Separation and Purification Technology 92 (2012) 11–16

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

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

Bioseparation of alpha-amylase by forming insoluble complexes with polyacrylate from a culture of *Aspergillus oryzae* grown in agricultural wastes

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ARTICLE INFO

Article history:
Received 25 January 2012
Received in revised form 6 March 2012
Accepted 8 March 2012
Available online 17 March 2012

Keywords:
Precipitation
Insoluble complexes
Alpha-amylase
Polyacrylic acid
Polyelectrolytes

ABSTRACT

Precipitation of insoluble complexes between alpha-amylase from *Aspergillus oryzae* and polyacrylic acid was studied by us in a previous work as a strategy of protein concentration and purification. Here we studied the effect of these polyelectrolytes on the stability of the enzyme at the pH of higher interaction (3.00), as well as the stability of the precipitates formed after applied the above methodology. The polymers showed a stabilizing effect on the activity of alpha-amylase at pH 3.00, given the kinetic constants achieved when adjusting the experimental data to a process of first order inactivation: $2.20e^{-2} \pm 2e^{-3}$ for alpha-amylase alone, $7.7e^{-3} \pm 3e^{-4}$ and $8.2e^{-3} \pm 2e^{-4}$ for polyacrylic acid 240,000 and 100,000, respectively.

Also, this stabilizing effect is intensified when complexes are in the solid precipitated form before redissolve, given the lower kinetic constants of inactivation obtained: $1.7e^{-3} \pm 2e^{-4}$ and $2.6e^{-3} \pm 2e^{-4}$ for polyacrylic acid 240,000 and 100,000, respectively.

In a subsequent step, a strain of *A. oryzae* was used as enzymatic source. In order to stimulate alpha-amylase production and secretion by the microorganism, we used a minimum culture medium with wheat processing residues as substrate. This has economic and environmental benefits, because of the recycling of agricultural wastes. This culture showed a maximum productivity at the fourth day of incubation.

When the technique of concentration and purification of alpha-amylase was implemented from this culture we obtained purification factors around two and recoveries around 70%. These results demonstrate that this methodology is suitable for the concentration and purification of alpha-amylase from *A. oryzae*. Besides, it is inexpensive since it uses agricultural materials without commercial value.

1. Introduction

Affinity precipitation has been subject of study for many years, but has received more attention in the last time due to the development of new materials for its implementation [1]. Both natural and synthetic polyelectrolytes can strongly interact with proteins of opposite charge and form insoluble complexes that are the bases for protein concentration and separation from a heterogeneous mixture [2–5]. Several techniques have been used to characterize polymer–protein complexes and have provided information about different aspects of them. These systems can be followed by turbidimetric techniques since these complexes can significantly increase the turbidity of the medium [6,7].

We have previously demonstrated that precipitation of insoluble complexes between purified alpha-amylase (α-Amy) and polyacrylic acid (PAA) can be used as a strategy of alpha-amylase concentration and purification [8]. We also concluded that the enzyme forms insoluble complexes with polyacrylic acid at pH under 5.00 (PAA 100,000 Da) and 4.00 (PAA 240,000 Da) with a molar ratio PAA/alpha-amylase 1/52 and 1/154, respectively [8]. Besides, we have previously designed a methodology to purify alpha-amylase by precipitating the enzyme with excess of polyelectrolyte at pH 3.00 and redissolving it at pH 6.00 (around mayor stability of the enzyme) with high tendency of the enzyme to precipitate (recoveries: 73.79% and 64.26% for PAA 240,000 Da and 100,000 Da, respectively) [8].

So that it is fully functional it is necessary that the enzyme is stable after applying the conditions of the designed methodology. This aspect was studied in the present paper by following the kinetics of inactivation of α-Amy at the pH of precipitation (3.00) in the presence of the polyelectrolytes and in the precipitated form.

Alpha-amylases are members of family 13 in the classification of glycoside hydrolases (according to Henrissat [9]). *Aspergillus*

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http://dx.doi.org/10.1016/j.seppur.2012.03.004

oryzae has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a variety of high-value proteins and industrial enzymes, e.g., alpha-amylase [10–12] The enzyme synthesized from *A. oryzae* is an extracellular endo-acting hydrolase that gives large oligosaccharides as products of starch degradation because of scission of internal alpha-1,4-linkages and its three-dimensional structure has been well investigated by X-ray crystallography [13].

Starch is a polysaccharide that is widely distributed in nature as a reserve of stored energy in many species of plants, and occurs extensively in waste materials produced from the processing of plant raw materials. Starch-processing waste is produced in large quantities and causes pollution problems [14].

The aims of this work were: 1 – to analyze the effect of the polyelectrolytes on the kinetics of inactivation of alpha-amylase at pH 3.00; 2 – to isolate the enzyme with PAA from a culture of *A. oryzae* grown at the expenses of residues of wheat processing as starch source.

2. Experimental

2.1. Chemical

Alpha-amylase (α-Amy) from *A. oryzae* was purchased from Sigma Chem. Co. (USA) and polyacrylic acid, sodium salt (PAA), sol. in water, molecular average mass 240 kDa, 25% (W/W) (PAA 240,000) and 100 kDa, 35% (W/W) (PAA 100,000) were purchased from Aldrich and used without further purification. Phosphate buffer solutions of different pH were prepared at concentration of 50 mM and were adjusted with NaOH or HCl.

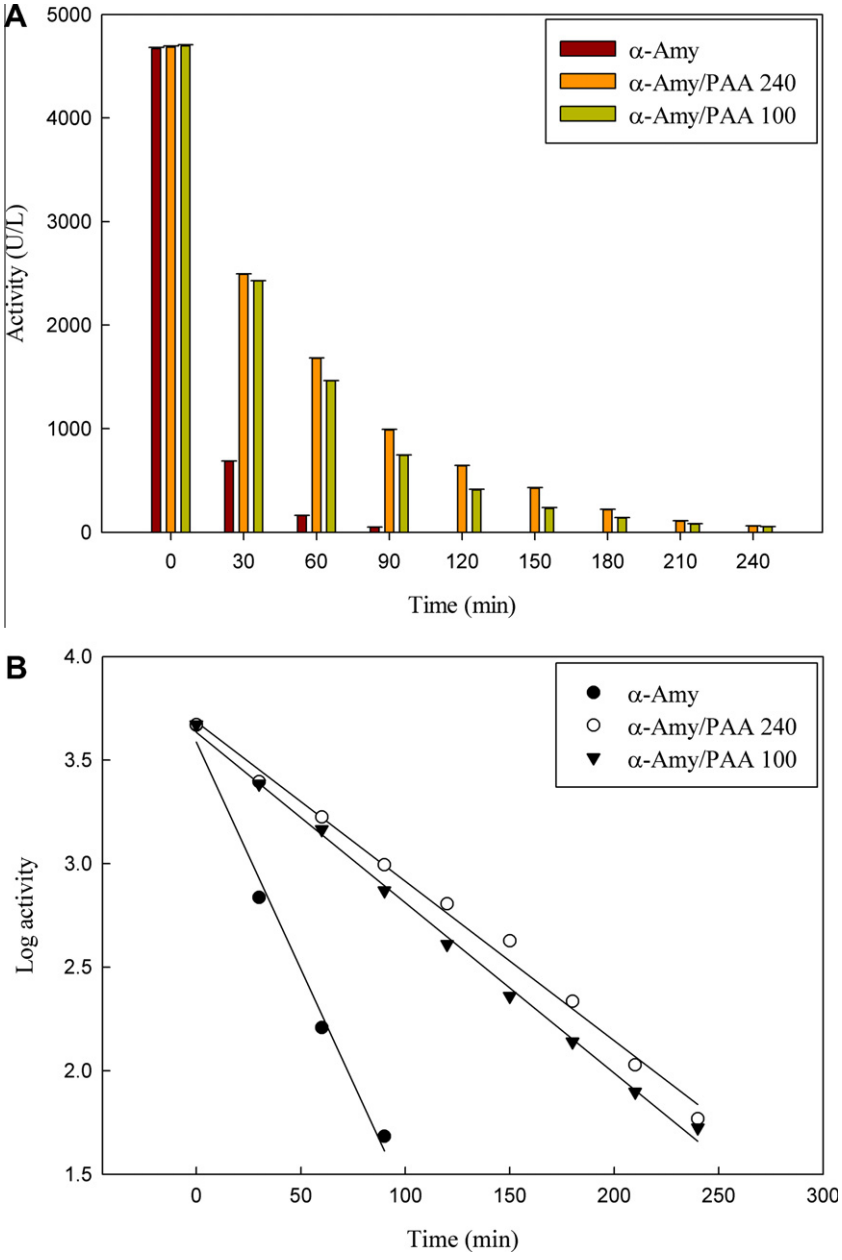

Fig. 1. A – Stability (activity vs. time) of alpha-amylase 2.75 mg/ml at pH 3.00 in the absence (—) and presence of polyacrylic acid 240,000 (—) and 100,000 (—) in a polymer:protein ratio 1:88 and 1:25, respectively. B – First-order kinetics of inactivation (log activity vs. time) of alpha-amylase 2.75 mg/ml at pH 3.00 in the absence (●) and presence of polyacrylic acid 240,000 (○) and 100,000 (▼) in a polymer:protein ratio 1:88 and 1:25, respectively.

Table 1
First-order kinetic constants (k, min^{-1}) of alpha-amylase acid inactivation at pH 3.00, in the absence and presence of PAA of molecular weight 240,000 and 100,000.

	Rate constants, k (min^{-1})
α-Amy	$2.20e^{-2} \pm 2e^{-3}$
α-Amy/PAA 240,000	$7.7e^{-3} \pm 3e^{-4}$
α-Amy/PAA 100,000	$8.2e^{-3} \pm 2e^{-4}$

Table 2
First-order kinetic constants (k, min^{-1}) of alpha-amylase inactivation in the precipitated form with PAA of molecular weight 240,000 and 100,000.

	Rate constants, k (min^{-1})
PAA 240,000	$1.7e^{-3} \pm 2e^{-4}$
PAA 100,000	$2.6e^{-3} \pm 2e^{-4}$

2.2. Enzyme assay

The measurements of alpha-amylase activity were carried out through the commercial kit *Amilase 405, kinetic unitest*, that was purchased from Winer 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 (ε_{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 (abs.) at 405 nm for 5 min and activities were calculated from the initial linear portion of the abs. vs. time curves [15] and expressed as "U". One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute.

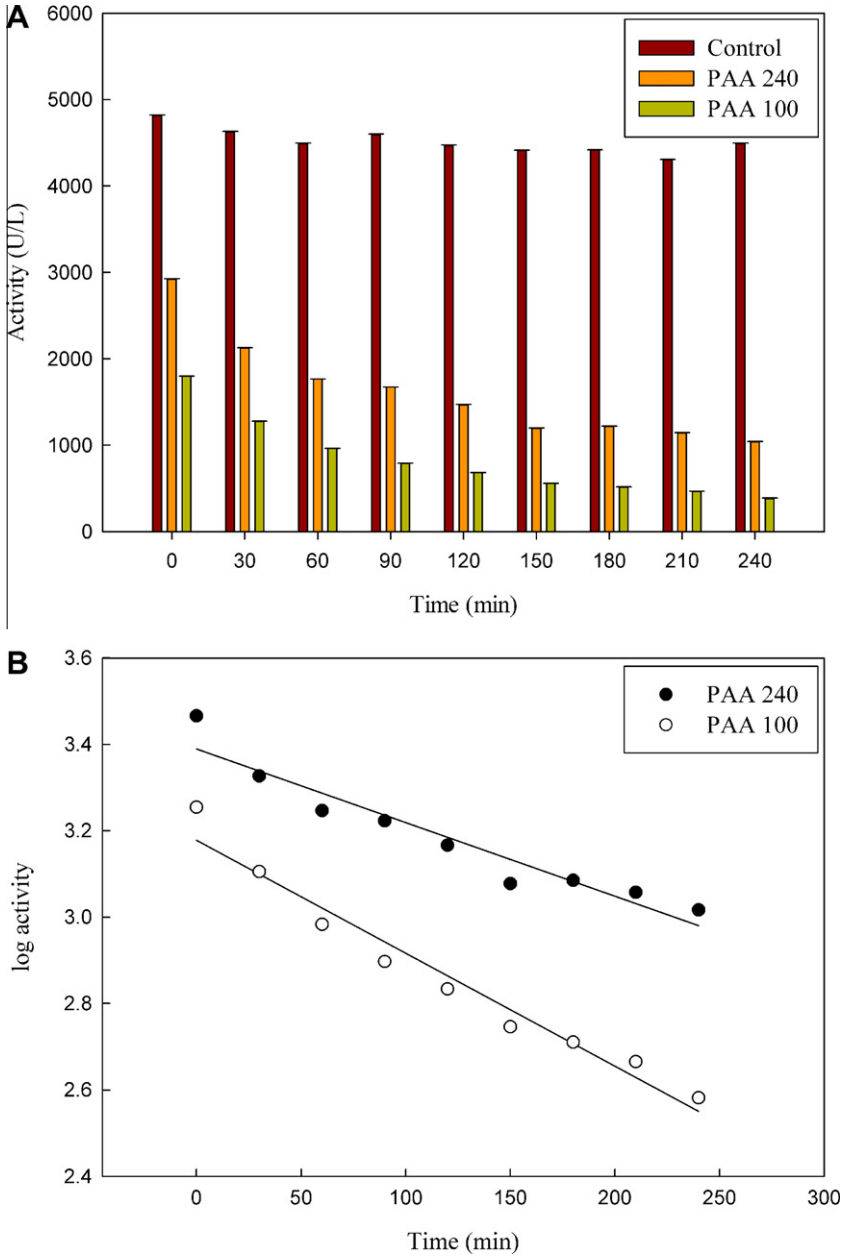

Fig. 2. A – Stability (activity vs. time) of alpha-amylase 2.75 mg/ml at pH 6.00 (—) and in the precipitated form with polyacrylic acid 240,000 (—) and 100,000 (—). B – First-order kinetics of inactivation (log activity vs. time) of alpha-amylase in the precipitates formed with polyacrylic acid 240,000 (●) and 100,000 (○).

The enzyme assays were performed at a constant temperature of 20 °C in medium phosphate buffer at the optimum pH of 6.00.

2.3. Effects of the polyelectrolytes on the kinetics of inactivation at pH 3.00

The inactivation of α-Amy at acid pH follows first-order kinetics, characterized by an exponential loss of activity through time (Eq. (1)):

$$\ln(act) = -kt + \ln(act_0) \quad (1)$$

where *act* is the enzyme activity at the time *t*, act_0 is the initial one and *k* the kinetic constant of inactivation [16].

The inactivation of α-Amy in phosphate buffer at pH 3.00 was analyzed by measurements of enzymatic activity after different periods of incubation in the absence and presence of PAA. Excessive amount of the polyelectrolytes respect α-Amy (1:88 for PAA 240,000 and 1:25 for PAA 100,000) were used. Measurements were performed after a shift in the pH of the medium from 3.00 to an optimum value of 6.00.

2.4. Stability of the precipitates

Stability of α-Amy in the precipitates formed with PAA was evaluated by monitoring the kinetics of inactivation of the enzyme after applying the strategy of concentration previously designed [8]. According to this methodology, α-Amy was precipitated at pH 3.00 by addition of PAA to reach the appropriate molar ratio polyelectrolyte/enzyme in each case. After incubation, centrifugation and phase separation steps, precipitates were redissolved at different periods of time (in phosphate buffer, pH 6.00). After redissolution α-Amy activity was determined and stability of the enzyme in the precipitates evaluated. These activities were compared to a control of the enzyme at pH 6.00.

2.5. 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 [17]. Spores suspensions in 10% glycerol were stored at −20 °C in 1 ml aliquots.

2.6. Inoculum preparation

Spores of *A. Oryzae* NRRL 695 from five-day-old in PGA plates were harvested by the addition of 15 ml distilled water. Then it was appropriately diluted for the required density of spores and used as the master suspension. The number of viable spores in the inoculum was determined by the counting technique using the Thomas Cell [17].

2.7. Substrate and culture media

Residues of wheat processing, obtained from local post-harvested fields, were used as substrates and sole carbohydrates source. Into 250 ml Erlenmeyer flask, 100 ml of enzyme production medium was prepared, containing (g/L): wheaťs wastes: 24.00, 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 spore suspension to a final concentration of $\sim 1.00 \times 10^6$ spores per 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 [17].

The content of proteins in the culture was characterized by electrophoretic separation on 10–13% SDS–polyacrylamide gel, on a Bio-Rad minigel apparatus, stained with coomassie blue.

2.8. Productivity in the culture

Productivity (alpha-amylase activity/time) was determined in the culture. Aliquots of this were taken after different periods of incubation of the inoculated medium at 30 °C. These were centrifuged for 5 min at 29,000*g* and α-Amy activity was measured. A graph of productivity (U/days) vs. time (days) allowed us to

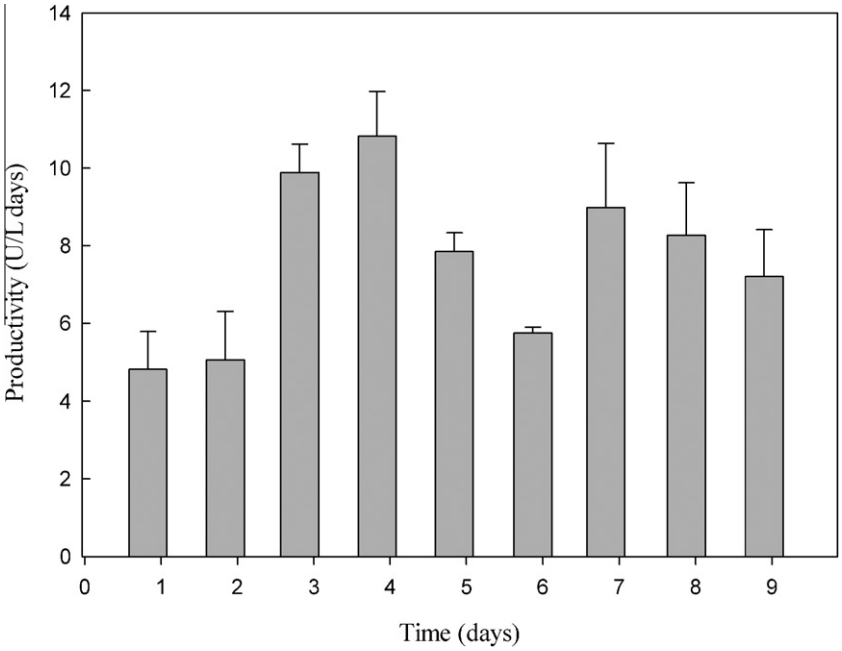

Fig. 3. Productivity of alpha-amylase (activity/time vs. time) in the culture of *Aspergillus oryzae*. Temperature of incubation: 30 °C.

determine the time in which higher enzymatic activity is achieved in the shortest time possible.

2.9. A. oryzae alpha-amylase precipitation with PAA of molecular weight 240,000 and 100,000

Different volumes of solution of PAA (240,000 or 100,000) were directly mixed with 2 ml of the culture at pH 3.00. The mixture was incubated for 30 min and centrifuged at 1274g for 15 min, at 8 °C. Precipitates were redissolved by the addition of a solution of buffer phosphate at pH 6.00. Alpha-amylase activity was determined in the supernatant (SN), redissolved precipitates (PP) and control (untreated culture). The concentrations of total proteins in all fractions were determined by Warburg method [18]. Specific activities (Sp. Act.), purification factors (PF) and recoveries (*R*%) were calculated by Eqs. (2)–(4), respectively:

$$\text{Sp.Act.} = \frac{\text{Enzymatic Activity}_{(\text{fraction})}}{[\text{Total Proteins}]_{(\text{fraction})}} \quad (2)$$

$$\text{PF} = \frac{\text{Sp.Act.}_{(\text{fraction})}}{\text{Sp.Act.}_{(\text{Control})}} \quad (3)$$

$$R\% = \frac{\text{Act}_{\propto\text{Amy(fraction)}}}{\text{Act}_{\propto\text{Amy(Control)}}} \cdot 100 \quad (4)$$

2.10. Statistical analysis

Experiments were done in duplicate and reported results represent the mean from two calculated values and their standard deviations. Statistical analysis of the results was carried out using the Marquardt–Levenberg algorithm.

3. Results and discussion

3.1. The effect of PAA on the kinetics of inactivation of a-Amy at pH 3.00

In a previous work Carlsen et al., have demonstrated that α-Amy from *A. oryzae* is extremely stable at neutral pH (between 5.00 and 8.00) but loses activity at lower values, with a substantial part of the enzyme irreversibly inactivated by first-order kinetics [16].

Here we corroborated the first-order kinetics of inactivation of the enzyme at pH 3.00 and evaluated the effects of the polyelectrolytes on its stability. Fig. 1A shows the activity (U/L) of α-Amy at pH 6.00 after incubation at pH 3.00 over time, in the absence and presence of PAA at a polymer:protein molar ratio of 1:88 (PAA 240,000 Da) and 1:25 (PAA 100,000 Da). From the semi-logarithmic plots (Fig. 1B) it is obvious that acid inactivation of α-Amy follows first-order kinetics even in the presence of PAA. The rate constants (k) obtained from the slope of the plots (Table 1) demonstrates that the polyelectrolytes stabilize the enzyme. These values also suggest that the stabilization is more significant for the PAA of higher molecular weight.

The mechanism behind acid-inactivation of α-Amy is described in terms of an unfolding of its three dimensional structure. So, the unfolded or partly unfolded molecules lose their activity [16]. Thus, the effects of PAA on the protein can be understood as stabilization of its three-dimensional structure, which delays the unfolding process.

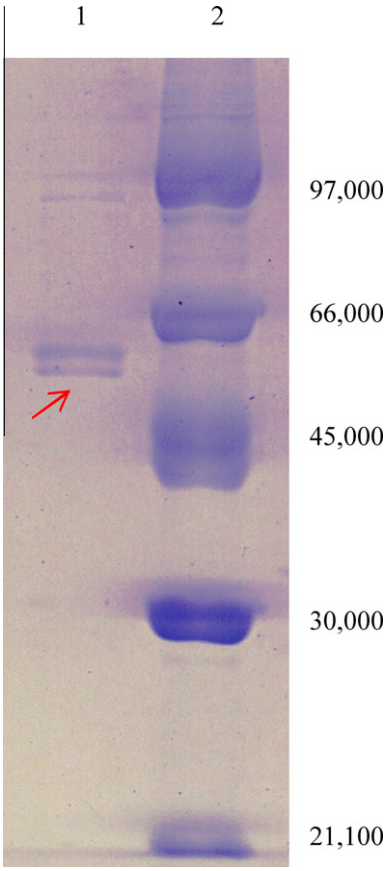

Fig. 4. SDS–PAGE of the culture of *Aspergillus oryzae* grown in agricultural wastes (lane 1) and molecular weight markers (lane 2).

3.2. Kinetics of inactivation of the precipitates

Fig. 2A shows the activities (U/L) of α-Amy in the control and precipitates obtained with PAA 240,000 and 100,000. High stability of the enzyme was achieved in the control at pH 6.00, but again the same pattern of inactivation was observed in the precipitates (as for the protein at pH 3.00, Section 3.1). The semi-logarithmic plots (Fig. 2B) demonstrate fist-order kinetics of inactivation of the enzyme in the precipitates. Table 2 shows the rate constants obtained in each case. These values indicate higher stability of α-Amy in the precipitated form than the enzyme at pH 3.00 before precipitate (Table 1). However, time of redissolution is an important factor to be taken into account and minimize it is an important event to optimize the recoveries of the process.

3.3. Productivity in the culture

Productivity through time (Fig. 3) shows two different stages in maximum α-Amy production: around fourth and seventh day of

Table 3
Purification table of alpha-amylase with PAA. Specific activity, purification factor (PF) and recovery (*R*%) achieved in the precipitates and control.

Vol. PAA (μl)	[PAA] %*P*/*P*	Specific activity (U/mg)	PF	*R*%
PAA 240,000				
20	2.50	$0.0454 \pm 4e^{-4}$	2.06 ± 0.07	70.9 ± 0.7
PAA 100,000				
10	3.50	$0.045 \pm 2e^{-3}$	2.1 ± 0.1	73.2 ± 0.7
Control				
		$0.0220 \pm 5e^{-4}$	1.00	100

incubation. We selected the first one to stop the growth of the culture and filtrate because of the benefits from the standpoint of the integrity of the enzyme and practical ones.

3.4. Electrophoresis in SDS–PAGE of the culture of A. oryzae

The content of proteins in the culture was analyzed by SDS–PAGE (Fig. 4) prior to applying precipitation protocol. A principal band appeared around 55 kDa (corresponding to alpha-amylase) and a minor one of higher molecular weight.

These results suggest that the sample is clean enough, with low content of contaminant. Thus, it is expected a successful concentration in α-Amy after precipitation with the polyelectrolytes, and a low purification factor since the starting sample is sufficiently pure.

3.5. Precipitation of alpha-amylase from A. oryzae with PAA of molecular weight 240,000 and 100,000

Specific activities, purification factors and recoveries achieved in the precipitates (PP) and control are shown in Table 3. Activity per mg of total proteins was two and a half fold higher in redissolved precipitates than control. The low purification factors were consistent with the assumption given by the electrophoresis (Section 3.4). However, recoveries show a strong tendency of the enzyme to precipitate with both polyelectrolytes, which suggests that this technique is appropriate to concentrate the sample in the enzyme of interest.

4. Conclusions

In this work we have examined the effect of polyacrylic acid of different molecular weight on the kinetics of inactivation of alpha-amylase at acid pH (3.00). The presence of the polyelectrolytes delays the inactivation progress, both in complexes before and after precipitation. Besides, the precipitated form seems to be more stable due to the lower rate constants of inactivation achieved.

On the other hand, we have precipitated alpha-amylase from a culture of *A. oryzae*, grown at the expense of wheat processing wastes as sole starch source, with polyacrylic acid of different molecular weight. Recoveries indicate an efficient precipitation but the enzyme was purified only twice, which is consistent with the few protein impurities in the starting sample (according to SDS–PAGE). So, these results suggest that this technique is suitable for concentration and primary purification of alpha-amylase from *A. oryzae*.

Acknowledgements

This work was supported by FonCyT No. PICT 2008-0186. We thank Lic. María Victoria González Eusevi for correcting the language of the manuscript. We also thank the National Centre for Agricultural Utilization Research (ARS), USDA, USA for donating the strain of *Aspergillus oryzae*.

References

[1] F. Hilbrig, R. Freitag, Protein purification by affinity precipitation, J. Chromatogr. B 790 (2003) 79–90.
[2] K. Clark, C. Glatz, Foreword recovery and purification of biological products, ACS Symp. Ser. (1990) i–iv.
[3] A. Kumar, A. Srivastava, I.Y. Galaev, B. Mattiasson, Smart polymers: Physical forms and bioengineering applications, Prog. Polym. Sci. 32 (2007) 1205–1237.
[4] D. Takahashi, Y. Kubota, K. Kokai, T. Izumi, M. Hirata, E. Kokufuta, Effects of surface charge distribution of proteins in their complexation with polyelectrolytes in an aqueous salt-free system, Langmuir 16 (2000) 3133–3140.
[5] C.S. Patrickios, L.R. Sharma, S.P. Armes, N.C. 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.
[6] C. Cooper, P. Dubin, A. Kayitmazer, S. Turksen, Polyelectrolyte–protein complexes, Curr. Opin. Colloid Interface Sci. 10 (2005) 52–78.
[7] H. Wang, J. Wang, X. Cao, Preparation of a pH-sensitive affinity precipitation polymer and its application in purification of trypsin, Sep. Purif. Technol. 68 (2009) 172–177.
[8] M.C. Porfiri, B. Farruggia, G. Picó, D. Romanini, Insoluble complex formation between alpha-amylase from *Aspergillus oryzae* and polyacrylic acid of different molecular weight, Process Biochem. 45 (2010) 1753–1756.
[9] B. Henrissat, G. Davies, Structural and sequence-based classification of glycoside hydrolases, Curr. Opin. Struct. Biol. 7 (1997) 637–644.
[10] Y. Rahardjoa, F. Weber, S. Haemersb, J. Tramperb, A. Rinzemaa, Aerial mycelia of *Aspergillus oryzae* accelerate α-amylase production in a model solid-state fermentation system, Enzyme Microb. Technol. 36 (2005) 900–902.
[11] Y. Imai, M. Suzuki1, M. Masamoto, K. Nagayasu, M. Kishimoto, Glucoamylase production of *Aspergillus oryzae* in fed-batch culture using a statistical regression model, J. Ferment. Bioeng. 78 (1994) 310–314.
[12] T. Zangirolami, M. Carlsen, J. Nielsen, S. Bay Jørgensen, Selection and characterization of a high (alpha-tegn)-amylase-producing variant in glucose-limited continuous cultures of *Aspegillus oryzae*, Mycol. Res. 104 (2000) 1241–1249.
[13] H. Swift, L. Brady, Z. Derewenda, E. Dodson, G. Dodson, J. Turkenburg, A. Wilkinson, Structure and molecular model refinement of *Aspergillus oryzae* (TAKA)-amylase: an application of the simulated-annealing method, Acta Crystallogr. Sect. B 47 (1991) 535–544.
[14] C.T. Li, O.J. Hao, K.P. Hsueh, Starch waste treatment by the activated sludge system, Water Res. 20 (1986) 1281–1289.
[15] 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.
[16] M. Carlsen, J. Nielsen, J. Villadsen, Kinetic studies of acid-inactivation of α-amylase from *Aspergillus oryzae*, Chem. Eng. Sci. 51 (1996) 37–43.
[17] 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.
[18] O. Warburg, W. Christian, Isolation and crystallization of enolase, Biochemistry Z 310 (1942) 384–421.