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New approach for separating *Bacillus subtilis* metalloprotease and α-amylase by affinity chromatography and for purifying neutral protease by hydrophobic chromatography

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Abstract

Proteases are commonly used in the biscuit and cracker industry as processing aids. They cause moderate hydrolysis of gluten proteins and improve dough rheology to better control product texture and crunchiness. Commercial bacterial proteases are derived from *Bacillus* fermentation broth. As filtration and ultrafiltration are carried out as the only recovery steps, these preparations contain also α-amylase and β-glucanase as the main side activities. The aim of this study is to purify and characterize the *Bacillus subtilis* metalloprotease from a commercial preparation, in order to study separately the impact of the protease activity with regards to its functionality on biscuit properties. Purification was achieved by means of affinity chromatography on Cibacron Blue and HIC as a polishing step. Affinity appeared to be the most appropriate matrix for large scale purification while ion exchange chromatography was inefficient in terms of recovery yields. The crude product was first loaded on a Hi Trap Blue column (34 μm, Pharmacia Biotech); elution was carried out with a gradient of NaCl in the presence of 1 m*M* $ZnCl_2$. This step was only efficient in the presence of Zn cations, because this salt promoted both protease stabilization resulting in high recovery yields and also complexation of amylase units into dimers resulting in amylase retention on the column and a better separation of the 3 activities. β-Glucanase was mostly non retained on the column and a part was coeluted with the protease. This protease fraction was then loaded on a Resource Phe column (15 μm, Pharmacia Biotech) in a last step of polishing. Elution was carried out with a linear gradient of 100–0% ammonium sulfate 1.3 *M*; protease was eluted at the beginning of the gradient and well separated from amylase and glucanase trace impurities. The homogeneity of the purified protease was confirmed by SDS–PAGE, which showed that its MW was about 38. pH and temperature optima were also determined on the fraction.

Keywords: Purification; *Bacillus subtilis*; Enzymes; Metalloprotease; α-Amylase; Protease

1. Introduction

Proteases and α-amylases are commonly used in the biscuit and cracker industry as processing aids. Proteases cause moderate hydrolysis of gluten proteins and improve dough rheology by reducing the dough retraction, so that the technologist can control the biscuit dimension [1]. They particularly decrease dough development time and resistance to extension [2]. It has been shown that both amylases and proteases improve the crispness of biscuits, particularly those made from medium-hard wheat flour [3]. Protease also reduces extrusion time, consistency as well as hardness.

Commercial bacterial proteases are derived from

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Bacillus fermentation broth. As filtration and ultrafiltration are carried out as the only recovery steps, these preparations contain also α-amylase (EC 3.2.1.1), alkaline protease (EC 3.4.21) and β (1–3), (1–4) glucanase (EC 3.2.1.6) as the main side enzyme activities.

It has been demonstrated from a commercial starting material that *Bacillus subtilis* neutral protease (EC 3.4.24.4) was a zinc enzyme, thanks to the direct proportionality between the zinc content and specific activity of the enzyme during the purification procedure [4]. Zinc is essential for catalytic activity and calcium is required to maintain the structural rigidity of the enzyme molecule [5]. The molecule contains 1 g of zinc per mole of enzyme [6]. It has also been shown that zinc ions were always the most effective in reactivating the apoenzyme followed by cobalt and manganese, but little or no reactivation occurred when calcium salts were added. This protease is particularly unstable, compared with thermolysin and subtilisin [7]. According to McConn et al. [8], it is stabilized against autolysis by many ions, but most markedly by alkaline earth metals. The peptide bonds susceptible to the action of the protease are mainly those involving hydrophobic amino acids and tyrosine [9].

All amylases from Bacillus species are believed to be metalloenzymes having calcium as a co-factor [10]. It has been established that they are resistant to proteolysis when combined with calcium ions [11]. Zinc has been found in significant amounts in *Bacillus subtilis* amylase only and is bound much less firmly than calcium [12].

Several protocols have been applied to purify the *B. subtilis* neutral protease and/or the amylase coproduced by this bacteria. Some authors have worked on a process using successively DEAE cellulose, ammonium sulphate precipitation and CM cellulose chromatography to purify the neutral protease but without giving attention to amylase and glucanase side activities [4].

Neutral protease was also purified and separated by gel permeation with several runs on Biogel P 60 [13]. But no mention was made of the β-glucanase and another process was used to purify α-amylase.

Purification of neutral protease was also tested by chromatography on hydroxylapatite. Amylase was removed by pulverising wheat starch and by filtering the solution to recover the protease since amylase coeluted with neutral protease [14]. After this step, they did not follow α-amylase nor β (1–3), (1–4) glucanase activities during purification. As a drawback, they had to use a phosphate buffer to elute neutral protease although phosphate is well known to inhibit metalloproteases, because of its metal chelating properties. According to this author, the method was found to be more reproducible than those using ion-exchange resins.

Partitioning in aqueous two-phase systems [15] and fraction precipitation with organic solvents have been mentioned to purify *B. subtilis* amylase [16].

Interaction of amylase with its substrate was also proposed for its purification by affinity chromatography but degree of adsorption decreases with temperature rising. In that purpose, Stredansky et al. [17] have shown that polyethylene glycol has the property to enhance adsorption of *Bacillus subtilis* amylase on starch.

However, liquid–liquid reversed micellar systems for the selective recovery of neutral protease and α-amylase are the only works to carry out both activities recovery in a single step [18].

Aiming to study separately the impact of protease and amylase activities with regards to their functionality on biscuit process, the *Bacillus subtilis* metalloprotease and α-amylase were purified and characterized from a commercial enzyme preparation. In order to achieve separation of both activities in a single step, we have developed a chromatographic procedure allowing simultaneous amylase and protease fractionation with high yields, low costs, good selectivity, resulting in an attractive alternative scaling-up procedure.

Then a polishing step using hydrophobic chromatography was applied to the neutral protease fraction to achieve purification to homogeneity and to study its properties.

2. Experimental

2.1. Materials

The crude powdered form of the commercial enzyme was purchased from Quest International Ireland (Dublin, Ireland).

EDTA, Trizma base, MOPS, zinc chloride, ammonium sulfate and calcium chloride were Sigma products (St. Louis, MO, USA).

2.2. Purification procedure

All chromatography steps were carried out at 4°C. The investigation was carried out on an Akta purifier 10 system from Pharmacia Biotech (Uppsala, Sweden). Detection was accomplished at 280 and 215 nm.

2.2.1. Affinity chromatography

This was performed on a Hi Trap Blue column (particle size 34 μm, volume 5 ml) obtained from Pharmacia Biotech.

2.2.2. Hydrophobic chromatography

This was performed on a Resource Phe (particle size 15 μm, volume 1 ml) obtained from Pharmacia Biotech.

2.3. Enzyme activity and protein assays

The protein content was determined according to Bradford with bovine serum albumin as a standard [19].

The protease activity was checked according to the modified Protazyme protocole (Megazyme, Co. Wicklow, Ireland), where phosphate buffer was replaced by 0.1 *M* Trizma Base, pH 7, to circumvent the inhibiting effect of phosphate ions. One unit is defined as the amount of enzyme which produces an equivalent of one μmole of tyrosine from soluble casein per min at 40°C and at pH 7.

The amylase activity was checked according to the Amylazyme protocol (Megazyme). One unit is defined as the amount of enzyme required to release one μmole of para nitrophenol from blocked nitrophenyl maltoheptaoside substrate per min at 40°C and at pH 7.

The glucanase activity was checked according to the Azo barley glucan method (Megazyme). One unit is defined as the amount of enzyme required to release one μmole of glucose reducing sugar equivalent per min at 30°C and at pH 7.

Determination of neutral protease optimum pH was realized with the modified Protazyme protocol by incubating the enzyme at 40°C with 1 m*M* calcium chloride for 10 min with buffers at different pH.

Determination of neutral protease optimum temperature was realised with the modified Protazyme protocole by incubating the enzyme in 0.1 *M* Trizma base pH 7, with 1 m*M* calcium chloride for 10 min at different temperatures.

2.4. Electrophoresis

SDS–PAGE was performed according to Laemmli [20] with the Ready gel cell system (Biorad, Hercules, USA) on a 4–15% ready gel. The migration conditions were those specified by Biorad. Proteins were stained with the silver staining kit of Biorad.

3. Results and discussion

3.1. Neutral protease and amylase separation by affinity chromatography

A 0.5 g of the commercial crude enzyme from Quest was dissolved in 5 ml of 0.01 *M* MOPS buffer pH 7, added with $ZnCl_2$ 2 m*M* and filtered through a 0.2 μm. The solution was charged on a column of Sepharose Cibacron Blue (Hi Trap Blue), equilibrated with the same buffer, at a flow rate of 2.5 ml/min. After washing unbound proteins, amylase and protease were eluted by 75 ml using a linear gradient from 0 to 150 m*M* NaCl followed by 20 ml using a linear gradient from 150 to 1000 m*M* NaCl.

Protease, α-amylase and β (1–3), (1–4) glucanase activities were determined in pooled fractions and a typical chromatogram is shown in Fig. 1a.

This step allowed to obtain one protease active fraction and two α-amylase active fractions, one of them being eluted later in the gradient (Table 1).

Protease activity was concentrated in fractions 8–14, while amylase activity was concentrated in fractions 16–21 and 22–25. High recovery yields were obtained: about 70% for protease and 87% for amylase activities.

A large degree of purification was obtained since amylase and protease fractions are scarcely contaminated by protease and amylase respectively. Protease fraction activity was totally inhibited by 1 m*M* EDTA, which confirms that it is a metalloprotease devoid of alkaline protease (data not shown).

3.1.1. Influence of salts as effectors on chromatography

3.1.1.1. Zinc ions. Addition of zinc ions resulted in a complete separation of both activities whereas with-

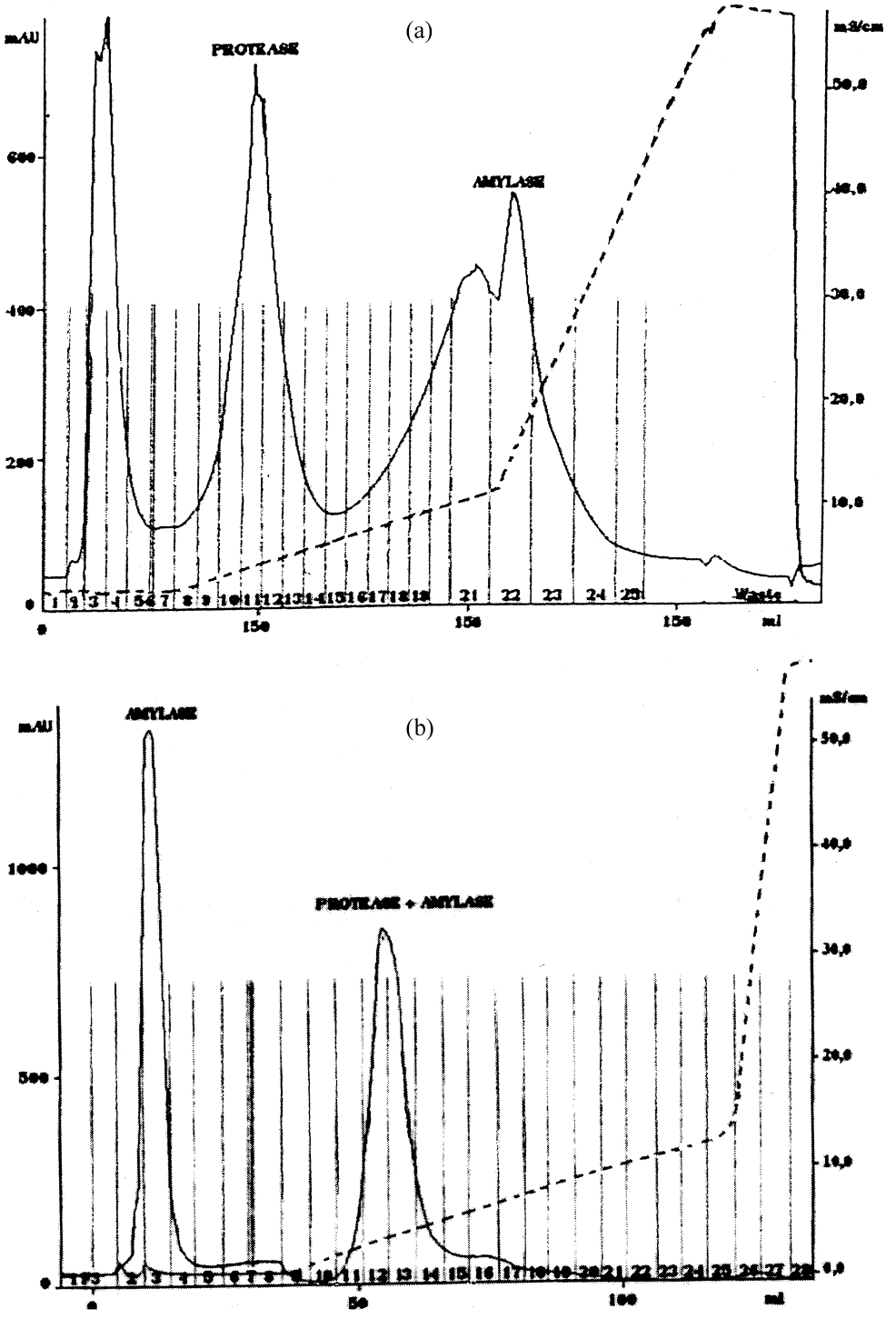

Fig. 1. (a) Separation of neutral protease and α-amylase from a commercial protease preparation on Cibacron Blue chromatography with $ZnCl_2$ in buffers. ———: UV absorbance at 280 nm; ---: conductivity. (b) Separation of neutral protease and α-amylase from a commercial protease preparation on Cibacron Blue chromatography without $ZnCl_2$ in buffers. ———: UV absorbance at 280 nm; ---: conductivity.

Table 1
Protease, α-amylase and β (1–3), (1–4) glucanase separation after affinity chromatography on Cibacron Blue

Fractions	Volume (ml)	Total protease activity (U)	Total amylase activity (U)	Total β (1–3), (1–4) glucanase activity (U)	Total proteins (μg)
Starting material	5	510	19 000	2016	5759
Unbound proteins, 3–5	15	0.75	1.65	202	68
8–14	35	362	15.05	1064	1508
16–21	35	12.1	8855	126.9	1409
22–25	38	4.3	7790	41.4	1067

out zinc, amylase and protease coeluted in a single peak (Fig. 1a and b, elution conditions were the same in the two chromatograms). This zinc effect is attributed to a change of amylase behavior during the chromatography. This phenomenon may be explained by zinc promoted amylase dimerization as described in the literature to be peculiar to *Bacillus subtilis* amylase [16,21]: in this interaction, one atom of zinc per molecule of dimer is taken up by the protein during dimerization.

3.1.1.2. Calcium ions. We have tested calcium influence during the chromatography (1 m*M* in buffers), as calcium is recognized to prevent neutral protease autolysis [4] and to protect amylase from proteolysis [10]. Our results showed that protease yield was unaffected whereas amylase yield decreased strongly from 87 to 4% (Table 2). Due to the loss of recovered proteins in amylase fraction, one concludes that calcium promotes amylase proteolysis during chromatography.

To conclude, the association of affinity chromatography on Cibacron Blue with the presence of zinc was used with success to separate in one step amylase and neutral protease. This procedure has been advantageously applied in a scaling-up and may therefore be proposed for an industrial scale.

3.2. Hydrophobic chromatography as a polishing step for neutral protease

To eliminate the side activities still remaining in the fraction, the protease active fraction was then adjusted to 1.4 *M* $(NH_4)_2$ SO_4, and to 1 m*M* $CaCl_2$ and loaded on a Phenyl sepharose column (Resource Phe) equilibrated with 10 m*M* MOPS buffer (pH 7) containing 1.4 *M* $(NH4)_2$ SO_4 and 1 m*M* $CaCl_2$, at a flow rate of 2 ml/min. After unbound proteins washing, protease was eluted with a linear gradient decrease from 1.4 to 0 *M* of $(NH4)_2$ SO_4. Protease, α-amylase and β (1–3), (1–4) glucanase activities were determined in pooled dialysed fractions (Table 3). A typical chromatogram is shown in Fig. 2.

Neutral protease was close to no longer being contaminated by amylase and contained a few glucanase activity. Loss of proteins showed that proteolysis had occurred, which confirmed the neutral protease sensitivity to autolysis.

3.2.1. Influence of salt type and concentration on hydrophobic interaction

According to Melander and Horvath [22], the type of salt used can have a profound effect on hydrophobic interactions. Use of NaCl up to 2 *M* instead of ammonium sulphate in the mobile phase was inefficient in retaining neutral protease on the column (data not shown). Finally, it appeared necessary to use ammonium sulphate at 1.4 *M* to promote strong adsorption of protease. This phenomenon is probably due to the higher molal surface tension effect of $(NH4)_2SO_4$, compared to the NaCl one.

3.2.2. Influence of calcium

Use of 1 m*M* calcium in chromatographic buffer

Table 2
Influence of calcium on α-amylase yields after affinity chromatography on Cibacron Blue

Amylase fractions	Total amylase activity (U)	Amylase yield (%)	Proteins (μg)
Without calcium	16645	87.6	2476
With 1 m*M* calcium	759.8	3.9	870

Table 3
Final purification of neutral protease by HIC on phenyl sepharose column

Fractions	Total protease activity (U)	Total amylase activity (U)	Total β (1–3), (1–4) glucanase activity (U)	Total proteins (μg)
2–5	0	16.8	0.25	6.8
9–10	130.4	0.7	33.1	358.4
14–15	0.9	2.8	1	0.6

was justified as stabilizing agent. This was confirmed by a test with and without calcium where protease yield dropped from 25 to 5% respectively.

3.3. Electrophoresis by SDS–PAGE

Homogeneity of the protease fraction (after step 2) and amylase (after step 1) was confirmed by SDS–PAGE (Fig. 3).

The neutral protease showed an apparent MW of about 38 000 (lanes Paff and Phic), which is comparable to the MW reported for *B. subtilis* neutral protease by Signor and al. [23]. Purity is improved between the first and the second step, as a small molecular weight protein band disappeared after hydrophobic chromatography.

The two amylase fractions, eluted at different salt concentrations, had the same apparent molecular weight of about 60 000. This result was comparable to the MW usually reported for *B. subtilis* amylase [24,25].

Both fractions only differed in their monomer–dimer proportion: fraction 1 (lane A1aff) contained a mixture of the dimeric form (MW=60 000) and the monomeric form (MW=30 000) while fraction 2 (lane A2aff) only showed the dimeric form.

These two fractions were pooled together, for the final report of purification efficacy.

3.4. Purification efficacy

Amylase and neutral protease purification results are summarized in Tables 4 and 5.

Affinity chromatography allowed to obtain neutral protease nearly devoid of amylase with a 69% yield and a purification factor of 2.6. In the same run,

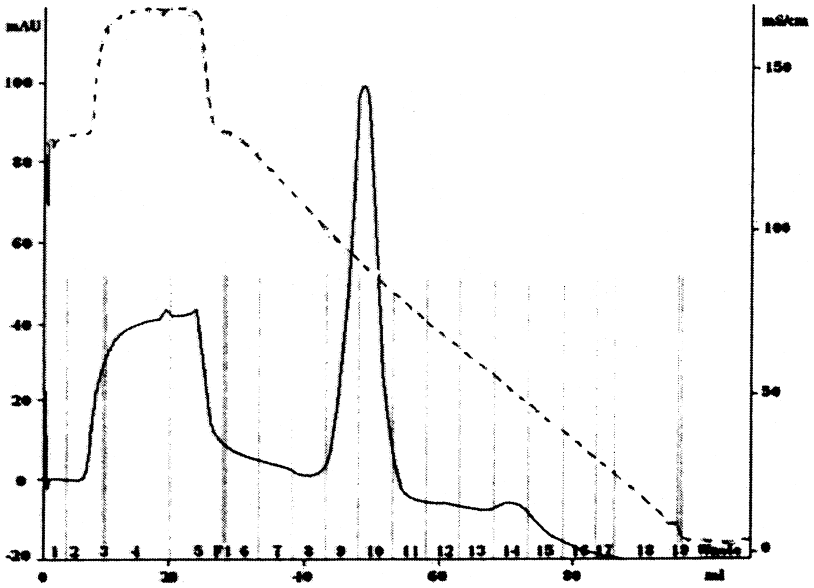

Fig. 2. Polishing of neutral protease from Cibacron Blue protease fractions on hydrophobic chromatography. ———: UV absorbance at 280 nm; ---: conductivity.

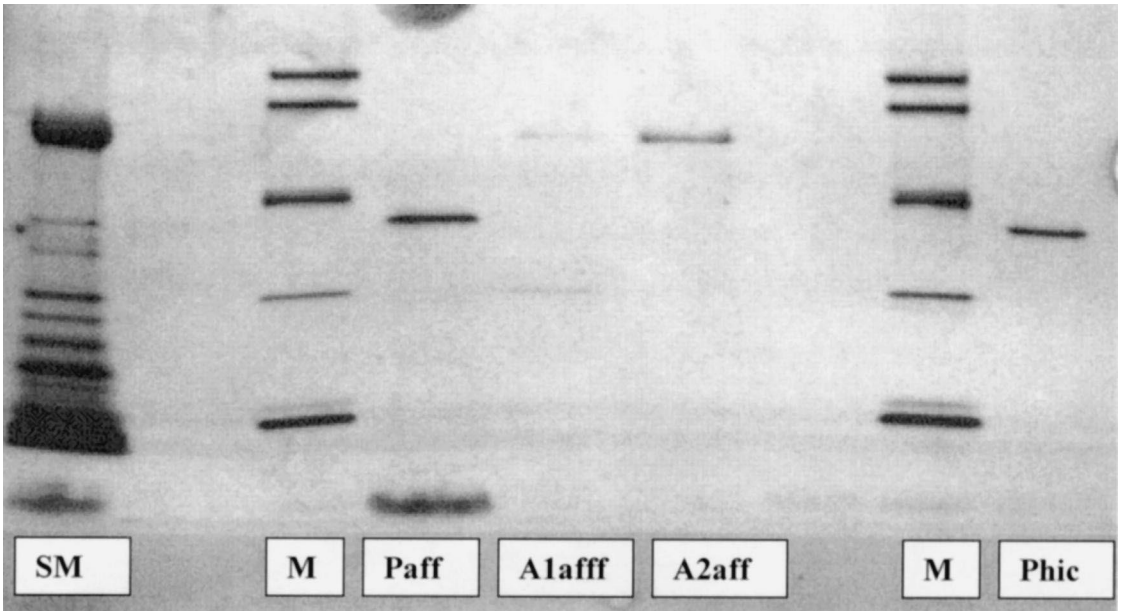

Fig. 3. SDS–PAGE electrophoresis profiles of amylase and protease fractions after affinity and hydrophobic chromatography. SM: starting material, Paff: neutral protease fraction after affinity step, A1aff: first amylase fraction after affinity chromatography, A2aff: second amylase fraction after affinity chromatography, Phic: neutral protease fraction after hydrophobic chromatography, M: marker proteins including, in order of decreasing molecular mass, ovotransferrin (78 kDa), bovin serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), myoglobin (17 kDa), cytochrome *c* (12 kDa).

Table 4
Purification of *B. subtilis* neutral protease by affinity and hydrophobic chromatography

Step	Total volume (ml)	Total activity (U)	Total protein (μg)	Specific activity (U/μg)	Purification factor	Yield (%)
Starting material	5	510	5759	0.088	1	100
Pooled fractions after affinity	35	352	1508	0.23	2.61	69
Pooled fractions after HIC	10	130.4	358.4	0.36	4.09	25.5

Table 5
Purification of *B. subtilis* α-amylase by affinity chromatography

Step	Total volume (ml)	Total activity (U)	Total protein (μg)	Specific activity (U/μg)	Purification factor	Yield (%)
Starting material	5	19 000	5759	3.29	1	100
Pooled fractions after affinity	73	16 645	2476	6.72	2.04	87.6

amylase was purified two-fold with a high yield of 87%.

In a further polishing step using hydrophobic chromatography, a purification of about four-fold compared to the starting material was obtained for protease.

3.5. pH and temperature optima of neutral protease

Figs. 4 and 5 illustrate pH and temperature effects on protease activity in the presence of 1 m*M* calcium chloride. A pH optimum near neutrality was identified and a quick activity decrease beyond 6.5 and

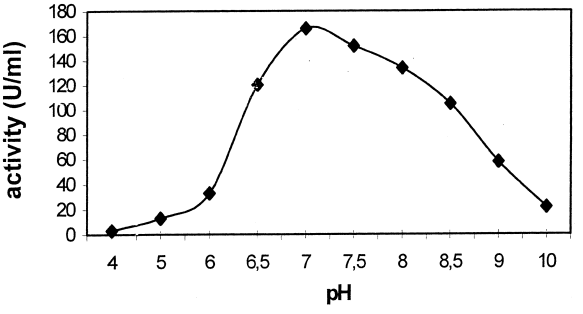

Fig. 4. Effect of pH on neutral protease activity.

8.5. The temperature optimum is about 55°C, which has also been shown by Tsuru et al. [26].

4. Conclusions

In this study, a new and attractive purification scheme was proposed to purify in a single step amylase and neutral protease from a *Bacillus subtilis* extract. We have demonstrated that affinity chromatography on Cibacron blue efficiency was dependent on the presence of zinc to achieve a complete separation of both activities with high recovery yields. This synergy is attributed to zinc functionality which promotes amylase dimerization resulting in a modification of protein–ligand interactions.

In order to improve purification degree, hydrophobicity chomatography on Phenyl sepharose was applied on the protease fraction. We confirmed in this step the stabilizing effect of calcium ions on neutral protease activity.

This innovative approach can be applied in an industrial downstream processing step to recover simultaneously *Bacillus subtilis* amylase and neutral protease.

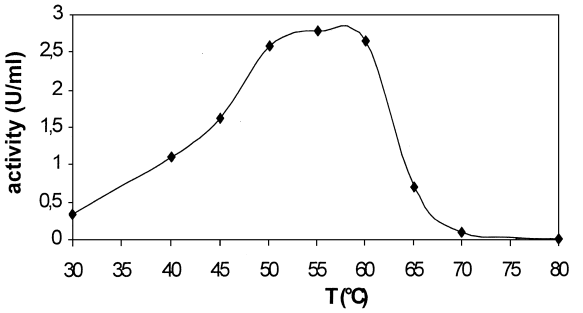

Fig. 5. Effect of temperature on neutral protease activity.

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