Partial Purification Of An Alkaline Protease From A New Strain Of Aspergillus Oryzae AWT 20 And Its Enhanced Stabilization In Entrapped Ca-Alginate Beads

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Citation

Abstract

An alkaline protease was produced and partially purified from a new strain of Aspergillus oryzae by using two chromatographies i.e. ion exchange chromatography on CM-Sephadex C-50 and gel filtration on Sephadex G-100 yielding an active major protein peak with ~29.29 purification fold. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) indicated that the purified alkaline protease is a monomeric enzyme with a molecular mass of ~33 kDa. The purified enzyme was completely inhibited by serine protease inhibitor, PMSF, and activated by Mg^{+2} & Ca^{+2} ions. The purified enzyme was immobilized by entrapment on Ca-alginate carrier. The optimum pH of the immobilized enzyme shifted to a more alkaline range (9.5) as compared with the free enzyme (9.0). It showed enhanced stability in acidic as well as alkaline environments in comparison to the free enzyme. The K_m' value (5.0 mg casein/ml) for the immobilized enzyme was one and a half-fold higher than for the soluble enzyme but there was no significant effect on V_{max} value (750 ± 2.45 µg tyrosine/min/ml) for both forms of the enzyme.

INTRODUCTION

Industrial enzymes have seen a spectacular rise in their production in the last three decades. The growth of industrial enzyme market has expanded to nearly 85 enzymes, which are currently in commercial production. With the discovery of a variety of new and more active enzymes, the enzyme market has been forecasted to go upto US $ 1.7-2.0 billion by 2006 [1].

Proteases, which account for about 60% of total enzyme market and among the most valuable commercial enzymes, are the single largest class of enzymes occupying a pivotal position due their wide application in the industrial processes [2]. Plants, animals and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties [2]. Microbial extracellular alkaline proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. They account for approximately 40 % of the total worldwide enzyme sale.

Though a good number of bacterial alkaline proteases such as subtilisin Carlsberg, subtilisin BPN and Savinase are commercially available, having their major application as detergents enzymes but alkaline proteases of fungal origin offer an advantage over bacterial proteases because (i) the mycelium can be easily removed from the final product by simple filtration, (ii) ability of the fungus to grow on cheaper substrate, (iii) easy immobilization of mycelium for repeated use, (iv) broad range of pH (4-11) and substrate specificity and hence low cost of production. As only few reports are available on the use of fungal proteases in detergent industry, therefore there is a growing need to exploit fungal proteases for commercial exploitation in detergent industry.

For industrial use enzyme must be produced at low cost and should be reused reproduce result with consistent efficiency. To achieve this many techniques for immobilization of enzymes on different types of supports have been developed [3,4]. The immobilization of proteases on solid supports has been widely used in many investigations [5,6]. When a

protease is immobilized, enzyme autolysis is minimized. For industrial applications, immobilization of the enzyme in gel or solid supports may offer several advantages such as repeated use of the enzyme, ease of product separation and improvement of enzyme stability [5,6].

In the present study, the alkaline protease produced by A. oryzae has been purified and characterized to exploit its use in detergent industry. The effect of immobilization on the obtained enzyme was also taken into consideration as an important biotechnological aspect.

MATERIALS AND METHODS

CHEMICALS

Bovine serum albumin (BSA), 1,10-phenanthroline, ℓ-N-tosyllysine chloromethyl ketone (TLCK), ℓ-N-tosylphenylalanine chloromethyl ketone (TPCK), phenylmethane sulphonyl fluoride (PMSF), ℓ-casein, sodium alginate (medium viscosity), Coomassie brilliant blue G and Blue Dextran-2000 ®were supplied by Sigma chemical Co., St. Louis, MO USA. Cysteine, calcium chloride were brought from Hi-Media (Banglore, India). The chromatographic media like Sephadex G-100 and CM-Sephadex C-50 were supplied by Pharmacia (Uppsala, Sweden). Sodium dodecyl sulfate (SDS) was procured from Hi-Media Laboratories, India. Other chemicals used were of highest quality available. Refrigerated centrifuge IEC-25 and table top centrifuge Remi R8C were used for routine centrifugation. Systronics Spectrophotometer 108 was used for recording the absorbance in UV/VIS range. Protein samples were concentrated using Ultrafiltration Amicon Cell Model 8200 having YM10 membrane under compressed nitrogen pressure of 4-5 psi.

ISOLATION AND SCREENING OF ALKALINE PROTEASE PRODUCING FUNGI

Proteases producing fungal strains were isolated from different samples such as garden soil, decaying wheat straw, spoiled cheese etc. Proteolytic fungi were screened on skim milk agar medium containing skim milk powder, 100 g; peptone, 5 g and agar, 20 g per litre at pH 8.0. Fungal isolates showing zone of clearance were picked up, purified by repeated streaking on the same medium and finally transferred to PDA slants and maintained at 4 °C. Fungi were further screened for alkaline protease production on modified Reese medium (pH 9.0) containing KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4.7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; Urea, 0.3 g; trace element solution 1.0 ml; Tween 80, 0.5 ml and

supplemented with 0.30 % glucose, 0.5 % casein and 0.05 % yeast extract. Trace element solution contained $MnSO_4.H_2O$, 1.56 g; $FeSO_4.7H_2O$, 5.00 g; $ZnCl_2$, 1.67 g; $CoCl_2$, 2.00 g per liter [7]. The best producing strain identified as Aspergillus oryzae was isolated from spoiled cheese and was used for further studies.

PROTEIN DETERMINATION

The amount of protein was estimated by the method of Lowry et al [8] with bovine serum albumin as the standard. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the protein originally added.

DETERMINATION OF ALKALINE PROTEASE ACTIVITY

Activity for alkaline protease was determined spectrophotometrically by the Anson method [9], with a slight modification. Enzyme (0.1 ml) was incubated with 1.0 ml (0.5 % casein) and 1.9 ml 0.1 M Tris-HCl buffer, pH 9.0 at 37 °C for 30 min. and then the reaction was arrested by the addition of 2.0 ml of 5 % trichloroacetic acid (TCA). This mixture was centrifuged and the released amino acids were measured as tyrosine by Lowry method. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate one µg of tyrosine per min per ml under the standard assay conditions.

ALKALINE PROTEASE PRODUCTION

Two ml spore suspension (10^4 to 10^6 spore/ml) was added to 250 ml Erlenmeyer flask containing 100 ml Reese medium (modified) pH 9.0 supplemented with 0.25% glucose, 0.5% casein and 0.5% yeast extract and the flasks were incubated at 30 °C for 72 h in an incubator (200 rpm) on rotary shaker. The culture medium was centrifuged at 5,000 rpm to remove the fungal mycelia and medium debris; the supernatant was used as crude enzyme solution.

PURIFICATION OF ALKALINE PROTEASES

All the purification steps were carried out at temperatures from 0° to 4 °C unless otherwise stated.

AMMONIUM SULFATE FRACTIONATION

The crude enzyme was first saturated upto 30% with solid $(NH_4)_2SO_4$ and then centrifuged at $5,000 \times g$ for 15 min. The supernatant obtained was further saturated upto 70% with solid $(NH_4)_2SO_4$ and again centrifuged. The pellets obtained were dissolved in minimum volume of 0.1 M phosphate

buffer, pH 6.0 (buffer A), and dialyzed against the same buffer extensively and then concentrated through Amicon Diaflo Ultra-filtration cell using YM-10 membrane.

ION-EXCHANGE CHROMATOGRAPHY ON CM SEPHADEX C-50

The dialyzed enzyme resulting from ammonium sulfate fractionation was loaded on CM-Sephadex C-50 column (35 × 2.5 cm) equilibrated with buffer A. After washing the column free from unadsorbed proteins, the bound proteins were eluted by applying a linear NaCl gradient (0.0-1.0 M NaCl), at a flow rate of 40 ml/h. In this step, alkaline protease was eluted at 0.3 M NaCl concentration. The fractions (27-37) showing alkaline protease activity were pooled, concentrated and dialysed against 0.1 M Tris-HCl buffer, pH 9.0 containing 0.2 M NaCl and 1 mM EDTA ((buffer B).

GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-100 COLUMN

The concentrated pool of activity obtained above was fractionated on Sephadex G-100 column (120 × 2.0 cm) with eluent buffer B. The fractions eluted from the column were assayed for alkaline protease. The fractions (41-55) having high alkaline protease activity were concentrated and stored at 4 °C and used further to check the homogeneity of the enzyme by electrophoresis and characterization studies.

POLYACRYLAMIDE GEL ELECTROPHORESIS

The purity of the enzyme was established by gel electrophoresis at pH 8.4 [10] and the molecular mass of the enzyme was determined by gel filtration on analytical Sephadex G-100 column and by SDS–PAGE [11].

IMMOBILIZATION METHODS ENTRAPMENT

The purified alkaline protease was immobilized in the calcium alginate beads through entrapment by the method of Banerjee et al [12] Bovine serum albumin (5.0 mg) was added to 5.0 ml of enzyme solution containing 2.5 mg proteins. To this 1.5% of sodium alginate was added and stirred gently. The entrapment was carried out by dropping the mixture through a hypodermic needle (No.16) into 50 ml of 2.0% (w/v) $CaCl_2$ solution. The beads so formed were left for 1 h in calcium chloride solution and then stored in 0.1 M Tris-HCl buffer pH 9.0, at 4 °C.

EFFECT OF PH AND TEMPERATURE ON THE ALKALINE PROTEASE ACTIVITY

The effect of pH and temperature on the free and immobilized enzyme was determined under standard assay conditions using casein as substrate. Alkaline protease activity was studied in the pH range from 5.0 to 11.5 for free and immobilized form of enzymes and than their activity was measured at various temperatures (10 °C to 80 °C).

STABILITY OF IMMOBILIZED ALKALINE PROTEASE ENZYME

The stability of the enzyme in buffers at different pH values (0.1 M sodium acetate buffer pH 4.0 to pH 5.5; 0.1 M histidine-HCl buffer pH 6.0 to 7.5; 0.1 M tris-HCl buffer pH 7.5 to 9.0; 0.1 M glycine-NaOH buffer pH 9.5 to 11.5) was measured by incubating 10 beads (0.125 mg enzyme) in 1.0 ml of different solutions at 37 °C. The temperature stability (from 0 to 80 °C) was monitored by incubating the immobilized enzyme at different temperature for 10 min in 0.1 M tris-HCl buffer pH 9.0 and the residual activity was monitored under the standard assay procedure.

EFFECT OF SUBSTRATE CONCENTRATION ON IMMOBILIZED ALKALINE PROTEASE

The K_m' (apparent) value was determined for the hydrolysis of casein by immobilized alkaline protease. A Lineweaver-Burk plot was drawn between the inverse of different concentrations of casein and the reaction velocity to determine the apparent Michaelis constant, K_m', for the immobilized alkaline protease.

EFFECT OF VARIOUS METAL IONS AND INHIBITORS ON ALKALINE PROTEASE ACTIVITY

To investigate the effect of some metal ions and inhibitors on the free form of enzyme activity, the purified enzyme solution was pre-incubated along with various metal ions and inhibitors for 15 min at 37 °C. The concentration of metal ions and inhibitors used was 1 mM and 5 mM, respectively. The residual enzyme activity was measured by adding the substrate and carrying out the enzyme assay under the optimum conditions.

RESULTS AND DISCUSSION
PURIFICATION OF ALKALINE PROTEASE

The purification of the alkaline protease enzyme produced by A. oryzae was simple, economical, efficient, reliable and rapid. When crude enzyme was subjected to 30-70 %

ammonium sulfate fractionation, there was 3.34-fold purity with 90.6% yield. Precipitates obtained after ammonium sulfate saturation were dissolved, dialyzed and subjected to cation exchange chromatography on CM Sephadex C-50 column, pH 6.0. The elution profiles for alkaline protease and protein from the cation exchange as shown in Fig 1 have two peaks. The second peak contains the highest specific activity (312.5 U/mg protein). The most active fractions (26 to 37) eluted 0.25 M NaCl gradient from the CM Sephadex C-50 column were pooled and loaded on Sephadex G-100 column (Fig 2), pH 9.0 leading to an apparently pure form of alkaline protease. Enzyme was purified ~29.29-fold with 35.2 % yield having specific activity 750 U mg-1 after Sephadex G-100 gel filtration chromatography (Table 1). However Ogundero & Osunlaja [13] reported 26.2% recovery by using DEAE-cellulose and Sephadex G-200 columns for purification of alkaline protease from Aspergillus clavatus.

Figure 1

Figure 1: Cation-exchange chromatography on CM-Sephadex C-50. [Ammonium sulfate fraction (30-70%, 10 ml) of alkaline protease was loaded on CM-Sephadex C-50 column at pH 4.6. Protein (?---?); alkaline protease activity (?-?). The bound protein was eluted with a linear NaCl gradient (0-1.0)]
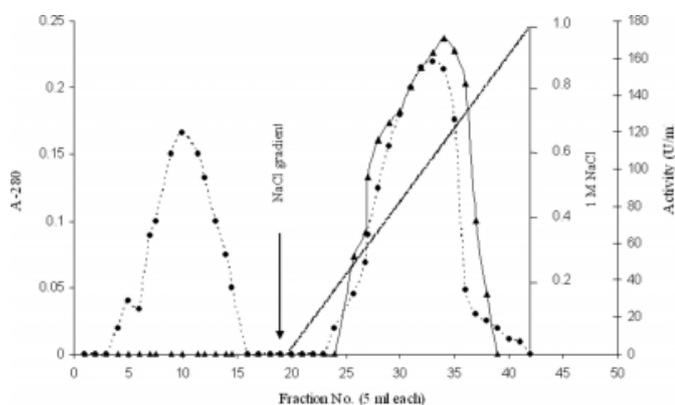

Figure 2

Figure 2: Molecular sieve chromatography of on Sephadex G-100 column [CM-Sephadex pool of DPP IV enzyme was loaded on Sephadex G-100 column equilibrated and eluted with 0.1 Tris-HCl buffer pH 9.0 containing 1m EDTA and 0.2 NaCl. Protein (?---?); alkaline protease activity (?-?).
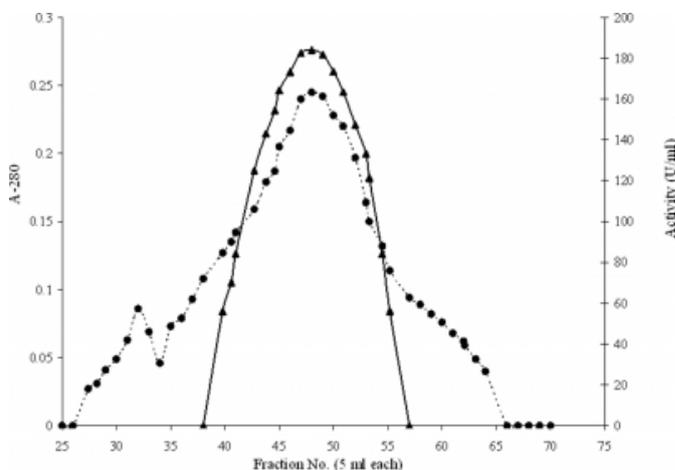

Figure 3

Table 1: Purification of alkaline protease from

Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	330.00	8500	25.70	100.00	1.00
(NH₄)₂SO₄ pellet (30-70 %)	90.00	7700	85.50	90.60	3.34
CM-Sephadex C-50, pH 6.0	20.00	6250	312.50	73.50	12.20
Sephadex G-100, pH 9.0	4.00	3000	750.00	35.20	29.29

[Casein was used as substrate to measure the activity of alkaline protease as described under 'Materials and Methods'. The activity units are expressed one µg of tyrosine liberated per min per ml under the standard assay conditions at 37 °C].
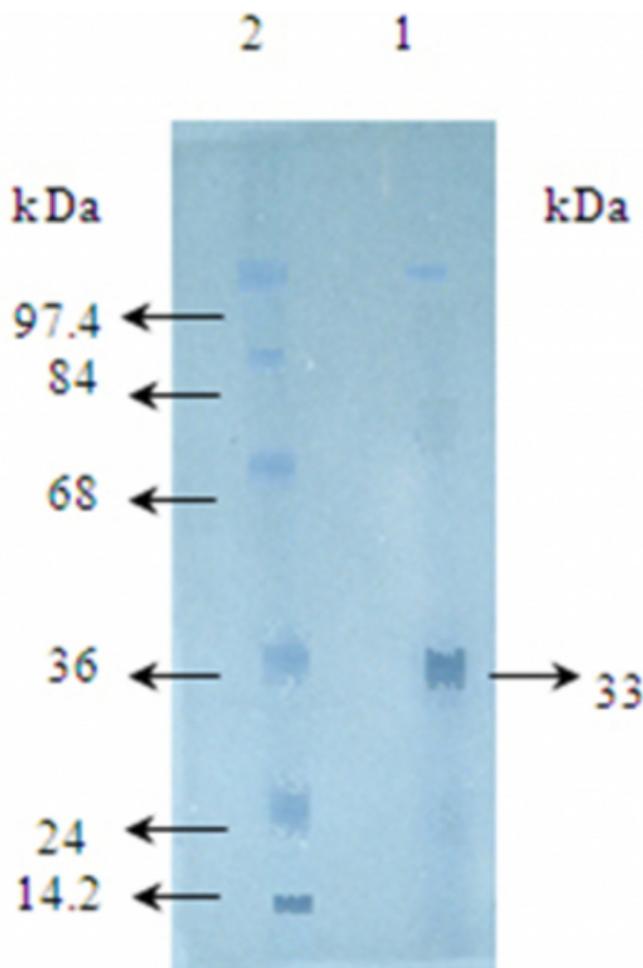
The purification fold of purified alkaline protease from Asperigillus oryzae is ~30-fold which is almost similar to that obtained from Nocardiopsis sp and T. koningii [14,15] but this factor is ~3 × higher than the enzyme purified from Bacillus and Pseudomonas sp.[16,17].

CHARACTERIZATION OF PARTIALLY PURIFIED ALKALINE PROTEASE

The apparent molecular mass of the partially purified alkaline protease was estimated to be ~33 kDa as measured on analytical Sephadex G-100 column and SDS–PAGE (Fig. 3). The native enzyme is thought to be a monomer, which is composed of only one subunit. This result is very similar for alkaline protease purified from Bacillus and Pseudomonas spp [14,15] while other workers have reported molecular mass ~48 kDa for alkaline protease from Aspergillus sps [18,19] .

Figure 4

Figure 3: Determination of molecular mass of purified alkaline protease by SDS-PAGE: [Lane 1, Alkaline protease enzyme (40 Âµg); lane 2, protein markers, (20 Âµg each of phosphorylase , 97.4 kDa; , Fructose 6 phosphate Kinase, 84 kDa; bovine serum albumin, 68 kDa; Glyceraldehydes 3 phosphate dehydrogenase, 36 kDa; trypsinogen, 24 kDa and ?-Lactoalbumin, 14.2 kDa)]

EFFECT OF SOME CHEMICALS AND INHIBITORS ON ALKALINE PROTEASE ACTIVITY

The different concentrations of each of the tested ions and inhibitors significantly affected the activity of the purified alkaline protease from A. oryzae. This protease was strongly inhibited by PMSF and moderately by trypsin inhibitor, TLCK and TPCK (Table 3). However, it was not inhibited by proteinase inhibitors such as EDTA, 1,10-phenanthroline (inhibitor of metalloproteinases). Neither the sulfhydryl reagents like benzamidine nor the thiol activators like ⏘-ME and cysteine had any effect on the enzyme activity. These findings indicated that a SH-residue is not involved in the

expression or regulation of the activity of alkaline protease. Among the metal ions, Hg2+ was found to e the strongest inhibitor whereas Li+ and Mn2+ inhibited the enzyme slightly. However, the enzyme activated by Ca2+ and Mg2+ by 35 % and 19 % at 1 mM concentration, respectively, indicated that the enzyme might require metal ions for its activity (Table 2). These observations indicate that metal-binding region on alkaline protease molecule is important for the regulation of the enzyme activity. These results and particularly strong inhibition by PMSF indicated that the active-centre of alkaline protease contains serine residues. Similar result of PMSF inhibition has also been reported by Hossan et al [19] for protease produced by Aspergillus flavus. Alkaline proteases isolated from different fungal and bacterial sources behaved differently in the presence of metals ions. At a concentration of 1 mM, Fe^{2+} , Co^{2+} and Ca^{2+} increased the activity of alkaline protease from T. koningii, whereas K^+ , Na^+ , Ag^+ and Pb^{2+} inhibited its activity [20]. Cu^{2+} strongly stimulated activity of the alkaline protease produced by ABacillus spp [21]. Some metal ions such as Ca^{2+} , Mg^{2+} and Mn^{2+} increased and stabilized the protease activity of both the proteases (AP-1 and AP-2) of Bacillus sp. NCDC 180, and Hg2+ ion (1mM) resulted in maximum inhibition of both the enzymes AP-1 and AP-2 by 74% and 73 % respectively [22]. In case of B. pumilus protease, Ca^{2+} , Mg^{2+} and Na^+ slightly increased enzyme activity whereas Cu^{2+} and Zn^{2+} inhibited the enzyme activity. Similarly Kunert et al [23] observed the stabilization effect of Ca^{+2} ions on the activity of serine protease Alp isolated from Aspergillus fumigatus.

Figure 5

Table 2: Effect of metal ions on activity of alkaline protease from A AWT 20.

Metal ion (1mM)	Residual activity (%)
Control	100
Cu^{2+}	100
Sn^{2+}	100
Ba^{2+}	100
Co^{2+}	100
Li^+	100
Mn^{2+}	80
Hg^{2+}	21
Mg^{2+}	135
Ca^{2+}	119

[Activity of alkaline protease was measured with casein as substrate at pH 9.0. Enzyme was pre-incubated with the respective metal ions for 10 min and then the residual enzyme activity was determined by the standard assay

procedure for alkaline protease. The inhibition was taken as 0% when no metal ion was added]

Figure 6

Table 3: Effect of various inhibitors on the activity of alkaline protease from AWT 20.

Inhibitors (5 mM)	Inhibition (%)
Control	0
1,10-Phenanthroline	0
EDTA	0
Benzamidine	0
Cysteine	0
□-ME	0
NaN$_3$	8
TLCK	35
TPCK	42
Trypsin Inhibitor	48
PMSF	100

[Activitiy of alkaline protease was measured with casein as susbtrate at pH 9.0. Enzyme was pre-incubated with the respective inhibitor for 10 min and then the residual enzyme activity was determined by the standard assay procedure for alkaline protease. The inhibition was taken as 0% when no inhibitor was added]

Figure 7

Table 4: Immobilization of alkaline protease in the presence of different concentration of sodium alginate in 2.0% CaCl concentrations

Alginate (%)	Beads/ml	Activity (%)
1.5	40	37.56
2.0	42	47.72
2.5	34	26.14
3.0	30	27.76
3.5	27	30.08

IMMOBILIZATION OF ALKALINE PROTEASE ENZYME

Table 1 shows the effect of varying concentrations of sodium alginate (1.5-3.5%) on alkaline protease immobilization. The percent-entrapped activity was maximal (~48%) at 2.0% (w/v) of sodium alginate concentration. Leakage of enzyme occurred at 1.5% (w/v) sodium alginate concentrations owing to the larger pores of the less tightly crosslinked gel. But, the amount of entrapped activity is comparatively very low at 2.5-3.5% (w/v) sodium alginate concentrations. It may be because of the high viscosity of the enzyme-BSA-sodium alginate mixture that results less diffusion of high molecular weight of substrates into the alginate beads. Thus, 2.0% concentration of alginate was selected for entrapment of alkaline protease to study its

altered physico-chemical behavior. The sodium alginate in the concentration range of 2-3% has been used for the immobilization of keratinase [24], lipase [25], Teredinobacter turnirae [26] and Saccharomyces cerevisiae [27] cells. There was no significant effect of varying concentration of CaCl$_2$ on the immobilization of alkaline protease when the sodium alginate concentration was maintained at 2.0% (w/v).

EFFECT OF PH AND TEMPERATURE ON THE ENZYME ACTIVITY

The optimum pH value of the immobilized alkaline protease shifted to pH 9.5 from pH 9.0, which was the optimum pH of the free enzyme (Fig 4). This may be due to the anionic nature of the alginate support used for immobilization and change in the microenvironment of the immobilized enzyme. Similar observations were reported in annase-immobilization studies [28]. The stability of immobilized alkaline protease was compared with the free enzyme in acidic and alkaline environment (Fig 5). The free alkaline protease was stable over the pH range 7.0-9.0, whereas the immobilized alkaline protease was stable over a wider range of pH (5.5-10.0). Even at pH 10.5, the immobilized enzyme could retain ~80% activity whereas free enzyme was totally inactivated at this pH. The stability of the two forms was also compared by their incubation for 10 min at various temperatures. At 55 °C, the immobilized alkaline protease retained ~86% of its activity while free enzyme could retain ~10% activity. The thermostability of the enzyme increased very significantly after entrapment (Fig 6). This probably reflects the fact that the entrapped enzyme is not chemically modified but remains in its native form in the gel matrix. Tanriseven & Dogan [29] have also reported the similar type of result with invertase immobilized in Ca-alginate capsules makes it a suitable candidate for detergent formulations.

Figure 8

Figure 5: Determination of pH stability of free and immobolized alkaline protease. After exposing the free and immobilized enzymes to various pH values for 10 min the residual activity were measured at pH 9.0.
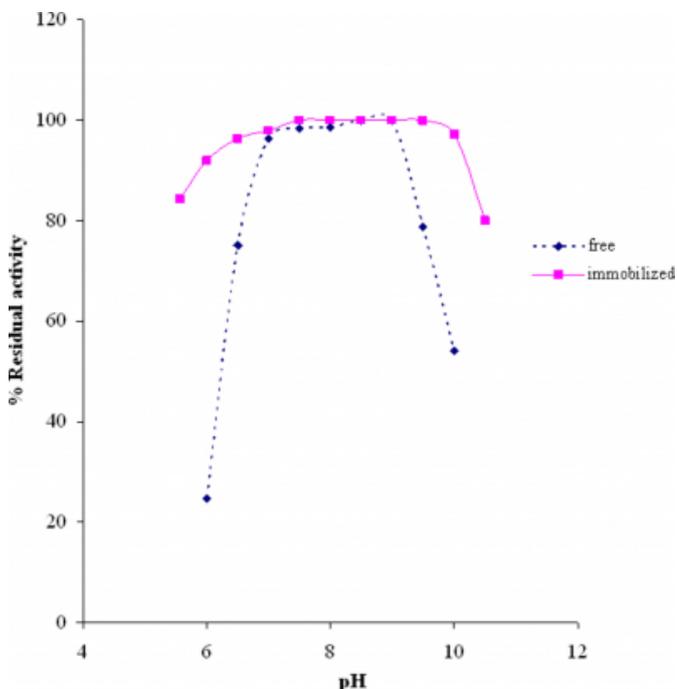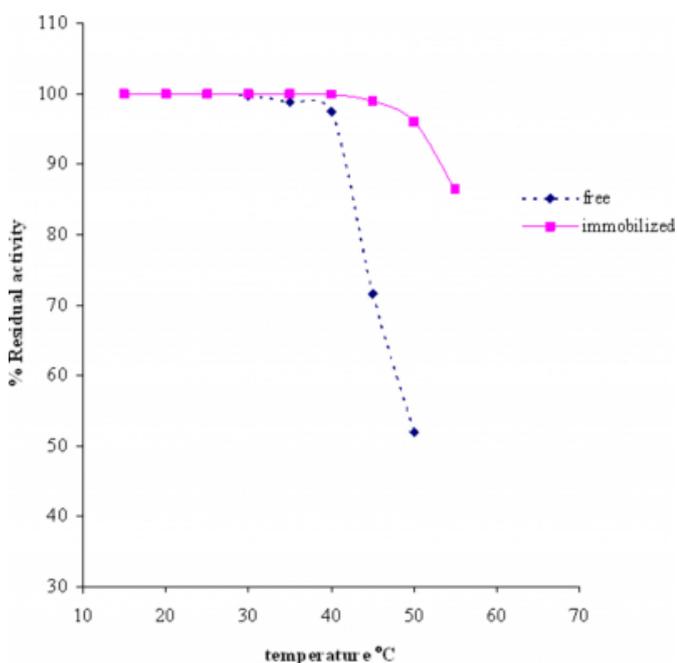

Figure 9

Figure 6: Determination of temperature stability of free and immobilized alkaline protease. The enzyme were first exposed to different temperature for 10 min and then immediately cooled to 4Â°C The residual activity were determined at 40Â°C

EFFECT OF SUBSTRATE CONCENTRATION ON IMMOBILIZED ALKALINE PROTEASE

To investigate further the catalytic activity of the immobilized alkaline protease, the Michaelis constant K_m was determined using casein as substrate. The values for the immobilized (apparent K_m' =5.0 mg casein/ml) and free form (K_m =3.3 mg casein/ml) of alkaline protease were different. The increase in the K_m' value caused by immobilization is due to the alginate matrix, which can offer significant resistance to the transport of substrates. The increase in K_m' has also been reported for glucose oxidase after immobilization within calcium alginate gel [30]. But the V_{max} value for both form of the enzyme were almost same i.e. 750 ± 2.45 μg tyrosine/min/ml.

CONCLUSIONS

In the present study, an alkaline protease has been partially purified using two chromatographies from a new strain of A. oryzae AWT 20. This is a serine protease as indicated by its inhibition studies. Proteases used in detergent industry are mainly derived from bacteria, which have some major limitations whereas fungal origin alkaline proteases offer advantages such as the ability of the fungus to grow on cheaper substrate and easy immobilization of mycelium for repeated use. The increase in stability of alkaline protease after immobilization in ca-alginate beads at high temperature as well as under acidic and basic conditions increases possibility of its use in various applications such as in peptide synthesis, for de-proteinization of shrimp and crab shell waste and in cleaning of Ultrafiltration membrane systems. Further the pH and temperature stability of this enzyme makes it a suitable candidate for detergent formulations.

ACKNOWLEDGEMENT

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