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Prita S Borkar
 Department of Microbiology,
 NES Science College, Nanded,
 Maharashtra, India

Purification and immobilization of thermostable serine alkaline protease from *Bacillus subtilis*

Prita S Borkar

Abstract

A protease producing bacteria was isolated from soil and identified as *Bacillus subtilis*. Of the 42 isolate screened, isolate S-8 was identified as thermostable alkaline protease producer. The protease was purified by ion exchange chromatography, and showed apparent molecular weight of 19,000 kD and an isoelectric point of 9.0. The enzyme had optimal proteolytic activities over a broad pH range (8-11) and exhibited temperature optimum of 60 °C. The protease was immobilized on tri (4- formyl phenoxy) cyanurate to form Schiff's base. The native and immobilized protease was used for catalyzing the hydrolysis of proteins in aqueous medium. The immobilized protease exhibited shift in optimal pH from 10 to 10.5 and optimal temperature from 60 °C to 65 °C. The immobilized protease revealed 10-15% increase in thermal stability and retained 70% of its initial activity after 3 cycles.

Keywords: Protease, *Bacillus subtilis*, multipoint binding, immobilization

Introduction

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. And among bacteria, *Bacillus* species are specific producers of extracellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Paster *et al.* 2001) [17]. Alkaline proteases from high yielding strains have been studied extensively; one of the major drawbacks affecting the stability at alkaline pH of enzymes recovered from thermophiles is that enzymes from alkalophiles confer stability in a wide pH range but are also usually thermolabile. Thus, it is desirable to search for new proteases with novel properties from as many sources as possible. Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two-thirds (Beg and Gupta, 2003; Gupta *et al.* 2002) [2, 8]. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed resulting in faster reaction rates, increase in solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms (Folasade and Joshua, 2005) [5]. The enzyme immobilization technologies have been developed based mostly on the consideration of stability and reusability of biocatalysts (Tischer and Wedekind, 1999) [22]. The previous efforts for the multiple binding of enzymes have been conducted mostly with organic synthetic polymers because of the ease of fabrication of desirable structures and the availability of reactive functional groups. Organic polymers can achieve considerably high enzyme loading (Borros *et al.* 2003; Balcao *et al.* 2001; Wang *et al.* 1997) [3, 1, 24]. The covalent coupling of enzyme can produce a loss of activity due to the influence of the coupling conditions and to conformational changes in enzyme structure. However, irreversible binding of the carrier during covalent coupling does not allow the recovery of the carrier from the carrier-enzyme complex (Huang *et al.* 1997) [9].

In the present work we report certain physical and biochemical properties of protease produced by newly isolated strain of *Bacillus subtilis* S-8. An attempt was also made to explore the proteolytic activity of *B. subtilis* protease covalently bound to tri (4-formyl phenoxy) cyanurate via multipoint attachment. The catalytic behavior of the immobilized enzyme was examined on casein in terms of enzyme activity, stability and reuse in an aqueous medium at variable pH and temperatures.

Correspondence

Prita S Borkar
 Department of Microbiology,
 NES Science College, Nanded,
 Maharashtra, India

Materials and Methods

Bovine serum albumin, casein, DEAE-cellulose (Diethyl amino ethyl cellulose) and PMSF (phenyl methyl sulfonyl fluoride), were purchased from HiMedia Laboratories Ltd., Mumbai, India. Molecular mass markers purchased from Bangalore Genie, Bangalore, India. The 4-hydroxy benzaldehyde, cyanuric chloride was purified by recrystallization from petroleum ether (80 - 100 °C). All chemicals were of analytical grade.

Screening of microorganisms

The soil samples were collected from milk processing plant and drainage of slaughter houses of Nanded, India. Isolation and screening was carried out by the modified method of (Kunamneni *et al.* 2003) [11]. A total of 42 isolates were screened for protease production by casein digestion method depending upon the zone of clearance and isolate S-8 was selected for further experimental studies. The isolated proteolytic strain was a spore-forming gram-positive rod, identified as *B. subtilis* on the basis of cultural and morphological characteristics as per The Bergey's Manual of Systematic Bacteriology (Sneath and Halt, 1986) [19].

Extracellular enzyme production

Production of protease from *B subtilis* was carried out in a medium containing glucose, 0.5% (wt/vol); peptone, 0.75% (wt/vol); and salt solution, 5% (vol/vol); [MgSO₄.7H₂O, 0.5% (wt/vol); KH₂PO₄, 0.5% (wt/vol); and FeSO₄.7H₂O, 0.01% (wt/vol)] and maintained at 37 °C for 48 h in a shaker incubator (160 rpm). At the end of the fermentation, the culture medium was centrifuged at 10,000 rpm for 20 min to obtain a clear supernatant, that was used as the enzyme source.

Protein estimation and enzyme assay

Protein content was determined according to the method of Lowry *et al.* (1951) [13] using crystalline bovine serum albumin as a standard. Alkaline protease activity was assayed by the modified procedure based on the method of Tsuchida *et al.* (1986) [23]. Casein, 2% in 0.2 M carbonate buffer, pH 10 was used as substrate. Casein solution 0.5 ml with an equal volume of suitably diluted enzyme solution was incubated at 40 °C. After 10 min, the reaction was terminated by adding 1 ml of 10% trichloroacetic acid. The mixture was centrifuged and 5 ml 0.44M Na₂CO₃ and 1 ml two-fold diluted folin ciocalteau reagent were added to the supernatant. After 30 min the color developed was read at 660nm (UV- vis Shimadzu) against blank solution in which no enzyme was present. One unit of enzyme activity is defined as the amount of the enzyme that releases 1µg of tyrosine/ml/min under above assay conditions. The esterase activity of the enzyme with *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester was determined spectrophotometrically at 253 nm and 256 nm respectively.

Enzyme purification

Enzyme from the crude culture filtrate was precipitated by addition of 80% (vol/vol) ice cold acetone, followed by centrifugation. The protein pellet was suspended in a volume of 0.05 M Tris HCl buffer pH 8.0 and brought to about 80% saturation by addition of solid ammonium sulphate. The precipitated protein was collected by centrifugation, suspended in small volume and dialyzed against same buffer. The dialysate was applied to sephadex G-100 column (1.2×15

cm) equilibrated with same buffer and the protein was eluted with 1:1 volume gradient. The elute containing all protease activity was pooled; applied to DEAE- cellulose column (1.5×24cm) previously equilibrated with Tris HCl buffer pH 8.0. The column was eluted at a flow rate of 1ml/min with a 1:1 volume gradient from 0.1 to 0.6 M NaCl in the same buffer. Fractions of 10 ml were collected and monitored for protein and enzyme activity.

Molecular weight determination and Isoelectric point

Molecular mass values were determined by SDS-PAGE using 10% gels according to the method of Laemmli (1970) [12]. The isoelectric focussing was carried out over the pH range 3-10.

Immobilization of enzyme

The *B. subtilis* protease was immobilized by carrier binding method, in which enzyme is made to bind to the support material covalently. For immobilization, 30mg of tri (4-formyl phenoxy) cyanurate and 90mg of enzyme was taken in 5ml of 10 mM phosphate buffer pH 8.0 and stirred continuously for 30 min at room temperature. The amino group of enzyme was made to bind reversibly to the aromatic aldehyde group of tri (4-formyl phenoxy) cyanurate to form Schiff's base (Gacche *et al.* 2002) [6]. The formed immobilized product was designated as 'B'.

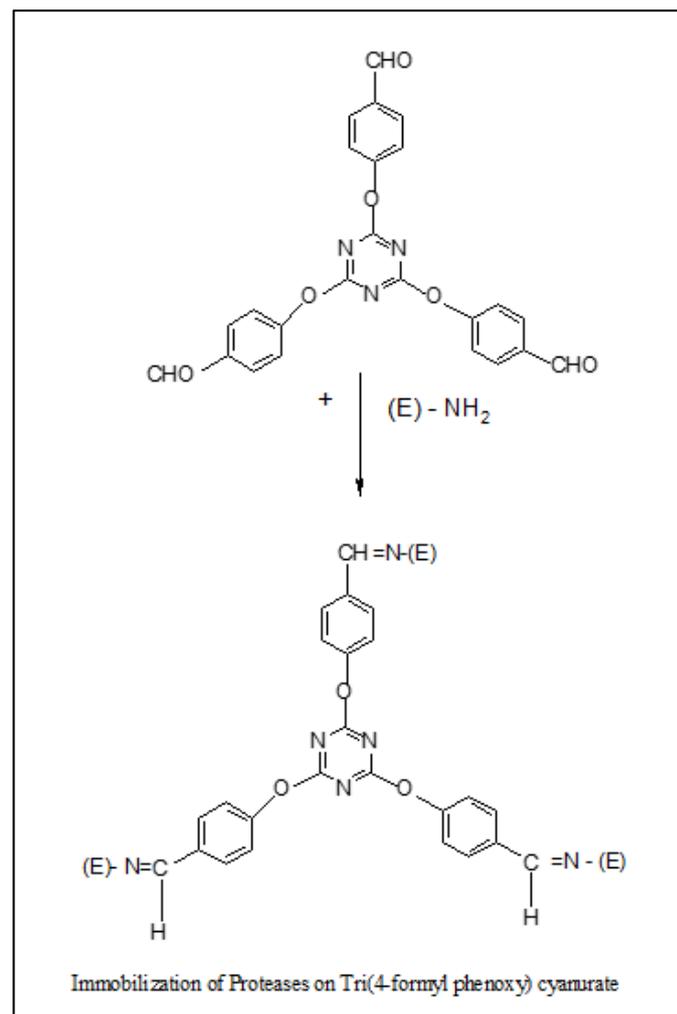


Fig 1

Enzyme loading capacity of tri (4-formyl phenoxy) cyanurate

Enzyme was loaded to the supporting material giving a

stoichiometry of 3:1 ratio [enzyme 90 mg (0.204 m mol) and 30 mg (0.068 m mol) support]. The three enzyme molecules are bound to three binding sites present on the one molecule of support. After immobilization the amount of unbound and bound (immobilized) enzymes were subjected for protein estimation. The amount of protein content of native (free) enzyme 2 mg/ml was used for immobilization. The protein content of unbound enzyme was estimated to be 1.8 mg/ml, while the protein concentration in bound (immobilized) enzyme for 1 mg of support was estimated to be 0.8 mg/mg.

Kinetic assays

Assays were performed by using the better physicochemical conditions for optimization of enzyme activity (free and immobilized) at various pH ranging from 6 to 12 and temperature ranging from 20 to 80 °C. The optimum pH and temperature for both the native and immobilized enzymes was determined by taking casein as substrate.

Results and Discussion

B. subtilis S-8, showed maximum growth and protease production at 48 h; at the end of fermentation time the extracellular protease was recovered from crude broth and purified by ion-exchange chromatography (Table 1) and used for further studies. The molecular weight of protease estimated by SDS-PAGE was 19000 Daltons and an isoelectric point of 9.0 (data not shown). The metal ions Ca^{2+} , and Mg^{2+} (5mM) had stimulatory effect on enzyme activity, while the other ions such as Cu^{2+} , Zn^{2+} , Hg^{2+} , Co^{2+} , Na^+ , Cd^{2+} , Al^{3+} (5mM) did not shown any appreciable effect. Enzyme was insensitive to EDTA upto 5 mM concentration and totally inhibited by 0.1 mM PMSF indicating the presence of a serine residue at the active site. The esterase activity of the enzyme indicated by hydrolysis of synthetic substrates like *N*-benzoyl-L-arginine ethyl ester (activity 1.8 U/mg) and *N*-benzoyl-L-tyrosine ethyl ester (activity 4.82 U/mg) respectively (Table 3).

The formation of the product 'B' was determined from IR (Bomem Canada, model MB-104) spectra of the product. The prominent peaks obtained in IR spectra of compound 'A' are 1102, 1602, 2741, 1702 and 2833 cm^{-1} can be assigned to C–O, C=C, C–H of aldehyde, C=O and C–H (aromatic) stretching frequencies. However IR spectra of the compound 'B' indicated the presence of peaks at 1100, 1606, 1569, 1702 and 2851 cm^{-1} . These can be assigned to C–O, C=C, CH=N (imine), C=O and C–H (aromatic) stretching frequencies. The appearance of peak at 1569 cm^{-1} for imine, disappearance of peak at 2741 and reduction of peak height at 1702 cm^{-1} clearly indicated the 80% immobilization of *B. subtilis* protease on tri (4-formyl phenoxy) cyanurate. The immobilized efficiencies changed with each enzyme and varied between 53 and 80% (Magnin and Dumitriu, 2003) [14].

The enzyme activities of both native and immobilized *B. subtilis* protease on tri (4-formyl phenoxy) cyanurate was studied at variable pH and temperatures. In native form this enzyme displayed maximal activity at pH 10.0 and was stable over pH 8 to 10 for 2 h while, after immobilization the optimum pH was shifted from 10.0 to 10.5 and stable over pH range 8-11 for 4h and retained full activity (Table 2). The shift in pH optimum may be due to the change in the H^+ ion concentration of the microenvironment of the immobilized proteases on tri (4-formyl phenoxy) cyanurate. The H^+ ion concentration in the immobilized enzyme and support

medium may be high as compared with the H^+ ion concentration around the immobilized enzyme. Hence to balance the identical H^+ ion concentration in both the environments, H^+ ion may be released from inside towards the outer environment and concentrate OH^+ ions around it, thus increasing the pH around the enzyme, there by shifting optimum pH towards the alkaline side (Carla *et al.* 2006) [4]. Another reason for increase in activity at pH 10.5 was seems to be related mainly to the formation of an enzyme support complex with a very suitable open conformation with little restriction to the access of substrates.

At temperature 60 °C the optimal activity was recorded for native form while after immobilization, temperature profile of this enzyme revealed shift in optimum temperature from 60-65 °C (Table 2). The shift in optimal temperature towards higher temperature may be due to the immobilization of the enzyme to the support providing stability resulting in formation of the enzyme substrate complex without any hindrance for the access of substrates to the active site and higher stereospecificity at 65 °C. The rise in temperature above 65 °C, might have denatured the enzyme and decrease in activity was therefore obtained at higher temperatures (Gupta, 1993) [7]. The thermal stability of native and immobilized *B. subtilis* protease was examined by heating at 65 °C. It appeared that the immobilized enzyme was remarkably more stable than the respective native form. In the immobilized state this enzyme retained 70 % of its original activity after 3 h incubation at 65 °C, while in the native form lost >50% of its original activity after 1 h. The estimated half life of immobilized enzyme was > 300 min at 65 °C.

The higher stability of the immobilized (bound) *B. subtilis* protease could be due to the diminished autoproteolysis of the enzyme fixed to the support (Figure 2). The auto proteolysis of protease in aqueous solutions may significantly inactivate the enzyme. The restricted interactions among the tri (4-formyl phenoxy) cyanurate immobilized enzyme molecules, as opposed to the effect of structural stabilization could play an important role in retaining the enzymes activity in aqueous solution (Novick and Dordick, 1998; Ping *et al.* 2001; Rao *et al.* 2006) [16, 18, 21].

Upon repeated use, the tri (4-formyl phenoxy) cyanurate immobilized protease retained 70% of its initial activity after 3 cycles (Mohsen *et al.* 2005) [15]. The decreased in activity after 3 cycles can be correlated to slight inactivation of enzymes after every use as evidenced by leaching in supernatant.

As the immobilized enzyme is stable at alkaline pH and at high temperatures, the unique configuration of tri (4-formyl phenoxy) cyanurate seems to provide some advantages such as high enzyme loading, multipoint covalent attachment sites for reversible binding of the enzyme and reusability therefore could be better carrier material for immobilization. The decrease in activity after 3 cycles can be correlated to slight inactivation of enzyme after every use as evidenced by leaching in supernatant. The tri (4-formyl phenoxy) cyanurate immobilized protease exhibited improved activity and stability in an aqueous medium, as compared with its native parent enzyme. As this supporting material is highly insoluble in water, crystalline in nature and thermally stable (mp 174 °C) it is expected that the present immobilization technology can be used as ideal system for long term chemical processing involving reactions at elevated pH and temperatures in an aqueous media in terms of caseinolytic activity.

Table 1: Purification steps of alkaline protease from *Bacillus subtilis* S8.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	1000	10500	35000	3.3	1.0	100
Ammonium sulphate precipitation	20	700	20000	28.5	8.6	57.1
Sephadex G-100	200	220	8600	39.0	11.8	24.5
DEAE – cellulose chromatography	18	120	5850	48.7	14.5	16.7

Table 2: Effect of pH, temperature and thermal stability on protease activity

Enzyme	pH				Temperature °C		
	Optimum	Stability range	Stability time (h)	Enhancement	Optimum	Thermal Stability (h)	Enhancement
Protease (Native)	10	8-10	2	-	60	>1	-
Protease (Immobilized)	10.5	8-11	4	>2.0	65	>3	>2.5

Thermal stability for native and immobilized protease was examined at 65 °C

Table 3: Effect of metal ions on alkaline protease activity

Conc. of metal ions (5mM)	Protease activity (%)
Control	100
Mg ²⁺	112
Zn ²⁺	98
Cu ²⁺	96
Hg ²⁺	93
Co ²⁺	92
Na ²⁺	98
Ca ²⁺	128
Cd ²⁺	91
Al ³⁺	99
Mn ²⁺	97

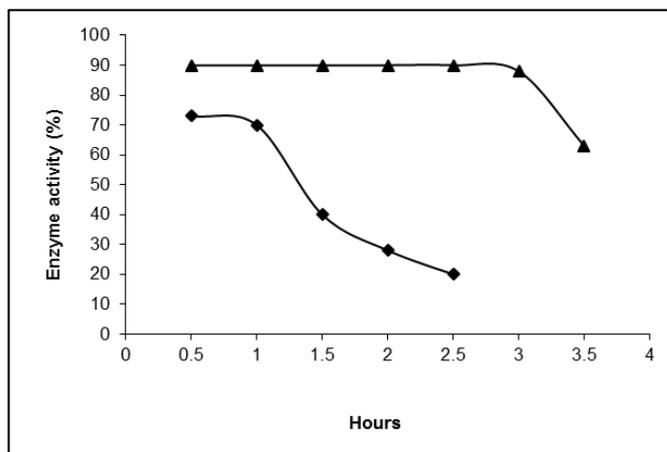
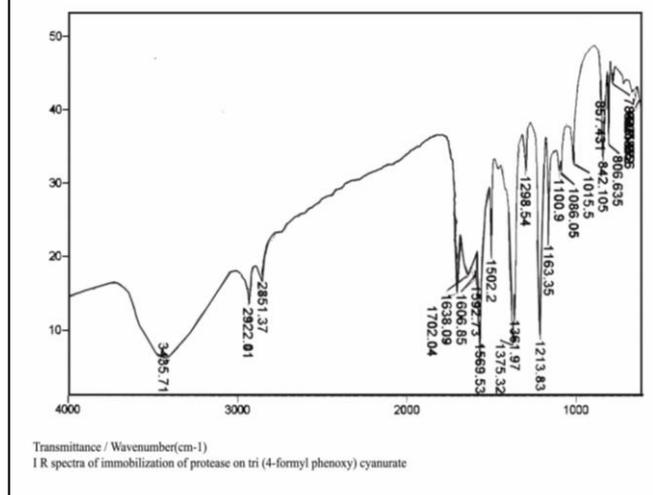
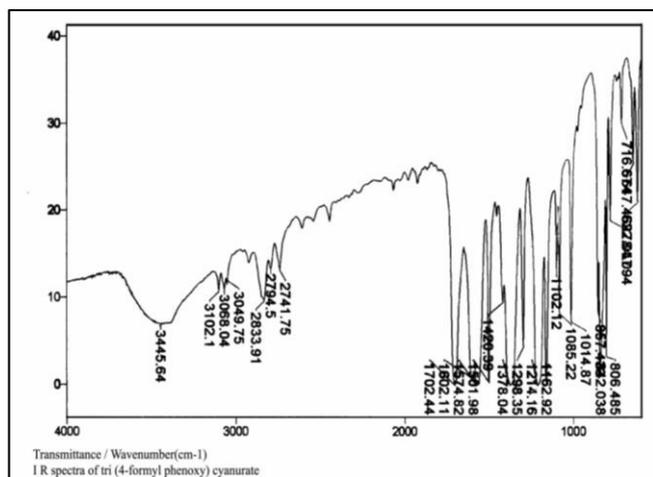


Fig 2: Thermal stability of native (●) and immobilized (▲) enzymes at 65 °C



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