Journal of Chromatography B, 1022 (2016) 87–92

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Production of alpha-amylase from *Aspergillus oryzae* for several industrial applications in a single step

María C. Porfirif, Esteban J. Milatich, Beatriz M. Farruggia, Diana Romanini*

Departamento de Tecnología, Facultad de Ciencias Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario, IPROBYQ-CONICET, Suipacha 531 (S2002RLK), Rosario, Argentina

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 5 April 2016
Accepted 8 April 2016
Available online 8 April 2016

Keywords:
Alpha-amylase
Intelligent polymer
Bioseparation
Enzymes
Aspergillus oryzae

ABSTRACT

A one-step method as a strategy of alpha-amylase concentration and purification was developed in this work. This methodology requires the use of a very low concentration of biodegradable polyelectrolyte (Eudragit® E-PO) and represents a low cost, fast, easy to scale up and non-polluting technology. Besides, this methodology allows recycling the polymer after precipitation.

The formation of reversible soluble/insoluble complexes between alpha-amylase and the polymer Eudragit® E-PO was studied, and their precipitation in selected conditions was applied with bioseparation purposes. Turbidimetric assays allowed to determine the pH range where the complexes are insoluble (4.50-7.00); pH 5.50 yielded the highest turbidity of the system. The presence of NaCl (0.05 M) in the medium totally dissociates the protein-polymer complexes.

When the adequate concentration of polymer was added under these conditions to a liquid culture of *Aspergillus oryzae*, purification factors of alpha-amylase up to 7.43 and recoveries of 88% were obtained in a simple step without previous clarification. These results demonstrate that this methodology is suitable for the concentration and production of alpha-amylase from this source and could be applied at the beginning of downstream processing.

1. Introduction

In their natural sources, proteins are normally found in very low concentrations or as part of a complex mixture of components. Besides, they can be unstable, thermolabile or sensitive to changes in variables of the medium such as pH, ionic strength, presence of cosolutes, etc.

The increasing demand of enzymes in different industrial activities (chemistry, food, textile, pharmaceutical, etc.) has promoted the development of purification technologies mainly if they are simple, low cost, fast, and clean. Alpha-amylase (α-Amy) is one of the enzymes with high demand for industrial applications. Starch depolymerisation by amylases is the basis for several processes such as glucose syrups production, bread making and brewing. It is also used as an additive in soaps, detergents and animal feeds, and in the treatment of paper, textiles, etc. [1,2]. Amylases are a class of hydrolases widely distributed in bacteria, fungi, plants and animals. They are members of family 13 in the classification of glycoside hydrolases according to Henrissat [3] which can specifically cleave the *O*-glycosidic bonds in starch.

Aspergillus oryzae is a filamentous fungus extensively used in fermentation industry because of its ability to secrete a variety of high-value industrial enzymes such as α-Amy, pectinase β-galactosydase, etc. [4–6]. The α-Amy synthesized from *A. oryzae* is an extracellular endoacting hydrolase that gives large oligosaccharides as products of starch degradation due to scission of internal α-1,4-linkages.

The complex formation between proteins and polyelectrolytes has been extensively studied [7–9]. due to its applications in the purification of proteins, control of protein release, enzyme immobilization and/or stabilization, etc. [10–18]. Precipitation of insoluble protein-polyelectrolyte complexes offers the basis for protein concentration and purification from a heterogeneous mixture. Furthermore, satisfying the requirements above mentioned, it has several advantages: it is easily scaled up, it requires simple equipment and a wide variety of alternative precipitants can be used [19].

A wide range of synthetic and natural [20–22] polyelectrolytes can interact with proteins to form stable protein-polyelectrolyte complexes that can be either soluble or insoluble depending on different experimental parameters such as concentration, number

* Corresponding author.
E-mail addresses: dromanini@conicet.gov.ar, romanini@iprobyq-conicet.gob.ar (D. Romanini).

http://dx.doi.org/10.1016/j.jchromb.2016.04.015
1570-0232/

and distribution of charged sites on the components, protein-polyelectrolyte ratio, pH and ionic strength of the medium, etc. This kind of polymers is often called "intelligent polymers". The insoluble complexes can be easily separated by centrifugation or simple decantation [23–25].

Eudragits® are different types of enteric copolymers widely used in controlled drug delivery. Depending on the pH, these copolymers act as polyelectrolytes which make them suitable for different purposes from gastric or intestinal soluble drug formulations to insoluble but swellable delivery forms, regulated by the number of charged and non-ionized (ether) groups in the structure of these copolymers. Some of them, which are soluble in aqueous media with different pH values, can be considered as polycations (Eudragit® type E) and others as polyanions (Eudragit® types L and S). Eudragit® E-PO (EPO) is a cationic copolymer based on 2-dimethylaminoethyl methacrylate, methyl methacrylate and *n*-butyl methacrylate [26]. It is an enteric polymer which is commercially available and it is an interesting alternative to form reversible soluble/insoluble complexes with proteins, as a strategy of enzyme isolation.

The aim of this work was to develop a strategy of recovery of α-Amy from a culture supernatant of *A. oryzae* by one simple step by insoluble complex formation with the polymer EPO.

2. Materials and methods

2.1. Chemicals

α-Amy from *A. oryzae* was purchased from Sigma Chem. Co. (USA) and EPO molecular average mass 47 kDa was gently donated by Evonick, Argentina. Phosphate buffer solutions of different pH were prepared at concentration of 50 mM and were adjusted with NaOH or HCl.

2.2. Enzyme assay

The measurements of α-Amy activity were carried out through the commercial kit *Amylase 405, kinetic unitest*, which was purchased from Winner Lab., Rosario, Argentina. This kit makes use of a specific substrate of α-Amy: 2-chloro-*p*-nitrophenyl-α-D-maltotriose (CNP-G3). The enzyme hydrolyzes the substrate releasing 2-chloro-*p*-nitrophenol (CNP) which absorbs at 405 nm (λ_{405} = 12.9 mM^{-1} cm^{-1}) and the color development is directly proportional to enzymatic activity. Thus, the reaction was followed by measuring the absorbance at 405 nm for 5 min and activities were calculated from the initial linear portion of the Abs. vs. time curves [27] and expressed as "U". One unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute. The enzyme assays were performed at a constant temperature of 20 °C in medium phosphate buffer at the optimum pH of 6.00.

The enzymatic activity of α-Amy was evaluated in the absence and presence of EPO at different molar ratios chosen from the plateau of titration curves. In order to evaluate the stability of α-Amy in the presence of EPO, the enzyme was incubated at pH 5.50 with the polyelectrolyte and the activity was measured for 24 h.

2.3. Microorganism, maintenance of culture

A. oryzae NRRL 695, donated by the National Centre for Agricultural Utilization Research (ARS), USDA, USA, was used in this study. It was propagated on Potato-Glucose-Agar (PGA) medium at 30 °C. The plates were grown for five days [28]. Conidia suspensions in 10% glycerol were stored at −20 °C in 1 mL aliquots.

2.4. Inoculum preparation

Conidia of *A. oryzae* NRRL 695 from five-day-old cultures in PGA plates were harvested by the addition of 15 mL distilled water. Then it was appropriately diluted to the required density of conidia and used as the master suspension. The number of viable conidia in the inoculum was determined by the counting technique using the Thomas Cell [28].

2.5. Substrate and culture media

Starch was used as substrate and sole carbohydrate source. 100 mL of enzyme production medium was prepared in 250 mL Erlenmeyer flask, containing (g/L): starch: 17.00, peptone: 1.90, urea: 1.25, glycerol: 0.60, KH_2PO_4: 0.50, $MgSO_4$: 0.25, $(NH_4)_2SO_4$: 0.025 and distilled water. The initial pH was adjusted to a value of 5.00 after which the flasks were autoclaved at 121 °C for 20 min. After cooling, the medium was inoculated with the master conidia suspension to a final concentration of $\sim 1.00 \times 10^6$ conidia/mL. Then, the inoculated medium was kept on rotary shaker (150 rpm) at 30 °C for 96 h. At the end of the incubation, the suspension was filtrated and used as the crude enzyme for precipitation experiments [28]. The content of proteins in the culture supernatant was characterized by electrophoretic separation on 1013% SDS-polyacrylamide gel (SDS-PAGE), on a Bio-Rad minigel apparatus, stained with coomassie blue.

2.6. α-Amy turbidimetric titration curves with EPO at different pH and ionic strengths

The formation of the insoluble polymer-protein complex was followed by means of turbidimetric titration [7]. Buffer sodium phosphate solutions with a fixed protein concentration (40 μM) were titrated at 20 °C in a cubic 1 cm path length glass cell with the polymer stock solution, in the total volume of 2.5 mL. The concentration of EPO solution was 0.5% w/w. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The complex formation was followed through a plot of absorbance (Abs.) at 400 nm vs. molar ratio EPO/α-Amy. We defined "stoichiometric polyelectrolyte/enzyme molar ratio" as the minimal EPO/α-Amy molar ratio in which the protein has been precipitated as an insoluble complex. It was calculated from the plot at the lowest polyelectrolyte concentration necessary to get a plateau. These values are important because they allow us to calculate the minimal polyelectrolyte amount necessary to fully precipitate the protein. The data have been expressed as the number of α-Amy moles bound per polyelectrolyte mol. The same was repeated at different ionic strengths by adding NaCl to phosphate buffer [24].

The time needed to form the complex was evaluated by measuring the time required to obtain the maximal absorbance at a fixed molar ratio [10].

2.7. Turbidimetric titration curves vs pH

Three different molar ratios from the plateau region of the titrations curves were selected. They were titrated with alkali and acid, at 20 °C. The protein concentration was 40 μM in the total volume of 8 mL. The Abs. at 400 nm was measured in a cubic 1 cm path length glass cell and plotted vs. pH. The same was repeated in the absence of α-Amy. These phase diagrams show the pH range where the polyelectrolyte-protein complex or the polyelectrolyte alone is soluble or insoluble.

2.8. α-Amy precipitation with EPO

Both commercial α-Amy and the crude enzyme obtained from the culture of *A. oryzae* were precipitated with EPO. For the commercial protein, solutions of α-Amy 75 μM and EPO to a relation of 0.106, 0.142, 0.177, 0.266 and 0.354 mol of polyelectrolyte per mol of enzyme were prepared in buffer Pi 50 mM at pH 5.50. These were incubated for five minutes at 20 °C and centrifuged at 1274 g for 10 min at 8 °C. The supernatant (SN) was separated from the precipitate (PP). Then, the PP was redissolved by adding phosphate buffer solution at pH 4.00 up to the initial volume. The enzymatic activity of α-Amy was measured in the SN, the redissolved PP and in a control (solution of α-Amy at the same concentration but in the absence of EPO, prepared in buffer Pi 50 mM at pH 6.00). The recoveries (R%) of the precipitation process were calculated using the following equation:

$$R\% = \frac{\text{Enzymatic Activity}_{(PP)}}{\text{Enzymatic Activity}_{(CONTROL)}} \times 100 \quad (1)$$

Different volumes of EPO 1% w/w were directly mixed with 2.5 mL of the crude enzyme, in order to precipitate the α-Amy from the culture supernatant. Then, the pH was adjusted to 5.50. These mixtures were incubated for five minutes at 20 °C and centrifuged at 1274 g for 10 min at 8 °C. The SN was separated from the PP, which was redissolved by adding phosphate buffer solution at pH 4.00 up to the initial volume. α-Amy activity was measured in the SN, redissolved PP and control (untreated culture). The concentrations of total proteins in all fractions were determined by the Warburg method [29]. Specific activities (Sp. Act.), purification factors (PF) and recoveries (R%) were calculated by Eqs. (2)–(4), respectively.

$$Sp.Act. : \frac{\text{Enzymatic Activity}_{(FRACTION)}}{[\text{Total Protein}]_{(FRACTION)}} \quad (2)$$

$$PF = \frac{Sp.Act._{(FRACTION)}}{Sp.Act._{(CONTROL)}} \quad (3)$$

$$R\% = \frac{\text{Enzymatic Activity}_{(FRACTION)}}{\text{Enzymatic Activity}_{(CONTROL)}} \times 100 \quad (4)$$

2.9. Statistical analysis

Experiments were done in triplicate and reported results represent the mean from three calculated values and their standard deviations.

3. Results and discussion

3.1. α-Amy/EPO complex formation

Fig. 1A shows different titration curves of α-Amy with EPO as Abs. at 400 nm vs. mol EPO/mol α-Amy, at different pH values of the medium. It can be observed that at pH 5–6, the absorbance increases with the increase of total polymer concentration, reaching a plateau. On the contrary, at pH 3.00 and 4.00 the turbidity of the medium was not affected. EPO is a cationic polyelectrolyte and α-Amy has an isoelectrical pH around 4.20, therefore, above this pH protein and polyelectrolyte have opposite net charge and the formation of α-Amy-EPO insoluble complexes was observed. The highest absorbance was obtained at pH 5.50, which suggests a greater size of the precipitate particles than other pHs. The stoichiometric polymer/protein molar ratio at this pH, chosen from the plateau of Fig. 1A, was 1:24 (0.0416). The time needed to form the complex at pH 5.50 was evaluated by measuring the time required to obtain the maximal absorbance at the stoichiometric molar ratio.

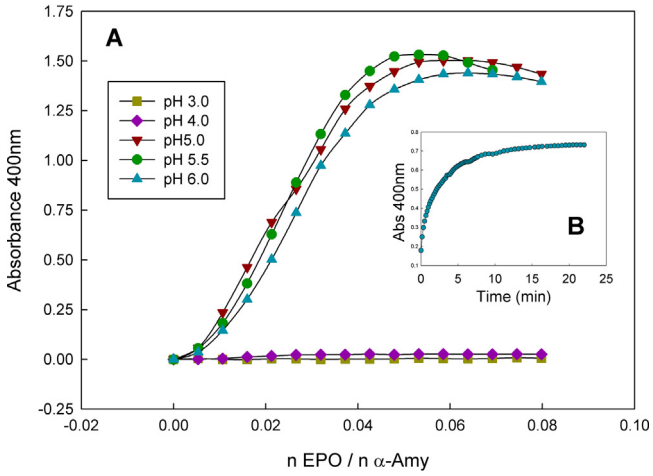

Fig. 1. Turbidimetric titration curves of α-Amy solution (40 μM) with EPO 0.5% w/w at different pHs (A). Kinetics of α-Amy/EPO complex formation (B). Temperature 20 °C, S.D. n = 3.

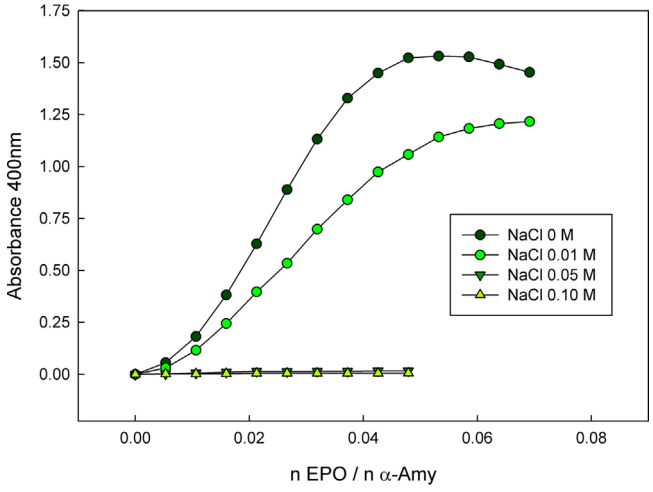

Fig. 2. Turbidimetric titration curves of α-Amy solution (40 μM) with EPO 0.5% w/w in a medium with phosphate buffer 50 mM at different ionic strength given by NaCl, pH 5.50. Temperature 20 °C, S.D. n = 3.

It was observed that only 5 min was required to form the complex (Fig. 1B).

Fig. 2 shows the α-Amy turbidimetric titration curves with EPO at different ionic strengths of the medium at pH 5.50. The insoluble complex formation was dramatically affected by ionic strength. An increase in ionic strength in the medium causes a decrease in turbidity of the system. This fact is consistent with the presence of coulombic forces involved in the interaction of insoluble complexes [25].

3.2. Solubility diagrams of α-Amy with EPO

Fig. 3A shows the phase diagrams of α-Amy with EPO and the polymer alone. The increase of pH above 7.5 indicates insolubilization of the polymer, while below this value it is completely soluble. In the presence of α-Amy, the dramatic increase in the maximum absorbance in the pH range between 4 and 7 suggests the formation of insoluble polymer/protein complexes, with a maximum of turbidity around 5.50. Fig. 3B shows the behavior of EPO along pH range in the absence of α-Amy at the same concentrations of polymer that Fig. 3A.

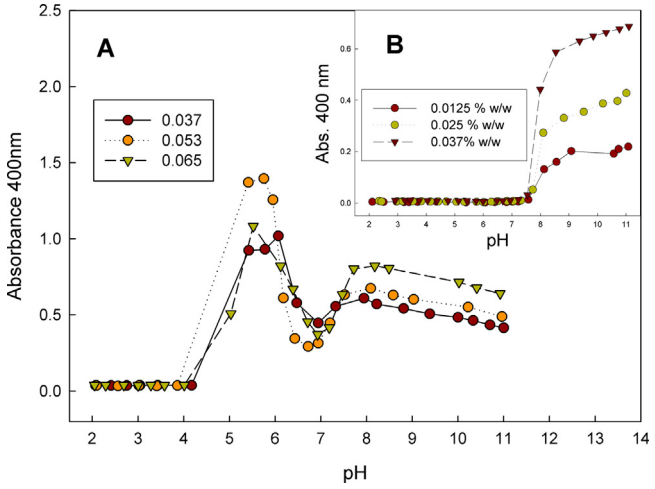

Fig. 3. Solubility diagrams: dependence of the absorbance at 400 nm vs. pH of the medium at different constant EPO/α-Amy molar ratios (A). Dependence of the absorbance at 400 nm vs. pH of the medium at different concentrations of EPO (% w/w)(B). Temperature 20 °C, S.D. n = 3.

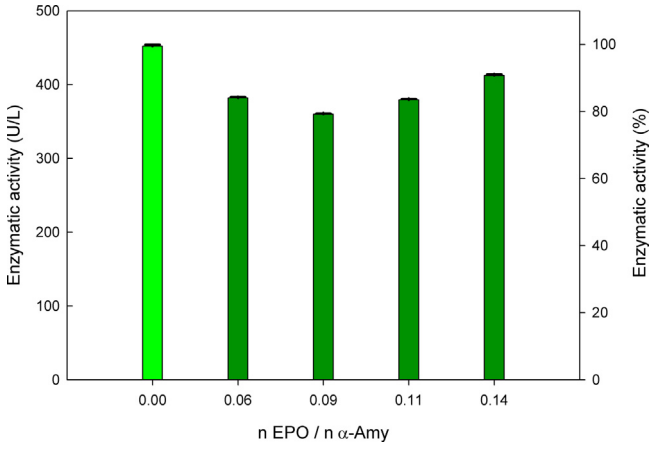

Fig. 4. Enzymatic activity of α-Amy in the absence and presence of EPO at different EPO/α-Amy molar ratios. [α-Amy] = 48 μM, pH 5.50. Temperature 20 °C, S.D. n = 3.

These results highlight the fact that the polyelectrolyte-protein interactions have an important electrostatic component, and this is reason why the complex was dissolved by pH change (Figs. 2 and 3). According to Clark and Glanz [30], the ionic strength was reported to reduce the electrostatic attraction between a protein and an oppositely charged polymer due to the shielding effect. This means that the protein is surrounded by the counterions existing in the solution, thereby reducing the precipitation efficiency [31]. This is the case for the interaction between α-Amy and EPO, given that insoluble complexes are completely solubilized in the presence of low concentrations of NaCl.

3.3. α-Amy biological activity and stability in the absence and presence of EPO

Fig. 4 compares the catalytic activity of α-Amy in the absence and presence of EPO. The molar ratios chosen correspond to an excess of polymer with respect to the stoichiometric value, to check the possible influence of an excess of EPO in the sample. The presence of increasing concentrations of EPO did not significantly affect the enzymatic activity.

To verify the effect of the polymer on the stability of the enzyme through time, α-Amy activity was measured for 24 h (Fig. 5). The

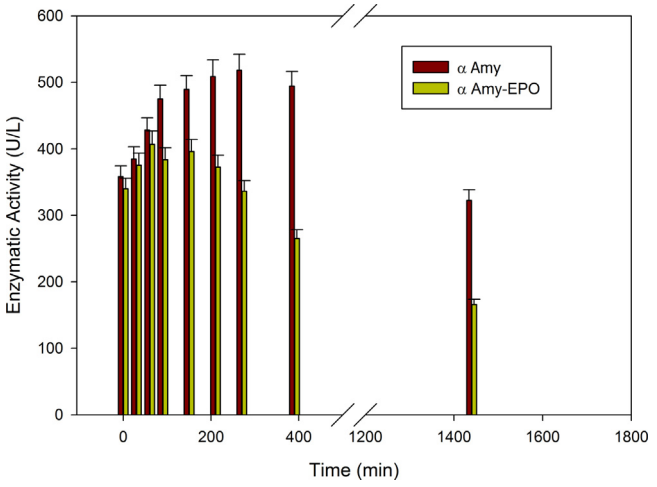

Fig. 5. Stability of α-Amy in the absence and presence of EPO at a constant EPO/α-Amy molar ratio of 0.142. [α-Amy] = 48 μM, pH 5.50. Temperature 20 °C, S.D. n = 3.

presence of EPO induced a significant drop on the enzymatic activity after approximately three hours of incubation, reaching the 50% with respect to the initial value after 24 h. These results indicate that an incubation of the enzyme in the presence of polyelectrolyte for over three hours risks enzymatic integrity.

3.4. Electrophoresis in SDS-PAGE

The content of proteins in the culture supernatant was analyzed by SDS-PAGE (Fig. 6) prior to applying precipitation protocol. A principal band appeared around 55 kDa (corresponding to α-Amy). Also, a contaminant of higher molecular weight was present. SDS-PAGE of the PP fraction was not possible due to interference of the polyelectrolyte.

The enzymatic activity remained stable in the presence of increasing concentrations of the polyelectrolyte, but it is affected at longer periods of incubation. Fortunately, binding and precipitation of the enzyme are completed in few minutes.

3.5. α-Amy precipitation with EPO

Fig. 7 shows the biological activities of commercial α-Amy after precipitation with EPO in the redissolved PP and SN, and they were compared with a control which was prepared at identical medium conditions. The R% reached around 60% when the EPO/α-Amy ratio was 0.142, and the lower supernatant activity confirmed the tendency of the enzyme to precipitate when bound to EPO. However, the addition of higher concentrations of polymer was unfavorable for the R%, given that the activities in the SN were higher than in the PP. This suggests that there is an optimum ratio which generates bigger polymer-protein particles with higher tendency to precipitate; also this effect can be seen by the decrease of turbidity after plateau in the turbidimetric curve (Fig. 1).

Parameters of precipitation, enzymatic activities and total protein concentration of α-Amy purification from the culture of *A. oryzae* are shown in Table 1. In all cases, specific activities after precipitation (PP) were higher than in the culture (control), suggesting a good purification which is reflected by the high purification factors. Besides, the R% reaches values that demonstrate a strong tendency of the enzyme to precipitate, which reflects low enzymatic loss during the precipitation process. These results suggest that the technique is appropriate for the purification of α-Amy from a complex medium, and also for the concentration of the enzyme of interest in the sample.

Table 1
Purification Table of α-Amy from the culture of *A. oryzae*, (S.D, n = 3).

Volume E-PO 1% w/w (μL)	Fracción	[Protein] (mg/mL)	Activity (U/L)	Sp. Act. (U/mg)	PF	R%
0	CONTROL	2.31 ± 0.1	51 ± 2	$22.08\ 10^{-3} \pm 2\ 10^{-5}$		
13.0	PP	0.32 ± 0.01	41.5 ± 2	$129.6\ 10^{-3} \pm 2\ 10^{-4}$	5.87 ± 0.01	82 ± 7
	SN	1.91 ± 0.09	9.9 ± 0.3	$5.18\ 10^{-3} \pm 3\ 10^{-5}$		
16.0	PP	0.28 ± 0.02	42 ± 2	$151.1\ 10^{-3} \pm 1\ 10^{-4}$	6.84 ± 0.01	84 ± 7
	SN	1.88 ± 0.08	10.3 ± 0.4	$5.479\ 10^{-3} \pm 5\ 10^{-6}$		
19.0	PP	0.27 ± 0.01	44.3 ± 2	$164.1\ 10^{-3} \pm 2\ 10^{-4}$	7.43 ± 0.02	88 ± 7
	SN	1.89 ± 0.09	9.5 ± 0.3	$5.026\ 10^{-3} \pm 3\ 10^{-6}$		

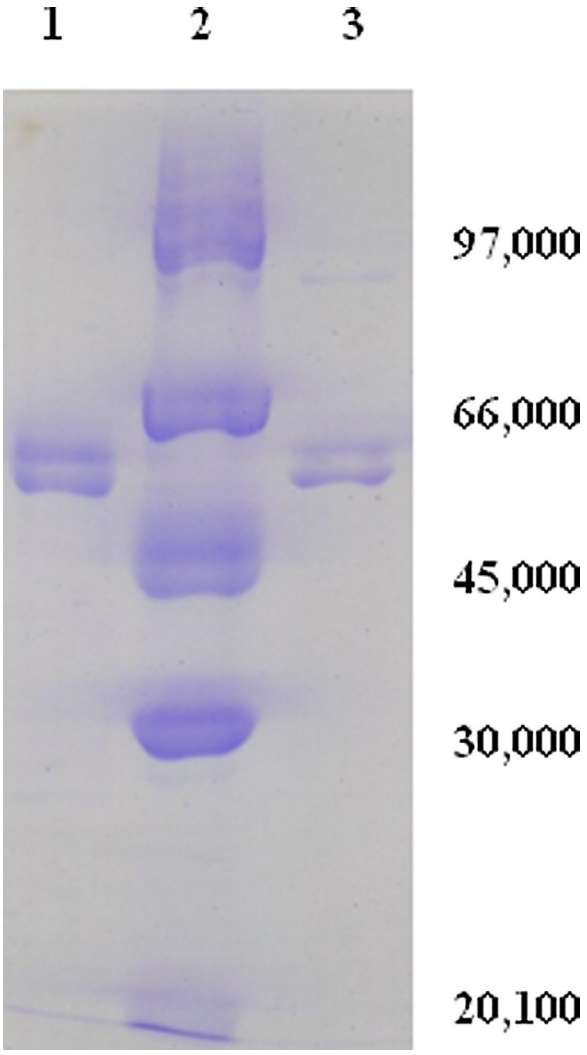

Fig. 6. SDS-PAGE of commercial α-Amy (lane 1), molecular weight markers (lane 2) and the culture supernatant of *A. oryzae* (lane 3).

According to Fig. 2 ionic strength affects the formation of insoluble complexes and increase the molar ratio n EPO/n α-Amy needed to achieve a maximum turbidity. Although the affinity between protein and polyelectrolyte could be very strong, the presence of ions in the culture supernatant partially inhibits the complex formation. This explains the higher amounts of polyelectrolyte required for α-Amy precipitation in the culture, which contains higher concentrations of salts than in assay with pure enzyme.

Other authors have recently reached similar or worse purification factors and recoveries using more purification steps and more expensive techniques [32,33]. High purification factors (7.43) and recovery (88%) shown by a simple step suggest that this technique

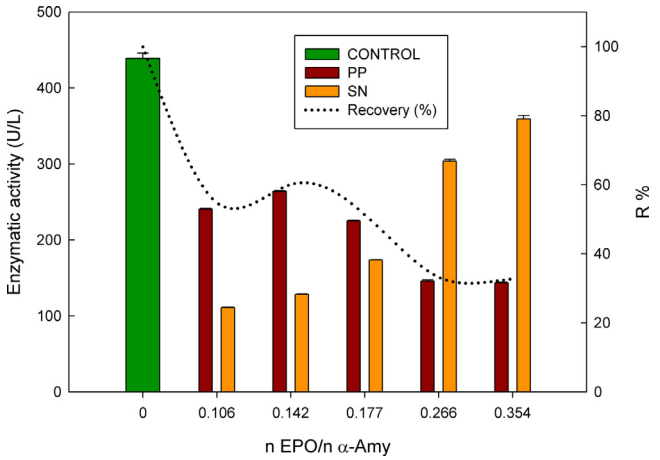

Fig. 7. Precipitation of α-Amy with EPO. Recoveries (R%) and activities measured in the redissolved precipitates (PP), supernatants (SN) and control, S.D. n = 3.

is suitable for the purification and concentration of α-Amy from this natural source without previous clarification step.

4. Conclusions

In this work a study on α-Amy/EPO insoluble complex formation as a novel industrial strategy in a single step for α-Amy yield from an *A. oryzae* culture is presented. Our experimental findings show that this is a simple, fast and low-cost methodology; moreover, previous clarification is not needed. On the other hand, EPO is cheaper than other charged polymers, offers the possibility to be used in food industry and its solubility profile enables it separation from the medium for recycling and reusing.

Acknowledgments

We would like to thank Evonik Degussa Argentina S.A for donating the EPO (Eudragit® E-PO) and the staff from the English Department (FCByF-UNR) for the language correction of the manuscript.

This work was supported by a grant from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-1730), CONICET (PIP 551/2012) and Secretaría de Ciencia y Tecnología, Universidad Nacional de Rosario (BIO 391), Argentina. We also thank the National Centre for Agricultural Utilization Research (ARS), USDA, USA for donating the strain of *Aspergillus oryzae*.

References

[1] G. Muralikrishna, M. Nirmala, Cereal α-amylases-an overview, Carbohydr. Polym. 60 (2005) 163–173.
[2] T. Bhanja Dey, R. Banerjee, Purification: biochemical characterization and application of α-amylase produced by *Aspergillus oryzae* IFO-30103, Biocatal. Agricult. Biotechnol. 4 (2015) 83–90.

[3] B. Henrissat, G. Davies, Structural and sequence-based classification of glycoside hydrolases, Curr. Opin. Struct. Biol. 7 (1997) 637–644.
[4] Y. Rahardjo, F. Weber, S. Haemers, J. Tramper, A. Rinzema, Aerial mycelia of *Aspergillus oryzae* accelerates α-amylase production in a model solid-state fermentation system, Enzyme Microb. Technol. 36 (2005) 900–902.
[5] L. Meneghel, G. Pellenz Reis, C. Reginatto, E. Malvessi, M. Moura da Silveira, Assessment of pectinase production by *Aspergillus oryzae* in growth-limiting liquid medium under limited and non-limited oxygen supply, Process Biochem. 49 (2014) 1800–1807.
[6] M. Carević, D. Veličković, M. Stojanović, N. Milosavić, H. Rogniaux, D. Ropartzc, D. Bezbradica, Insight in the regioselective enzymatic transgalactosylation of salicin catalyzed by β-galactosidase from *Aspergillus oryzae*, Process Biochem. 50 (2015) 782–788.
[7] D. Takahashi, Y. Kubota, K. Kokai, T. Izumi, M. Hirata, E. Kokufut, Effects of surface charge distribution of proteins in their complexation with polyelectrolytes in the aqueous salt-free systems, Langmuir 16 (2000) 3133–3140.
[8] N. Almeida, C. Oliveira, I. Torriani, W. Loh, Calorimetric and structural investigation of the interaction of lysozyme and bovine serum albumin with poly(ethylene oxide) and its copolymers, Colloid Surf. B 38 (2004) 67–76.
[9] A. Sediq, M. Nejadnik, I. El Bialy, G. Witkamp, W. Jiskoot, Protein-polyelectrolyte interactions: Monitoring particle formation and growth by nanoparticle tracking analysis and flow imaging microscopy, Eur. J. Pharm. Biopharm. 93 (2015) 339–345.
[10] C. Cooper, P. Dubin, A. Kayitmazer, S. Turksen, Polyelectrolyte-protein complexes, Curr. Opin. Colloid Interface Sci. 10 (2005) 52–78.
[11] F. Hilbrig, R. Freitag, Protein purification by affinity precipitation, J. Chromatogr. B 790 (2003) 79–90.
[12] T. Foreman, M. Khalil, P. Meier, J. Brainard, L. Vanderberg, N. Sauer, Effects of charged water-soluble polymers on the stability and activity of yeast alcohol dehydrogenase and subtilisin Carlsberg, Biotechnol. Bioeng. 76 (2001) 241–246.
[13] D. Djabali, N. Belhaneche, B. Nadjemi, V. Dulong, L. Picton, Relationship between potato starch isolation methods and kinetic parameters of hydrolysis by free and immobilized α-amylase on alginate (from *Laminaria digitata* algae), J. Food Comp. Anal. 22 (2009) 563–570.
[14] L. Cong, R. Kaul, U. Dissing, B. Mattiasson, A model study on Eudragit and polyethyleneimine as soluble carriers of α-amylase for repeated hydrolysis of starch, J. Biotechnol. 42 (1995) 75–84.
[15] A. Rodrigues, J. Cabral, M. Taip, Immobilization of *Chromobacterium viscosum* lipase on Eudragit S-100: coupling: characterization and kinetic application in organic and biphasic media, Enzyme Microb. Technol. 31 (2002) 133–141.
[16] M. Kahraman, G. Bayramoğlu, N. Kayaman-Apohan, A. Güngör, α-Amylase immobilization on functionalized glass beads by covalent attachment, Food Chem. 104 (2007) 1385–1392.
[17] G. Bayramoğlu, M. Yilmaz, M. Arica, Immobilization of a thermostable α-amylase onto reactive membranes: kinetics characterization and application to continuous starch hydrolysis, Food Chem. 84 (2004) 591–599.
[18] Y. Xu, M. Mazzawi, K. Chen, I. Sun, P. Dubin, Protein purification by polyelectrolyte coacervation: influence of protein charge anisotropy on selectivity, Biomacromolecules 12 (2011) 1512–1522.
[19] M. Vikelouda, V. Kiosseoglou, The use of carboxymethylcellulose to recover potato proteins and control their functional properties, Food Hydrocoll. 18 (2004) 21–27.
[20] A. Pessoa Jr., B. Kilikan, Purificación de produtos biotechnológicos, Editora Manole Ltda, Sao Paulo, 2005, pp. 89–113.
[21] A. Kumar, A. Srivastava, I. Galaev, B. Mattiasson, Smart polymers: physical forms and bioengineering applications, Prog. Polym. Sci. 32 (2007) 1205–1237.
[22] L. Arvind, N. Aruna, J. Roshnnie, T. Devika, Reversible precipitation of proteins on carboxymethyl cellulose, Process Biochem. 35 (2000) 777–785.
[23] M. Othman, A. Aschi, A. Gharbi, Polyacrylic acids–bovine serum albumin complexation: structure and dynamics, Mater. Sci. Eng. C 58 (2016) 316–323.
[24] M. Braia, M. Porfiri, B. Farruggia, G. Picó, D. Romanini, Complex formation between protein and poly vinyl sulfonate as a strategy of proteins isolation, J. Chrom. B 873 (2008) 139–143.
[25] C. Patrickios, L. Sharma, S. Armes, N. Billingham, Precipitation of a water-soluble ABC triblock methacrylic polyampholyte: effects of time, pH, polymer concentration, salt type and concentration and presence of a protein, Langmuir 15 (1999) 1613–1620.
[26] R. Moustafine, I. Zaharov, V. Kemenova, Physicochemical characterization and drug release properties of Eudragit® E PO/Eudragit® L 100-55 interpolyelectrolyte complexes, Eur. J. Pharm. Biopharm. 63 (2006) 26–36.
[27] K. Ishikawa, I. Matsui, K. Honda, H. Nakatani, Substrate-dependent shift of optimum pH in porcine pancreatic alpha-amylase-catalyzed reactions, Biochemistry 29 (1990) 7119–7123.
[28] R. Kammoun, B. Naili, S. Bejar, Application of a statistical design to the optimization of parameters and culture medium for α-amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product), Bioresour. Technol. 99 (2008) 5602–5609.
[29] O. Warburg, W. Christian, Isolation and crystallization of enolase, Biochemistry 310 (1942) 384–421.
[30] K. Clark, C. Glatz, Foreword recovery and purification of biological products, ACS Symp. Ser. (1990) 1–4.
[31] M. Porfiri, M. Braia, B. Farruggia, G. Picó, D. Romanini, Precipitation with poly acrylic acid as a trypsin bioseparation strategy, Process Biochem. 44 (2009) 1046–1049.
[32] T. Bhanja Dey, R. Banerjee, Purification, biochemical characterization and application of α-amylase produced by *Aspergillus oryzae* IFO-30103, Biocatal. Agricult. Biotechnol. 4 (2015) 83–90.
[33] M. Sahnoun, S. Bejar, A. Sayari, M.A. Triki, M. Kriaa, R. Kammoun, Production: purification and characterization of two α-amylase isoforms from a newly isolated *Aspergillus oryzae* strain S2, Process Biochem. 47 (2012) 18–25.