Separation and Purification Technology 111 (2013) 125–130

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

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

Interaction of catalase with carrageenan applied to its recovery from murine liver

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ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form 1 March 2013
Accepted 9 March 2013
Available online 20 March 2013

Keywords:
Catalase
Carrageenan
Precipitation
Purification

ABSTRACT

The complexes formation between an acid polyelectrolyte: carrageenan with catalase was evaluated in order to understand the mechanism of their interaction. Catalase was selected as the model enzyme since it is applied in several biotechnological processes. The study of this interaction had showed that the non-soluble complexes take place in a pH interval which depends on the salt presence. Carrageenan showed to be an appropriate option for the catalase precipitation, since the amount needed for the full precipitation of the enzyme was low, with a fast kinetic and a slight stabilization of the protein structure. After establishing the optimal conditions, we were able to purify catalase from murine liver, a natural source, with a purification factor of 6.4 and a yield of 19%, using only one step in the enzyme purification process.

1. Introduction

One of the most challenging issues about large scale biotechnological operation is protein purification. High performance protein purification is commercially interesting for several industrial sectors such as food, biotechnology and pharmaceutics [1]. However, commercial purification methods commonly involve combinations of familiar techniques, all of which have their virtues and drawbacks [2]. The selection of an appropriate technique and the right operating conditions is important to ensure a successful protein purification process [3] and allow to integrate clarification and concentration procedures [4].

Precipitation is one of the protein purification operations that can be easily scaled-up [5]. The precipitation based on the interaction between proteins with polyelectrolytes (PE) to form stable protein–polyelectrolyte complexes (PPC) has been used as a method of purification of several enzymes [6–9]. The process of PPC formation is driven by a kinetic that depends on several factors such as protein and polyelectrolyte concentrations, pH, ionic strength and temperature. As long as the protein–polyelectrolyte interaction is established the complexes tend to form aggregates and the turbidity of the mixture media increases [6].

Among the advantages of this technique it can be highlighted how easy is to recover PPC (by centrifugation or decantation), the low polyelectrolyte concentration employed (up to 1% w/v), and the opportunity of reusing the polyelectrolyte [10]. Therefore, it is possible to recover a concentrated and purified protein extract from a natural source as the first step of an overall downstream process.

Catalases [(CAT) E.C. 1.11.1.6] are enzymes of widespread application in different biotechnological processes (e.g. food and textile industry). The main use of catalases include decomposition of H_2O_2 added to process streams to act as an antimicrobial agent [11]. Therefore, it is important to achieve an economic and simple purification protocol. Bovine and murine liver catalases are tetrameric enzymes and have an isoelectric point around 5.5, and a molecular mass of 240 kDa [12,13].

This work is focused on two main objectives. The first one is to study the interaction between catalase and carrageenan (CARR), in order to understand the complex formation between them. The second goal is to achieve a high performance purification protocol of catalase by optimizing the process factors related to its precipitation. To address this purpose we used CARR. CARR is a naturally occurring family of polysaccharides extracted from *Rhodophyceae* (red seaweed), that consists in sulfated or non-sulfated galactose and 3–6 anhydro galactose units with a molecular weight of 300–400 kD [14]. It is an anionic non-toxic economic polymer used in processed foods [15,16], pharmaceutical industries [17] and purification techniques [18,19].

2. Materials and methods

2.1. Chemical

Catalase from bovine liver, ι Carrageenan and Trichloroacetic acid (TCA) were purchased from Sigma Chem. Co. (USA). Phosphate

Abbreviations: CAT, liver catalase; CARR, carrageenan; PE, polyelectrolyte; PPC, protein–polyelectrolyte complexes.

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http://dx.doi.org/10.1016/j.seppur.2013.03.027

and Acetate buffer solutions of different pH were prepared at concentration of 50 mM, and pH was adjusted with NaOH or HCl in each case.

2.2. Solubility diagram of CARR and CAT–CARR mixtures

CARR and CAT–CARR mixture's turbidity (absorbance at 420 nm) was measured [10]. For this purpose, three different ratios [CAT]/[CARR] were selected. The titration was performed adding NaOH or HCl aliquots in 50 mM acetate–phosphate buffer at 25 °C. After system reached equilibrium, turbidity was measured and plotted vs. pH. These titration curves were made in order to estimate the pH at which CARR becomes insoluble and to analyze how the presence of CAT affects the soluble-insoluble equilibrium [7]. $pH_{0.5}$ value corresponds to the pH where the absorbance is half of the maximum and was calculated from the fitting of the data to the following equation:

$$Abs = \frac{Abs_{\max}}{1 + e^{\frac{-pH - pH0.5}{b}}} \quad (1)$$

being *Abs* the absorbance at each pH, Abs_{max} the maximum absorbance and *b* a fitting parameter related to the cooperativity of the precipitation process.

2.3. Titration curves of CAT with CARR

The formation of the insoluble PPC was studied by turbidimetric titration. A solution of 1% (w/v) CARR was used as titrant and added to a CAT solution under constant agitation at 25 °C. To avoid changes in pH during titration, both the CAT and CARR solutions were adjusted to the same pH value. This value was carefully chosen to achieve the best PPC insolubility condition (acetate buffer pH 4.00). In order to determine the influence of the ionic strength in the PPC formation, titrations were performed as mentioned but with the addition of different concentrations of NaCl. The turbidity was plotted vs. the total concentration of CARR. In all cases, plots were fitted to a hyperbolic equation. Solution absorbance was measured using a Jasco V 550 spectrophotometer with cell of 1 cm of path length.

2.4. Determination of the kinetics of PPC formation

The time to reach the maximum interaction between CARR and CAT was determined by measuring the turbidity (420 nm) of the CAT–CARR mixture during a period of time. The data was fitted to the following exponential equation:

$$Abs = Abs_{\max}(1 - e^{-kt}) \quad (2)$$

were *k* is the first order constant of the PPC formation, *Abs* is the absorbance at 420 nm, *t* is the time in minutes and $Abs_{\max}$ is the absorbance value if the time tends to infinity.

2.5. CARR effect on the native CAT fluorescence emission

This effect was analyzed obtaining the fluorescence emission spectrum at 300–400 nm of CAT in non-precipitation conditions, at increasing CARR concentrations. The scanning rate was 1 nm/min and the data acquisition was performed every 0.1 nm with a slit of 0.1 nm. The fluorescence spectra were obtained in an Amico Browman spectrofluorometer series 2000 using a thermostatized cuvette of 1 cm pathlength, and they were corrected using a software provided by the instrument manufacturer.

2.6. CARR effect on CAT secondary structure

Circular dichroism (CD) scan of CAT with and without CARR was carried out using a Jasco spectropolarimeter, model J-8150. The ellipticity values [θ] were obtained in millidegrees (mdeg) directly from the instrument. The cell path length of 1 cm was used for the spectral range 200–250 nm. In all cases, five scans were made.

2.7. CAT thermal stability

Thermal denaturation studies were carried out in a Jasco V-560 UV/visible spectrophotometer equipped with a Peltier-type temperature controller (ETC-505T) at a heating rate of 1 °C/min. This scan rate was found to provide an adequate time for equilibration. Thermally induced unfolding was monitored between 20 and 80 °C by absorbance at 280 nm [12]. The data analysis was made assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated and the absorptivity coefficients of both protein states were different. The unfolded protein fraction was calculated as:

$$\alpha = \frac{A_i - A_D}{A_N - A_D} \quad (3)$$

where α is the unfolded protein fraction, A_N and A_D are the absorbances of the native and unfolded states respectively, A_i is the absorbance at a given temperature. A non-linear least squares fitting was used to adjust the unfolded protein fraction with the temperature data. After, the temperature at the mid-point of denaturation (T_m) was determined.

2.8. Determination of catalase activity

CAT activity was measured using a UV spectrophotometer equipped with magnetic stirrer (Jasco V-560), using 12 mM H_2O_2 as substrate in 50 mM phosphate buffer pH 7.00. The degradation of hydrogen peroxide was followed at 240 nm and the relative activity was calculated as ΔAbs/min [20]. 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 25 °C.

2.9. 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 [21].

2.10. Evaluation of the purification processś performance

CAT activity and the total protein concentration were determined in the homogenate (H) and re-dissolved precipitates (PP). The specific activities (Sp. Act.), purification factor (PF) end recovery (R%) of CAT were calculated as follows:

$$\mathrm{Sp \cdot Act} = \frac{\text{Enzymatic activity (fraction)}}{\text{Total proteins (fraction)}} \quad (4)$$

$$\mathrm{PF} = \frac{\mathrm{Sp \cdot Act}(PP)}{\mathrm{Sp \cdot Act} \cdot (H)} \quad (5)$$

$$R\,\% = \frac{\text{Total CAT activity (PP)}}{\text{Total CAT activity (H)}} \times 100 \quad (6)$$

3. Results and discussion

3.1. pH effect on the PPC formation

Due to the presence of sulfonic strong groups, CARR is soluble in all the pH range assayed, as shown in Fig. 1. No turbidity was observed when the pH was varied between 2 and 12 in the solution of CARR. In the presence of CAT, an important increase of the absorbance values was observed in the pH range from 3 to 7, and this increase was proportional to the increase in the CAT concentration, due to the insolubility of the CAT–CARR complex. For this PE, the optimum pH interval at which the PPC is insoluble was pH 4.00.

3.2. Titration curves of CAT with CARR

The formation of the insoluble PPC was studied by turbidimetric titration and turbidity vs. CARR total concentration plots were obtained. From the analysis of these curves, it was possible to determine the optimum CARR concentration for PPC formation. In all cases, the data fitted to a hyperbolic function, since typical saturation curves were observed. The plateau value, when the maximum amount of CAT is forming the insoluble complex, depends on the enzyme concentration, confirming that the turbidity of the medium is proportional to the PPC concentration. From these curves, by non-linear fitting of the turbidimetric titration data the stoichiometry CAT–CARR ratio necessary to precipitate the maximum amount of the enzyme in solution can be calculated, according previous reports [22]. The CAT–CARR value found was, in average, 4.0 ± 0.8 g CAT/g CARR. This result indicates that CARR has a high capacity to precipitate CAT. This is important in designing scaling-up methods to precipitate proteins by using a PE because the target proteins are present in high volume of solution, so small masses of these PE are enough to completely precipitate the desired enzyme.

3.3. Ionic strength effect on the PPC formation

In Section 3.1, we demonstrated that there is a pH influence on the PPC formation, which can be explained due to columbic interactions between the CARR and CAT. In order to quantify the electrostatic character of the CAT–CARR interaction, we analyzed the effect of ionic strength given by NaCl, as shown in Fig. 2. The results indicate that 300 mM NaCl inhibited around 25% the formation of the insoluble CAT–CARR complex. Therefore, a significant part of the CAT–CARR complexes cannot be re-dissolved by increasing medium's ionic strength. This finding can be explained by the presence of strong sulfonic groups of CARR that have strong interactions with the basic groups of CAT or by the presence of other type of molecular forces in the complex formation such us hydrophobic effect, which are not affected by the ionic strength. This fact has been found in the formation of other enzyme–PE complexes [12].

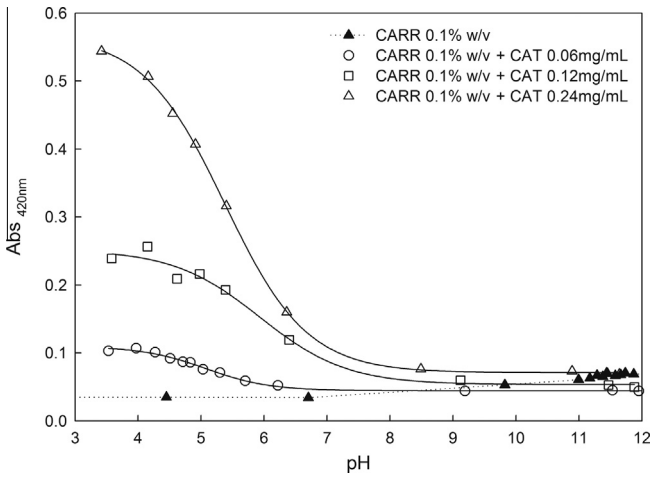

Fig. 1. Solubility diagrams of CARR (solid symbol) and CAT–CARR mixtures (empty symbols). Medium: 50 mM acetate–phosphate buffer. Temperature: 25 °C. CARR concentration: 0.1% (w/v).

3.4. Kinetics of the PPC formation

The formation of the protein–PE complexes follows a kinetics that depends on several factors such as protein and PE concentration, pH, ionic strength and temperature. The time necessary to achieve the best protein–PE interaction is characteristic for each protein–PE complex. From the kinetic curves of the mixtures CAT–CARR, the best conditions of the PPC formation were determined. The value of the kinetic parameters k and $t_{1/2}$ calculated from this curve were $(382 \pm 7)\ 10^{-3}\ \text{min}^{-1}$ and 1.81 ± 0.05 min. These results show that the kinetic for CAT–CARR precipitation is fast, and the maximum turbidity (or PPC concentration) is achieved in around 8 min.

3.5. Spectroscopic behavior of CAT in the CARR presence

To evaluate possible changes in the secondary and tertiary structure of the enzyme, the fluorescence and circular dichroism spectra of CAT in the absence and presence of CARR were carried out. The fluorescence emission spectra showed that the presence of CARR did not modify the emission band position (data not shown), suggesting that CARR did not change the polarizability of the TRP in the CAT. However, there was a decrease in the emission band intensity of CAT in the presence of CARR, and this decrease in the signal was proportional to the CARR concentration. This can be due to the enhancement of non-radiant emission, product of the greater number of molecules in the mixture as the CARR concentration increased.

On the other hand, Fig. 3 shows the CAT circular dichroism spectra in the absence and presence of CARR. Our data shows that CARR has a slightly stabilizing effect on CAT secondary structure. This result supports the idea that CARR can be used for the CAT purification.

3.6. CARR effect on CAT thermal stability

The interaction between a polymer and a protein may induce conformational changes in the protein's tertiary and secondary

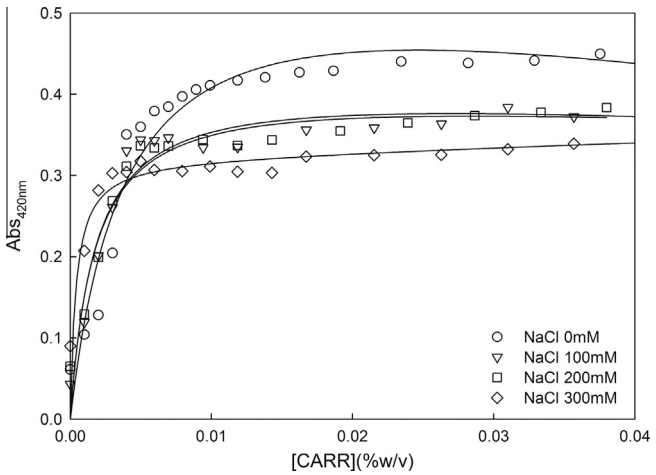

Fig. 2. Turbidity titration curves of CAT with CARR in 50 mM acetate buffer pH 4.0. Temperature: 25 °C. CAT concentration: 0.12 mg/mL.

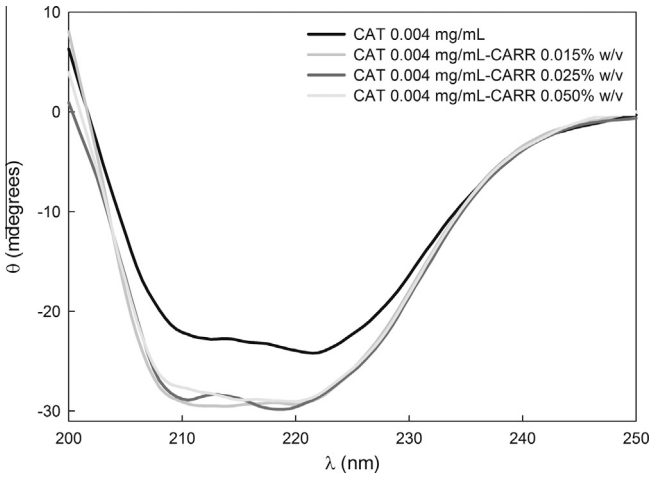

Fig. 3. Circular dichroism spectra of CAT in the absence and presence of CARR. CAT concentration: 0.004 mg/mL. Medium: 50 mM phosphate buffer pH 6.00. Temperature: 25 °C.

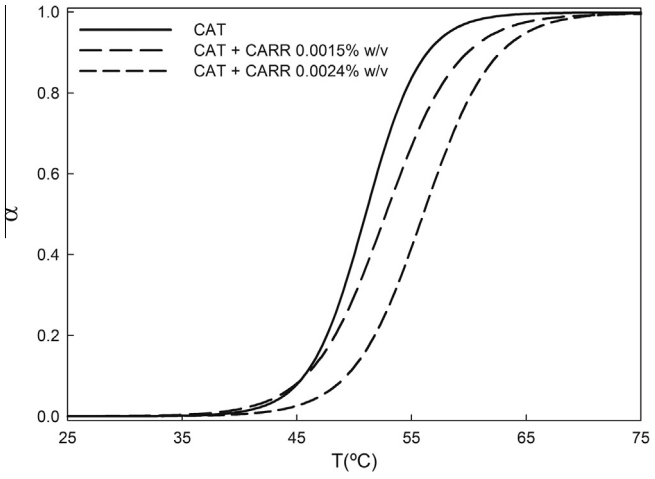

Fig. 4. Unfolding CAT fraction (α) vs. temperature in the absence and presence of CARR. Medium: 50 mM phosphate buffer pH 6.00. Temperature: 25 °C.

structure, with a loss of its thermodynamical stability and biological activity. The CAT thermal unfolding was analyzed by measuring the CAT absorbance at 280 nm as the temperature was increased. Fig. 4 shows the dependence of the unfolded CAT fraction (α) as function of the temperature in the presence of CARR. From these curves, the middle transition temperature (Tm) and the derivative at the inflection point of this thermal unfolding curve ($\partial\alpha/\partial T$) were calculated by a non-lineal fitting.

As shown in the figure, a shift of the curve to the right is produced as the CARR concentration increases. This fact indicates that CARR increases the thermal stability of the native state of CAT. The Tm values calculated were 323.99 ± 0.08 K for CAT in buffer and 329.0 ± 0.1 K for CAT in the presence of CARR, supporting the idea that CARR is a stabilizer of the enzyme. In addition, the $\partial\alpha/\partial T$ values (102 ± 2 10^{-3} K^{-1} for CAT in buffer) diminish in the presence of CARR (82 ± 2 10^{-3} K^{-1}), in agreement with a loss in the cooperativity of the unfolding process, being a proof of a stabilization of the enzyme.

3.7. CARR effect on the biological activity of CAT

In an enzyme purification protocol, it is vital to obtain a protein that not only conserves its secondary and tertiary structure, but also its enzymatic activity. The effect of CARR on the CAT activity was evaluated revealing that an increase in the CARR concentration induces a loss of the biological activity of around 20% at high CARR concentrations. This finding can be attributed to the strong interaction between the sulfonic groups of CARR and the amino groups of CAT which can produce a steric hindrance at the active site of the enzyme or also to the increase in the media viscosity as the CARR concentration increases.

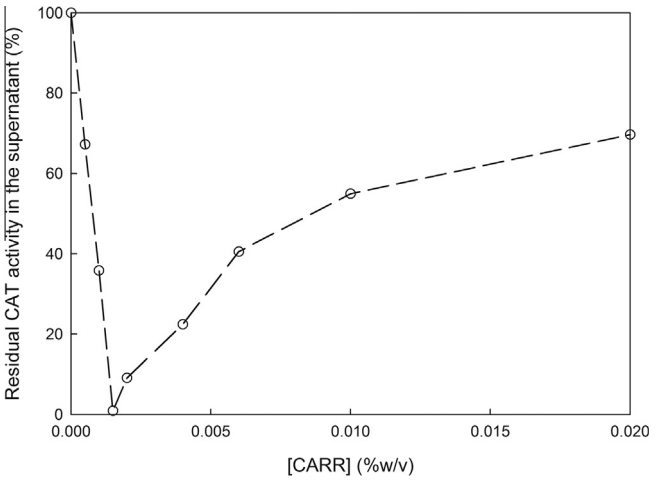

Fig. 5. CAT precipitation curves for CARR. CAT concentration: 0.12 mg/mL. Medium: 50 mM acetate buffer pH 4.00. Temperature: 25 °C.

3.8. CAT precipitation

The enzyme precipitation with CARR was performed in the pH condition previously determined. Fig. 5 corresponds to the precipitation curve, which shows the residual activity of the enzyme in the supernatant solution as the CARR concentration increases. CARR was able to quantitatively precipitate CAT. It is interesting to point out that there was a CARR concentration from which the extent of the CAT precipitation started to decrease. This fact can be due to the CARR–CARR interaction at high CARR concentration, instead of the CAT–CARR complexation, thus being the enzyme released to the media. These results indicate that the precipitation of CAT with CARR can be a suitable method of purification since the PE is non-expensive, natural and biodegradable. Taking this curve into account, the optimal CAT/CARR ratio for the precipitation process turned out to be 8 g CAT/1 g CARR.

With the precipitation conditions optimized, the re-dissolution of the PPC was assayed. The experiments were preformed in a pH range of 6.00–9.00 and ionic strength of 0–300 mM NaCl, and the CAT activity recovered was measured for each point. Fig. 6 shows the enzymatic activity of CAT after PPC re-dissolution in the assayed conditions. The higher activity value was obtained for pH values in the 7.00–7.50 range, for ionic strengths between 0 and 150 mM NaCl. Therefore, we choose as optimal experimental condition a pH of 7.00 (optimal pH for the enzymatic activity) without NaCl addition, avoiding the eventual CAT damages in a saline media as well as reducing the process cost.

Finally, the CAT precipitation with CARR was carried out from a natural source: mice liver homogenate. The purification results are shown in Table 1. It can be seen that even though the yield was not very high, the CAT was purified 6.4 times. In addition, the total protein concentration decreased, indicating that a considerable amount of the contaminant proteins were eliminated.

In order to verify the purity of CAT after the precipitation with CARR, polyacrylamide gel electrophoresis under denaturing

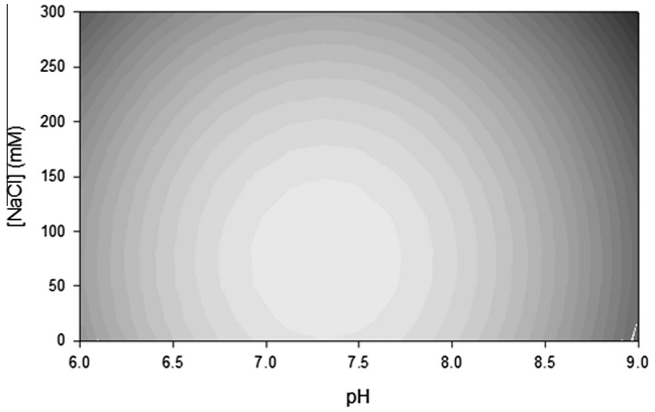

Fig. 6. Recovered activity after PPC re-dissolution. Precipitation conditions: Medium 50 mM acetate buffer pH 4.00. CAT concentration: 0.12 mg/mL. CARR concentration: 1.5 10^{-3}% (w/v). Temperature: 25 °C.

Table 1
Purification parameters obtained for the precipitation of CAT from murine liver homogenate with CARR 0.023% (w/v).

	Homogenate	Precipitation
Activity (UA)	$(384 \pm 9) \times 10^{-5}$	$(72.9 \pm 0.3) \times 10^{-5}$
Total protein (mg/mL)	17.8 ± 0.2	0.53 ± 0.03
Specific activity	$(216 \pm 5) \times 10^{-6}$	$(1385 \pm 6) \times 10^{-6}$
Yield (%)	100	19.0 ± 0.5
Purification factor	1	6.4 ± 0.6

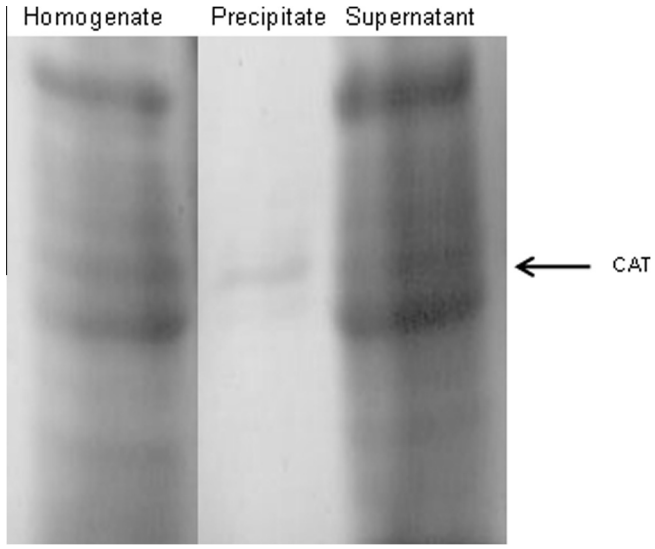

Fig. 7. SDS–PAGE of the murine liver homogenate (lane 1), the fraction obtained after the precipitation with CARR 0.023% (w/v) and re-dissolution in 50 mM phosphate buffer pH 7.00 (lane 2) and the supernatant obtained after the CARR addition to the murine liver homogenate (lane 3).

conditions with the anionic detergent sodium dodecylsulfate (SDS–PAGE) was carried out. Fig. 7 shows that most of the proteins contained in the homogenate (lane 1) are present in the supernatant (lane 3). A band corresponding to CAT is observed in the precipitated fraction (lane 2). From visual inspection it is clear that most of bands present in the crude homogenate disappeared after precipitation and that whose position corresponds to CAT remained practically unaltered in the precipitated fraction. Results corresponding to gel quantification analysis indicated that the purification factor and the recovery yield in the precipitated fraction calculated on the basis of enzymatic activity measurements differ significantly from that obtained from the gel imaging. This may indicate that the band observed in SDS–PAGE gel corresponds to a mixture of both active and non-active forms of CAT. This fact can explain the low recovery yield and the moderate purification factor obtained. The partial inactivation of other enzymes by sulphonated PE was previously observed [8].

However, this appears to be a satisfactory technique as a first step of a purification process, since it is possible to concentrate and purify the enzyme all at once.

4. Conclusion

In this work, CAT–CARR complexes formation was studied as a strategy for the catalase precipitation with this natural anionic polymer, commonly used in purification processes [9,18]. As expected, due to the electrostatic nature of the interaction, CAT–CARR precipitation takes place in acid media. From the turbidimetric titration of CAT with CARR the stoichiometry of interaction was calculated, indicating that CARR has a high capacity to precipitate CAT. Besides, PPC formation depends on the salt addition, corroborating the presence of some degree of electrostatic character of the CAT–CARR interaction. Moreover, the study of the PPC formation shows that CAT–CARR has a fast kinetic.

The spectroscopic study of CAT in the CARR presence revealed that CARR slightly stabilizes the secondary structure of CAT; in addition, CARR also thermally stabilizes the enzyme. However, the enzymatic activity of CAT decreased around 20% in the presence of CARR. The precipitation curves show that the extent of CAT precipitation by CARR was an appropriate option for CAT precipitation. The re-dissolution condition settled was: pH 7.00 without NaCl addition. The precipitation of catalase from murine liver homogenate under these conditions resulted in a purification factor of 6.4 and a yield of 19%, very reasonable result for a first step in a protein downstream process. The low recovery was attributed to a loss of the enzymatic activity due to the inactivation of the enzyme during the precipitation and re-dissolution operations.

5. Acknowledgements

This work was supported by a grant from CONICET PIP 0196/09-011. Dr. V. Boeris thanks CONICET for her fellowship.

References

[1] M. Pinelo, C. Ferrer, A.S. Meyer, G. Jonsson, Controlling the rejection of protein during membrane filtration by adding selected polyelectrolytes, Separation and Purification Technology 85 (2012) 54–60.
[2] Y. Xu, M. Mazzawi, K. Chen, L. Sun, P.L. Dubin, Protein purification by polyelectrolyte coacervation: influence of protein charge anisotropy on selectivity, Biomacromolecules 12 (2011) 1512–1522.
[3] F.C. Chong, W.S. Tan, D.R.A. Biak, T.C. Ling, B.T. Tey, A preparative hydrophobic interaction chromatography for purification of recombinant nucleocapsid protein of Nipah virus from clarified *Escherichia coli* homogenate, Separation and Purification Technology 71 (2010) 97–101.
[4] F. Hilbrig, R. Freitag, Protein purification by affinity precipitation, Journal of Chromatography B 790 (2003) 79–90.
[5] H. Wang, J. Wan, X. Cao, Preparation of a pH-sensitive affinity precipitation polymer and its application in purification of trypsin, Separation and Purification Technology 68 (2009) 172–177.
[6] M.C. Porfiri, B.M. Farruggia, D. Romanini, Bioseparation of alpha-amylase by forming insoluble complexes with polyacrylate from a culture of *Aspergillus oryzae* grown in agricultural wastes, Separation and Purification Technology 92 (2012) 11–16.
[7] V. Boeris, D. Spelzini, B. Farruggia, G. Picó, Aqueous two-phase extraction and polyelectrolyte precipitation combination: a simple and economically technologies for pepsin isolation from bovine abomasum homogenate, Process Biochemistry 44 (2009) 1260–1264.
[8] V. Boeris, D. Romanini, B. Farruggia, G. Picó, Purification of chymotrypsin from bovine pancreas using precipitation with a strong anionic polyelectrolyte, Process Biochemistry 44 (2009) 588–592.

[9] N. Woitovich Valetti, J. Lombardi, V. Boeris, G. Picó, Precipitation of chymotrypsin from fresh bovine pancreas using ι-carrageenan, Process Biochemistry 47 (2012) 2570–2574.
[10] T. Matsudo, K. Ogawa, E. Kokufuta, Complex formation of protein with different water-soluble synthetic polymers, Biomacromolecules 4 (2003) 1794–1799.
[11] J. Fiedurek, A. Gromada, Production of catalase and glucose oxidase by Aspergillus niger using unconventional oxygenation of culture, Journal of Applied Microbiology 89 (2000) 85–89.
[12] C.N. Pace, Measuring and increasing protein stability, Trends in Biotechnology 8 (1990) 93–98.
[13] S. Belluzo, V. Boeris, B. Farruggia, G. Picó, Influence of stabilizers cosolutes on catalase conformation, International Journal of Biological Macromolecules 49 (2011) 936–941.
[14] V.L. Campo, D.F. Kawano, D.B.d. Silva Jr, I. Carvalho, Carrageenans: biological properties, chemical modifications and structural analysis – a review, Carbohydrate Polymers 77 (2009) 167–180.
[15] K. Candogan, N. Kolsarici, Storage stability of low-fat beef frankfurters formulated with carrageenan or carrageenan with pectin, Meat Science 64 (2003) 207–214.
[16] Y.S. Gu, E.A. Decker, D.J. McClements, Influence of pH and ι-Carrageenan concentration on physicochemical properties and stability of β-lactoglobulin-stabilized oil-in-water emulsions, Journal of Agricultural and Food Chemistry 52 (2004) 3626–3632.
[17] A. Ellis, J.C. Jacquier, Manufacture of food grade κ-carrageenan microspheres, Journal of Food Engineering 94 (2009) 316–320.
[18] K. Mondal, I. Roy, M.N. Gupta, κ-Carrageenan as a carrier in affinity precipitation of yeast alcohol dehydrogenase, Protein Expression and Purification 32 (2003) 151–160.
[19] I. Roy, M.N. Gupta, κ-Carrageenan as a new smart macroaffinity ligand for the purification of pullulanase, Journal of Chromatography A 998 (2003) 103–108.
[20] S.A. Costa, T. Tzanov, A. Filipa Carneiro, A. Paar, G.M. Gübitz, A. Cavaco-Paulo, Studies of stabilization of native catalase using additives, Enzyme and Microbial Technology 30 (2002) 387–391.
[21] W. Choi, K. Chung, M. Chang, J. Chun, H. Lee, S. Hong, A turbidimetric determination of protein by trichloroacetic acid, Archives of Pharmacal Research 16 (1993) 57–61.
[22] D. Romanini, M. Braia, R.G. Angarten, W. Loh, G. Picó, Interaction of lysozyme with negatively charged flexible chain polymers, Journal of Chromatography B 857 (2007) 25–31.