic name for a family of polysaccharides obtained from certain species of red seaweeds. They are non-toxic, water soluble and widely used within the food, pharmaceutical, cosmetic, printing and textile industries. Carr are sulfated linear polysaccharides, their primary structure consists of alternating (1–3)-D-galactose-4-sulfate and β (1,4)-3,6-anhydro-D-galactose residues. There are 6 forms of these polymers: iota (ι)-, kappa (κ)-, lambda (λ)-, mu (μ)-, nu (ν)- and theta (θ)-carrageenan, being the most commercially important: iota-, kappa- and lambda-carrageenan [24]. Previous reports have demonstrated the use of carrageenan to isolate and to immobilize enzymes [25–27].

The main objective of this research was to extract and purify ChTRP from a crude filtrate of fresh bovine pancreas homogenate through precipitation with ι-carrageenan (Carr), a non-toxic polyelectrolyte, accepted for use in food industry. Also, Carr can be discarded in the environment without a negative impact on it. To achieve this goal, we first determined the optimum conditions of

Abbreviations: Carr, carrageenan; ChTRP, chymotrypsin.

* Corresponding author. Fax: +54 0341 480 4598.
E-mail address: gpico@fbioyf.unr.edu.ar (G. Picó).

1359-5113/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.procbio.2012.09.021

Please cite this article in press as: Woitovich Valetti N, et al. Precipitation of chymotrypsin from fresh bovine pancreas using ι-carrageenan. Process Biochem (2012), http://dx.doi.org/10.1016/j.procbio.2012.09.021

precipitation of the pure enzyme with Carr and then, we applied the ChTRP precipitation with Carr to the purification of the enzyme from its natural source.

2. Materials and methods

2.1. Chemicals

Chymotrypsin (ChTRP), ι-carrageenan (Carr) and N-benzoyl-l-tyrosine ethyl ester (BTEE) 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.

2.2. Methodology

2.2.1. Bovine pancreas homogenate preparation

The pancreas was removed from a recently killed bovine, washed with isotonical saline solution, cut into small pieces, mixed with $CaCl_2$ solution in final concentration of 50 mM and homogenized for 5 min in a Minipimer homogenizer. The resulting homogenate was divided in aliquots and frozen at −30 °C [18].

2.2.2. Chymotrypsin activation from fresh homogenate pancreas

Since ChTRP is produced as chymotrypsinogen in the pancreas, a previous activation step was required. The zymogen activation was initiated by adding a small aliquot of trypsin (0.0001%, w/w) in 90 mM Tris–HCl buffer, pH 8.2 and 45 mM $CaCl_2$. The time required to complete the activation process was determined by measuring the ChTRP activity at different intervals until a maximal value was reached [18].

2.2.3. Solubility diagram of Carr–ChTRP complex

Turbidity (absorbance at 420 nm) of solutions of 0.5 mg/mL of ChTRP with 0.005% (w/v) of Carr was measured and plotted against pH. The pH variations of the medium were obtained by adding NaOH or HCl aliquots and leaving the system to equilibrate before measuring the turbidity. These titration curves were made in order to estimate the pH range where the polymer–protein complex is soluble or insoluble [14,28].

2.2.4. ChTRP turbidimetric titration curves with Carr

The formation of the insoluble Carr–ChTRP complex was monitored by means of turbidimetric titration. A fixed ChTRP concentration (0.5 mg/mL) in acid acetic/acetate buffer was titrated at 25 °C in a glass cell using Carr solution as titrant. To avoid changes in pH during titration, both ChTRP and Carr solutions were adjusted to the same pH value. The absorbance of solution at 420 nm was used to follow the Carr–ChTRP complex formation and plotted vs. the total Carr concentration in the tube. The results were fitted with a 4-parameters sigmoidal function in order to determine the value of the Carr minimal concentration required to precipitate ChTRP. This parameter was calculated as the intersection of the tangent at the inflection point with the plateau of the plot. The [ChTRP]/[Carr] ratio can be calculated as the ratio between the ChTRP total concentration and the [Carr] calculated. Absorbance solutions were measured using a Jasco 520 spectrophotometer with constant agitation in a thermostatized cell of 1 cm of path length [14].

2.2.5. Determination of ChTRP activity

The ChTRP assay is based on the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE) [29]. The reaction rate was determined by measuring the absorbance increase at 256 nm, at 25 °C, which results from the hydrolysis of the substrate at 0.6 mM concentration in 200 mM Tris–HCl buffer, pH 8.2–200 mM $CaCl_2$. One ChTRP unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction and was calculated with the following equation: $U\,(\mu\text{mol/min}) = (\Delta\text{Abs}_{256\,\text{nm}}/\text{min}) \times 1000/964$, where 964 is the benzoyl-tyrosine molar extinction coefficient [5].

2.2.6. Determination of total protein concentration

It was carried out using the bicinchoninic assay [30,31]. 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 ($CuSO_4$ solution 4% (w/v) 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 down at room temperature, the absorbance was measured at 562 nm using a cell with a 1 cm path length. The calibration curve was performed using dilutions of a standard solution of BSA 1 mg/mL.

2.2.7. Evaluation of the performance of the purification process

The recovery of ChTRP (with respect to the initial total activity in the same mass of homogenate) and the purification factor were calculated. The ChTRP activity recovery yield was defined as:

$$\text{yield} = \frac{A_f}{A_i} \quad (1)$$

where A_f is the ChTRP activity after the precipitation and A_i is the initial enzymatic activity in the pancreas homogenate.

The purification factor was defined as:

$$\text{PF} = \frac{\text{SA}_f}{\text{SA}_i} \quad (2)$$

where SA_i is the ChTRP specific activity in the pancreas homogenate and SA_f the specific activity after precipitation. SA is the ratio between ChTRP activity and the total protein content.

2.2.7.1. Evaluation of the purification process by polyacrylamide gel electrophoresis.

Aliquots of activated pancreas homogenate, supernatant and redissolved precipitate were analyzed by sodium dodecyl sulfate (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.

3. Results and discussion

3.1. Influence of pH on the complex formation

Fig. 1 (inset) shows the solubility diagram of a 0.005% (w/v) Carr–0.5 mg/mL ChTRP mixture. When the pH decreases below 8.2, an increase in the turbidity is observed; this is consistent with the non-soluble Carr–ChTRP complex formation. The curves showed a sigmoidal behavior, reaching a maximum absorbance value at pH lower than 5. The pH region in which the absorbance variation is higher is around pH 6. At this pH value, the protonation/deprotonation equilibrium of the histidine residues has a remarkable importance since the positive net charge of the ChTRP abruptly increases as the pH decreases in a narrow range.

Fig. 1 (inset) also shows the effect of increasing NaCl concentration on the Carr–ChTRP complex solubility. The presence of salt increases the solubility of the complex, as it is observed by the decrease of the turbidity. Taking this into account, the interaction of ChTRP with Carr seems to be one of electrostatic nature, which explains the Carr–ChTRP complex formation at pH lower than 5 due to the increase in the ChTRP positive electrical charge.

Blank titration curves of Carr without ChTRP and ChTRP without Carr were carried out at the same pH range assayed and absorbance changes were not observed.

3.2. Titration of ChTRP with Carr

Fig. 1 shows the variation of the absorbance at 420 nm when ChTRP, at different concentrations, is titrated with Carr at pH 4.5. This pH was selected because the absorbance proved to be constant below pH 5.0. The Abs values at the plateau increase as the ChTRP initial concentration increases, indicating a higher extent of complex formation.

The titration curve was assayed not only at different enzyme concentrations but also at different pHs. From these curves, the stoichiometric ChTRP/Carr ratios, which correspond to the case in which most ChTRP has been precipitated as an insoluble complex, were determined and shown in Table 1. These values are important because they allow us to calculate the minimal Carr amount needed to precipitate ChTRP.

The increase in the total ChTRP concentration induced an increase in the formation of non-soluble complexes. At pH 5.0, the precipitation showed better results, i.e., a higher amount of ChTRP is precipitated per mass unit of Carr. However, as shown in Fig. 1 (inset), the turbidity in the pH range from 4.0 to 5.0 is constant and does not reflect the stoichiometry of Carr–ChTRP interaction.

High ChTRP/Carr ratios with values from 24 to 33 mg/mg were obtained for the range of pH tested considering an average concentration of ChTRP of 0.5 mg/mL. The mass of polyelectrolyte was expressed in grams because Carr is a polydisperse polymer since it is a natural product. The necessary amount of Carr to precipitate

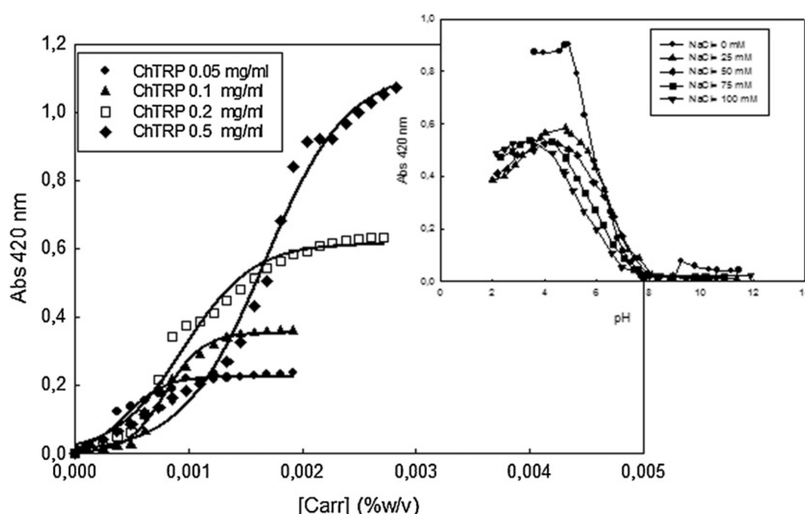


Fig. 1. Titration of ChTRP with increasing concentration of Carr. Medium: 10 mM acetate buffer, pH 4.5. Inset: acid–base titration of a mixture of Carr–ChTRP (0.5 mg/mL). Medium: 10 mM sodium acetate–phosphate buffer. Temperature 25 °C.

ChTRP is extremely small when compared with the values obtained by other authors for the same polymer [26] and the values obtained for other polyelectrolytes required to precipitate some proteins [32,33] or the traditional protein precipitants such as inorganic cations and anions [34]. This is important because of 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.

3.3. Carr effect on the ChTRP activity

Previous reports have demonstrated that polyelectrolytes influence enzymatic activity because they induce a modification of the secondary and tertiary protein structure [18,35]. ChTRP at constant concentration (0.5 mg/mL) was incubated for 30 min in media of increasing Carr concentrations (up to 0.1%, w/v) at the pH of the enzyme maximal activity (8.2). Under these conditions, the Carr–ChTRP complex remains in the soluble form. The enzymatic activity of the ChTRP was determined with respect to a medium reference in the absence of Carr. Comparing the ChTRP activity in the different media by ANOVA, it was found that there were significant differences between the levels ($p = 0.0005$): Carr induced a slight decrease in the enzyme activity. However, when Carr was present in the media, the ChTRP activity was independent of Carr concentration ($p = 0.153$). The loss in the ChTRP enzymatic activity was around 10% compared to the ChTRP enzymatic activity in a media free of Carr. This is observed in a media containing up to ten times the Carr concentration necessary to quantitatively precipitate ChTRP. The decrease in the enzymatic activity may be due to an increase in the media viscosity. A change in this hydrodynamic property can affect the kinetics of the enzyme. Despite this limitation, Carr seems to be appropriate to be used in a ChTRP purification protocol since we consider this slightly modification of the enzymatic activity acceptable.

3.4. ChTRP precipitation from the activated homogenate of bovine pancreas

It has been reported [36] that the polyelectrolyte concentration needed to precipitate a target enzyme from a complex mixture is higher than that needed to precipitate the same enzyme when it is pure in solution. This fact is due to the presence of other macromolecules in the sample with the same electrical charge as the target enzyme that consumes polyelectrolyte. Taking this into account, aliquots of 1 mL of activated homogenate were titrated with increasing concentration of Carr solution (0–0.08%, w/v) by adding small aliquots of Carr concentrated solution at different pH values (4.0, 4.5 and 5.0). The precipitate obtained was separated by centrifugation (10 min at 5000 × g) and then dissolved by addition of Tris–HCl buffer, pH 8.2. The ChTRP activity recovered and the total protein concentration were determined in the supernatant and in the redissolved precipitate.

Fig. 2 shows that an increase in the Carr total concentration favors the ChTRP recovery, reaching a maximum yield of 60%, around a Carr concentration of 0.06% (w/v), while the remaining enzyme in the supernatant was around 20%. Fig. 2 also shows that the maximal precipitation of the enzyme is reached at a Carr concentration ten times higher than that required to precipitate the pure enzyme. This difference may be explained on the basis of the interaction of Carr with other positively electrical charged proteins and also with cell membranes.

The total protein content was also determined in the redissolved precipitate in order to calculate the purification factor. The appropriate pH to carry out the precipitation was 4.5 since the ChTRP specific activity recovered in the precipitate was higher than the one observed in the other assayed media. The purification

Table 1
Stoichiometry for the Carr–ChTRP complex formation at different total concentration of ChTRP.

[ChTRP] (mg/mL)	pH 4.0 ChTRP mg/Carr mg	pH 4.5 ChTRP mg/Carr mg	pH 5.0 ChTRP mg/Carr mg
0.05	7.8 ± 0.2	6.4 ± 0.1	17.2 ± 0.7
0.1	12 ± 2	8 ± 1	17 ± 2
0.2	13.3 ± 0.4	12 ± 1	22 ± 2
0.5	24 ± 3	24 ± 2	33 ± 4

Please cite this article in press as: Woitovich Valetti N, et al. Precipitation of chymotrypsin from fresh bovine pancreas using ι-carrageenan. Process Biochem (2012), http://dx.doi.org/10.1016/j.procbio.2012.09.021

Table 2
Purification of ChTRP from bovine pancreas homogenate.

	Homogenate	Extract (precipitated with Carr 0.06%, w/v)
Volume (mL)	1	1
Total protein concentration (mg/mL)	7.7 ± 0.2	1.50 ± 0.03
ChTRP activity (U/mL)	58 ± 4	34 ± 2
ChTRP specific activity (U/mg)	7.5 ± 0.7	23.0 ± 0.9
ChTRP recovery (%)	100	60 ± 3
Fold purification	1	3.0 ± 0.4

The values are means of three independent measurements.

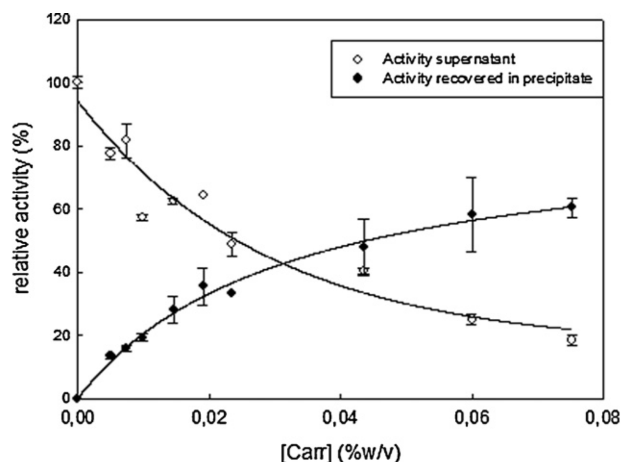


Fig. 2. Recovery of the ChTRP activity in the precipitate and in the supernatant at different initial concentrations of precipitant agent (Carr). Medium: 10 mM acetate buffer, pH 4.5. Enzyme activity was measured at pH 8.2.

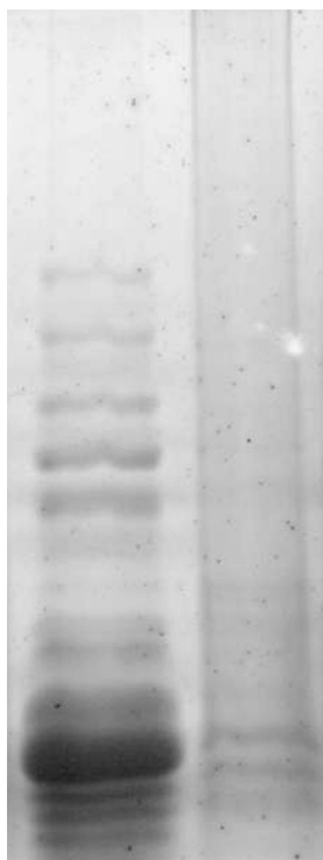


Fig. 3. SDS-polyacrylamide (13%) gel electrophoresis (Coomassie blue staining) of the proteins present in the activated pancreas homogenate (first lane) and in the redissolved precipitate (second lane).

performance is shown in Table 2. To verify the purity of the ChTRP in the final solution, a SDS-PAGE of the different fractions was made, as shown in Fig. 3. The lane corresponding to the redissolved precipitate shows a predominant band corresponding to ChTRP.

The effect of the final volume where the non-soluble complex was dissolved was also assayed. In this experiment, a constant volume of homogenate (5 mL) was treated with the same concentration of Carr (0.005%, w/v), the precipitate was separated and redissolved in different final volumes of 200 mM Tris–HCl buffer, pH 8.2, between 0.50 and 5 mL. Analyzing the results of the recovered ChTRP activity by ANOVA, the volume in which the precipitate was redissolved had no significant effect on the yield ($p = 0.1118$). Thus, we obtained a 10-times decrease in the volume of the sample without compromising the ChTRP recovery.

Finally, the effect of the incubation time of the enzyme in the presence of Carr was assayed. To do this, one aliquot of 1 mL of activated homogenate was mixed with Carr in a final concentration of 0.005% (w/v), the precipitate was separated and redissolved. The ChTRP enzymatic activity in the extract was determined after different incubation times. The statistical analysis of the results showed that ChTRP enzymatic activity was not significantly affected by the incubation time up to 10 days. After this period, the ChTRP enzymatic activity recovered decreased to 55% of the initial value. These results are encouraging since ChTRP seems to be stable over time in Carr-containing media. This fact comes as no surprise, since it is known that the addition of cosolutes such as polysaccharides and other hydrophilic substances to a protein solution leads to an enhanced structural stability [37,38].

4. Conclusion

In this work, the recovery of ChTRP from bovine pancreas was carried out by means of precipitation with Carr. This polyelectrolyte, a common food ingredient, was found to be effective in precipitating ChTRP from fresh bovine pancreas homogenate. Precipitation using this polysaccharide is more advantageous compared to other synthetic polyelectrolytes previously used with the same aim [18,19] that can be toxic or not allowed by the alimentary codex.

Our finding showed that this negative charged polysaccharide can precipitate around 60% of the ChTRP from a flesh pancreas homogenate using only one step (ideally effective in terms of cost and processing time), with a purification factor of 3.0. On the other hand, this polyelectrolyte modified only slightly the biological activity of the enzyme at the concentration used to form the complex. Also, this methodology allowed concentrating the ChTRP activity by reduction of the final volume where the precipitate is dissolved.

We are proposing an economical and environment-friendly method to obtain a ChTRP 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 more 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.

Acknowledgements

This work was supported by a grant from CONICET PIP00196. N. Woitovich Valetti and V. Boeris thank CONICET for their fellowship. We thank María Robson, Geraldine Raimundo, Mariana De Sanctis and Marcela Culasso for the language correction of the manuscript.

References

[1] Neville DM. Molecular weight determination of protein–dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem 1971;246:6328–34.
[2] Singh N, Jabeen T, Sharma S, Roy I, Gupta MN, Bilgrami S, et al. Detection of native peptides as potent inhibitors of enzymes. FEBS J 2005;272:562–72.
[3] Demanèche S, Chapel J-P, Monrozier LJ, Quiquampoix H. Dissimilar pH-dependent adsorption features of bovine serum albumin and α-chymotrypsin on mica probed by AFM. Colloids Surf B 2009;70:226–31.
[4] Appel W. Chymotrypsin: molecular and catalytic properties. Clin Biochem 1986;19:317–22.
[5] Castillo-Yáñez FJ, Pacheco-Aguilar R, García-Carreño FL, de los Ángeles Navarrete-Del Toro M, López MF. Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (Sardinops sagax caeruleus). Food Chem 2006;99:252–9.
[6] Joo H-S, Park G-C, Tae Kim K, Paik SR, Chang C-S. Simple methods for alkaline protease purification from the polychaeta, Periserrula leucophryna. Process Biochem 2001;37:299–303.
[7] Carrea G, Pasta P, Antonini E. Purification of chymotrypsin by subunit exchange chromatography on the denatured protein. Biotechnol Bioeng 1983;25:1331–9.
[8] Wolman FJ, Grasselli M, Cascone O. Rapid neutral protease purification by dye-affinity membrane chromatography. Process Biochem 2006;41:356–61.
[9] Cohen T, Gertler A, Birk Y. Pancreatic proteolytic enzymes from carp (Cyprinus carpio)—I purification and physical properties of trypsin, chymotrypsin, elastase and carboxypeptidase B. Comp Biochem Physiol B 1981;69:639–46.
[10] Gupta A, Roy I, Khare SK, Gupta MN. Purification and characterization of a solvent stable protease from Pseudomonas aeruginosa PseA. J Chromatogr A 2005;1069:155–61.
[11] Bhaskar N, Sudeepa ES, Rashmi HN, Tamil Selvi A. Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour Technol 2007;98:2758–64.
[12] Przybycien TM, Pujar NS, Steele LM. Alternative bioseparation operations: life beyond packed-bed chromatography. Curr Opin Biotechnol 2004;15:469–78.
[13] Scopes RK. Protein purification in the nineties. Biotechnol Appl Biochem 1996;23:197–204.
[14] Cooper CL, Dubin PL, Kayitmazer AB, Turksen S. Polyelectrolyte–protein complexes. Curr Opin Colloid Interface Sci 2005;10:52–78.
[15] Niederauer M, Glatz C. Selective precipitation. Bioseparation, vol. 47. Berlin/Heidelberg: Springer; 1992. p. 159–88.
[16] Sternberg M, Hershberger D. Separation of proteins with polyacrylic acids. Biochim Biophys Acta: Protein Struct 1974;342:195–206.
[17] Porfiri MC, Braia M, Farruggia B, Picó G, Romanini D. Precipitation with poly acrylic acid as a trypsin bioseparation strategy. Process Biochem 2009;44:1046–9.
[18] Boeris V, Romanini D, Farruggia B, Picó G. Purification of chymotrypsin from bovine pancreas using precipitation with a strong anionic polyelectrolyte. Process Biochem 2009;44:588–92.
[19] Cappella LV, Boeris V, Picó G. A simple method of chymotrypsin concentration and purification from pancreas homogenate using Eudragit® L100 and Eudragit® S100. J Chromatogr B 2011;879:1003–7.
[20] Gupta MN, Guoqiang D, Kaul R, Mattiasson B. Purification of xylanase from *Trichoderma viride* by precipitation with an anionic polymer Eudragit S 100. Biotechnol Tech 1994;8:117–22.
[21] Bassani G, Farruggia B, Picó G. Cationic polyelectrolytes–lipases complexes formation as tool for recovery of these enzymes from their natural sources. Int J Biol Macromol 2011;49:351–5.
[22] Boeris V, Spelzini D, Farruggia B, Picó G. Aqueous two-phase extraction and polyelectrolyte precipitation combination: a simple and economically technologies for pepsin isolation from bovine abomasum homogenate. Process Biochem 2009;44:1260–4.
[23] Spelzini D, Farruggia B, Picó G. Purification of chymotrypsin from pancreas homogenate by adsorption onto non-soluble alginate beads. Process Biochem 2011;46:801–5.
[24] Campo VL, Kawano DF, Silva Jr DBd, Carvalho I. Carrageenans: biological properties, chemical modifications and structural analysis—a review. Carbohydr Polym 2009;77:167–80.
[25] Roy I, Gupta MN. κ-Carrageenan as a new smart macroaffinity ligand for the purification of pullulanase. J Chromatogr A 2003;998:103–8.
[26] Fabian CB, Huynh LH, Ju YH. Precipitation of rice bran protein using carrageenan and alginate. LWT Food Sci Technol 2010;43:375–9.
[27] Mondal K, Roy I, Gupta MN. κ-Carrageenan as a carrier in affinity precipitation of yeast alcohol dehydrogenase. Protein Expr Purif 2003;32:151–60.
[28] Kaibara K, Okazaki T, Bohidar HB, Dubin PL. pH-induced coacervation in complexes of bovine serum albumin and cationic polyelectrolytes. Biomacromolecules 2000;1:100–7.
[29] Guyonnet V, Tłuscik F, Long PL, Polanowski A, Travis J. Purification and partial characterization of the pancreatic proteolytic enzymes trypsin, chymotrypsin and elastase from the chicken. J Chromatogr A 1999;852:217–25.
[30] Walker JM. The bicinchoninic acid (BCA) assay for protein quantitation. In: Walker JM, editor. The protein protocols handbook. New Jersey: Humana Press; 1996. p. 11–4.
[31] Brown RE, Jarvis KL, Hyland KJ. Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal Biochem 1989;180:136–9.
[32] Wibowo S, Velazquez G, Savant V, Torres JA. Surimi wash water treatment for protein recovery: effect of chitosan–alginate complex concentration and treatment time on protein adsorption. Bioresour Technol 2005;96:665–71.
[33] Montilla A, Casal E, Javier Moreno F, Belloque J, Olano A, Corzo N. Isolation of bovine β-lactoglobulin from complexes with chitosan. Int Dairy J 2007;17:459–64.
[34] Arakawa T, Timasheff SN. Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. Biochemistry 1984;23:5912–23.
[35] Mazzaferro L, Breccia JD, Andersson MM, Hitzmann B, Hatti-Kaul R. Polyethyleneimine–protein interactions and implications on protein stability. Int J Biol Macromol 2010;47:15–20.
[36] Zhang C, Lillie R, Cotter J, Vaughan D. Lysozyme purification from tobacco extract by polyelectrolyte precipitation. J Chromatogr A 2005;1069:107–12.
[37] Chaiklahan R, Chirasuwan N, Bunnag B. Stability of phycocyanin extracted from Spirulina sp.: influence of temperature, pH and preservatives. Process Biochem 2012;47:659–64.
[38] Robertson AJ, Conrad V, Moraes G. Stabilisation of carboxypeptidases in acid extracts of ovine pancreas. Process Biochem 2004;39:615–21.

Please cite this article in press as: Woitovich Valetti N, et al. Precipitation of chymotrypsin from fresh bovine pancreas using ι-carrageenan. Process Biochem (2012), http://dx.doi.org/10.1016/j.procbio.2012.09.021