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Purification and Characterization of Caseinolytic Extracellular protease from *Bacillus amyloliquefaciens* S94

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From the culture supernatant of the psychrotrophic strain of *Bacillus amyloliquefaciens* an extracellular serine protease was purified to apparent homogeneity by successive purification steps using QAE-Sephadex, SP-Sephadex and Sephacryl S-100 column chromatography. The protease is monomeric, with a relative molecular mass of 23,000. It is inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride, but not by EDTA. The enzyme is most active at pH 9-10, and at 45°C, although it is unstable at 60°C.

Key words: extracelluar protease, alkaline protease, psychrotrophic *Bacillus*

Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Rao *et al.*, 1998). Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. Vast numbers of purification procedures for proteases, involving affinity chromatography, ion-exchange chromatography, and gel filtration techniques have been well documented (Igarashi *et al.*, 1979; Rawlings and Barrett, 1993).

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms (Wandersman, 1989). Proteases play a critical role in many physiological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens (Delepelaire and Wandersman, 1989), release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Rawlings and Barrett, 1993).

Proteases are degradation enzymes which catalyze the total hydrolysis of proteins. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, whereas intracellular proteases play a critical role in the regulation of metabolism. Bacterial proteases are also important virulence factors in many diseases (Farrell and Crosa, 1991). They are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine protease, aspartic proteases, cysteine proteases, and metalloproteases (Beynon and Bond, 1989). The mechanism of action of proteases has been a subject of great interest to researchers.

In this study, the major caseinolytic protease from *Bacillus amyloliquefaciens* S94 has been purified from culture supernatant to apparent homogeneity by a combination of ammonium sulfate precipitation, QAE-Sephadex chromatography, SP-Sephadex chromatography, and Sephacryl S-100 chromatography.

Materials and Methods

For protease production, cells (*Bacillus amyloliquefaciens* S94) were grown aerobically in media containing 0.2% bactopeptone, 0.2% beef extract, 0.8% NaCl and 2% skim milk at 25°C for 20 h with constant shaking (150 rpm). After 20 hr, the culture medium was centrifuged at 12,000×g for 15 min at 4°C to pellet the cells; then the supernatant was chilled and concentrated by ultrafiltration Minitan (molecular size cut-off, 10 kDa, Millipore). The concentrate was used for further purification steps.

Protease purification

All steps were performed at 4°C unless otherwise indi-

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cated.

1) *Ammonium Sulfate Precipitation.* Fractions of 60~80% ammonium sulfate saturation contained comparatively high protease activities by preliminary result. Four liters of chilled culture medium were concentrated and ammonium sulfate was added to the supernatant to 60% saturation. After stirring gently for 1 hour, the medium was centrifuged at 12,000×g for 30 min. To the supernatant, solid ammonium sulfate was added to 80% saturation. After stirring gently for 1 hour, the pellets were collected by centrifugation (12,000×g, 30 min). The dark-brown pellets were resuspended by 20 mM Tris-HCl buffer (pH 7.0) and dialyzed overnight at 4°C against the same buffer. The insoluble material was separated by centrifugation. The suspended pellet was stored at 4°C.

2) *QAE A-25 column chromatography.* The column was prepared, degassed, and packed into a column following the manufacture's recommendations. The dialyzed supernatant was applied to a QAE A-25 column (2.5×20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was eluted with a linear gradient of 0 to 0.5 M KCl in 20 mM Tris-HCl buffer (pH 7.0) at a flow rate of 3 ml/min. Fractions of 4 ml were collected and pooled as indicated in the text.

3) *SP C-25 column chromatography.* The pooled fractions from the QAE Sephadex were dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 7.0) with two changes and applied to a SP C-25 Sephadex column (2.5×8 cm) equilibrated with the same buffer used for dialysis. The column was washed with 2 column volumes of 20 mM Tris-HCl buffer (pH 7.0) and then bound proteins were eluted with a linear gradient of 0 to 0.7 M KCl in 20 mM Tris-HCl buffer (pH 7.0) at 3 ml/min. Fractions of 3 ml were collected, and the fractions containing protease activity were collected and concentrated by ultrafiltration with an Amicon PM 10 membrane.

4) *Sephacryl S-100 Gel filtration chromatography.* The concentrated sample (2 ml) was applied to a Sephacryl S-100 gel filtration column (1.6×60 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted with 2 column volumes of 20 mM Tris-HCl buffer (pH 7.0) at 0.6 ml/min.

Protein assay

Protein determinations were performed by using the method of Bradford or total protein from each step of the purification was determined by using bicinchonic acid assay (BCA; Sigma) with bovine serum albumin as standard (Smith *et al.*, 1985).

Protease assay

For routine assays during purification, pH optimum determination, temperature effects, and the effects of inhibitors, the activity of protease was measured spectrophotometrically at 440 nm with using azocasein as substrate. The caseinolytic activity was determined by a method described previously (Jung *et al.*, 1999). The standard mixture (0.5 ml) contained 50 mM Tris-HCl (pH7.0), 0.2% (w/v) azocasein, and enzyme. The reaction mixture was incubated at 30°C for 60 min and was stopped by adding an equal volume of 10 %TCA (trichloroacetic acid) and standing on ice for 10 min. After removal of the precipitated azocasein by centrifugation (12,000 g, 3 min), the absorbance of the supernatant was read at 440 nm. One unit (U) of hydrolytic activity of the protease was defined as the amount of enzyme required to cause an increase of 0.001 A_{440} unit in 1 min.

Polyacrylamide Gel Electrophoresis

Samples taken from each of the purification steps were electrophoresed on 10% SDS-polyacrylamide gels as previously described according to the method of Laemmli (Laemmli, 1970). Standard protein markers were from Bio-Rad. Gels were stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma) and destained in 5% methanol/7% acetic acid.

Molecular Weight Determination

Molecular weight of the protease was determined by two methods: (1) SDS-PAGE, comparing its migration with those of standard molecular weight proteins, and (2) gel filtration on Sephacryl S-100 column (1.6×60 cm), comparing the elution position with those of standard proteins including albumin (66,000), carbonic anhydrase (29,000), cytochrome C (12,400), and aprotinin (6,500). The column was eluted with 20 mM Tris-HCl buffer (pH 7.0) at 0.6 ml/min. The elution volume of the enzyme was determined by A_{280} and enzyme activity, and the apparent molecular weight of the enzyme was calculated by comparison with the relative elution volumes of protein standards.

Results

Protease purification

Initial studies showed that *Bacillus amyloliquefaciens* S94 grown on casein-containing nutrient agar plates secreted extracellular protease which readily digested the casein. The secretion of this protease was not dependent on the presence of casein, and so could be harvested from cultures grown in nutrient broth. The conditions for the growth of *Bacillus amyloliquefaciens* S94 at 25°C for 20 h allowed for the production of enough protease activity to make purification feasible.

In the fractions with 60-80% ammonium sulfate saturation, the protease activity was maximally precipitated from the cleared growth medium. The dialyzed ammonium sulfate pellet was applied to a QAE-Sephadex column. A large amount of caseinolytic activity was detected

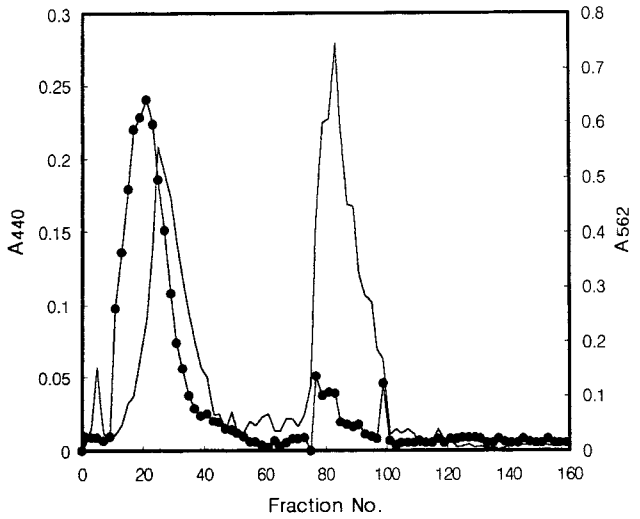

Fig. 1. QAE Sephadex A-25 chromatography. The crude extract obtained from the culture filtrate were loaded on a QAE A-25 column. Fractions were assayed for activity on azocasein (●) and monitored for protein content (–) at 562 nm by BCA assay.

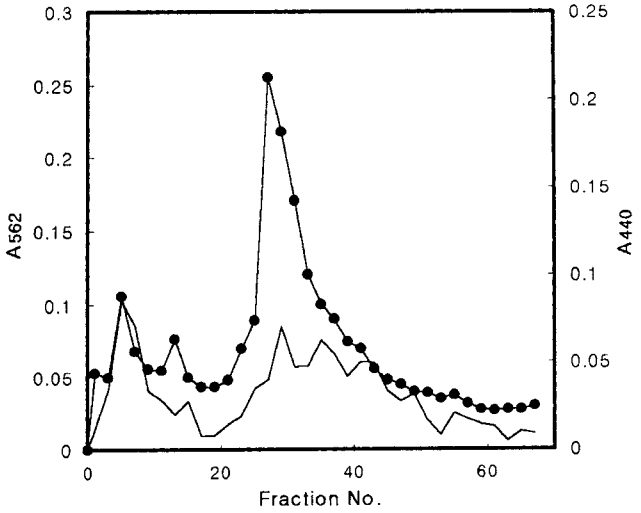

Fig. 2. SP Sephadex C-25 chromatography. The activity peaks purified by QAE A-25 column chromatography were concentrated and loaded onto a SP C-25 column. Fractions were assayed for activity on azocasein (●) and monitored for protein content (–) at 562 nm by BCA assay.

in the unadsorbed flow-through from the QAE-Sephadex column (Fig. 1), which indicated that the protease behaved as a neutral or alkaline molecule and unbound to QAE-Sephadex under near-neutral conditions. Some other protease activities were followed through the purification. A small amount of activity capable of cleaving the casein substrate was detected in the fractions adsorbed to QAE-Sephadex, which were eluted with 0.1 M KCl, were not further studied. Although the summary of the purification (Table 1) shows that chromatography of the protease on QAE-Sephadex resulted in a net loss of activity, this step was necessary to eliminate contaminating proteins or proteases which eluted with the protease on the next purification step. Fractions with high caseinolytic activity from QAE-Sephadex column were combined and loaded to the SP-Sephadex column. Bound proteins were eluted with a linear gradient of 0 to 0.7 M KCl in 20 mM Tris-HCl buffer (pH 7.0). The caseinolytic activities were detected in the unadsorbed fractions from the SP-Sephadex column. However, the major active fractions were eluted with 0.2 M KCl (Fig. 2). Sephacryl S-100 was used to further fractionate the eluate from the SP-Sephadex step. This step resulted in the highly effective step for the elimination of contaminants,

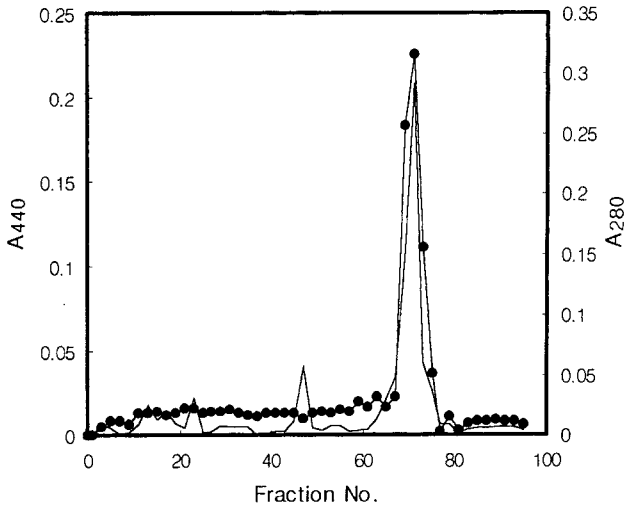

Fig. 3. Sephacryl S-100 chromatography. The activity peaks purified by SP C-25 column chromatography were concentrated and loaded onto a Sephacryl S-100 gel filtration column. The column was eluted with 2 column volumes of 20 mM Tris-HCl buffer (pH7.0) at 0.6 ml/min. Fractions were assayed for activity on azocasein (●) and monitored for protein content (–) at 280 nm.

Table 1. Purification and yield of the protease

Purification step	Total activity unit	Total protein mg	Specific activity unit/mg	Yield %	Purification fold
Crude extract	71,320	5,830	12	100	1.0
Ammonium Sulfate 60~80%	22,800	600	38	32.0	3.1
QAE A-25 (unbound)	7,420	90.7	110	10.4	9.0
SP C-25	9,980	37.0	272	14.0	22.2
Sephacyl S-100	2,262	3.9	586	3.2	48.0

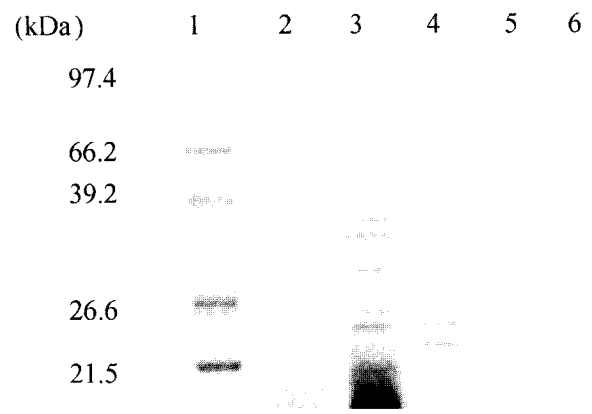

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purification steps. The pooled samples from each of the steps were subjected to electrophoresis and stained with Coomassie Blue R-250. The electrophoresis was performed according to the method of Laemmli using 10% gel. *Lane 1*, molecular markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; aldolase, 39.2 kDa; triose phosphate isomerase, 26.6 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa *Lane 2*, crude extract; *lane 3*, unbound eluent from QAE A-25 sephadex; *lane 4*, 0.1M KCl eluent from SP C-25 sephadex, *lane 5*, purified protease SE94, *lane 6*, active fractions from Sephacryl S-100.

with the bulk of the protein eluting to the protease activity (Fig. 3). Final purification to electrophoretic homogeneity was achieved by this step. The purity of the isolated enzyme was confirmed by SDS-PAGE (Fig. 4). By SDS-PAGE, a single band with a molecular mass of 23,000±2,000 Da was observed (Fig. 5A).

By successive chromatographic procedures utilizing QAE-Sephadex, SP-Sephadex, Sephacryl S-100, the caseinolytic protease was purified 48 fold. Table 1 summarizes the results of a typical purification from 4 liters of 20 hrs culture supernatant. The apparent yield of activity was only 0.5%. However, this is an approximation because of the possible presence of other proteases in the crude sample, which may degrade the substrate, and because of the difficulty in measuring the protein content of the purified enzyme.

The native molecular weight of the purified protease was determined on a calibrated column of Sephacryl S-100. As compared with a standard mixture of proteins of known molecular weight (albumin, carbonic anhydrase, cytochrome c, and aprotinin), the caseinolytic protease, which eluted as a single symmetrical peak, was determined to have a molecular mass of 23 kDa (Fig. 5B). This value was observed in the denaturing condition, determined from the SDS-polyacrylamide gel electrophoresis (Fig. 5) and clearly indicates that this protease is monomeric in its native state.

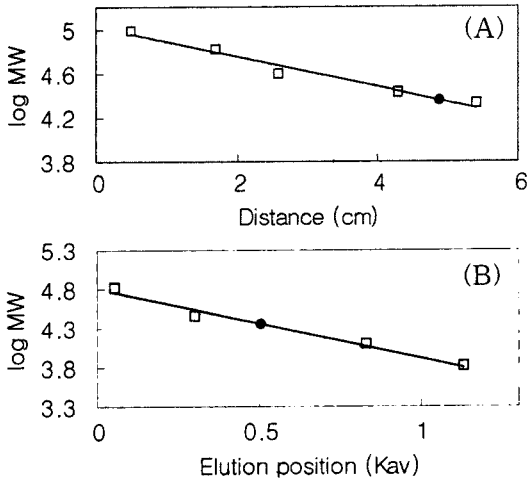

Fig. 5. Molecular weight determination of the purified protease. (A) Calibration curve for the migration of molecular marker proteins (□) and purified protease (●) on 10% SDS-PAGE. (B) Calibration curve for elution position of proteins on Sephacryl S-100. Molecular weight standards were phosphorylase B(97,400), bovine serum albumin (66,200), aldolase (39,200), triose-phosphate isomerase (26,600), and trypsin inhibitor (21,500).

Protease Activities

(1) pH profile. The proteolytic activity of the purified enzyme was determined at several pH values. The pH dependence of purified enzyme was measured by assaying at pH values from 5 to 11 in the presence of 50 mM buffers of MES, Tris-HCl, CAPS buffer. The protease displayed a broad pH activity profile in the neutral to basic range. Very little activity was seen at or below pH 6, but a gradual increase was seen above pH 7. The purified protease was maximally active at pH 10 against azocasein (Fig. 6). Approximately 63% of maximal activity was observed at pH 11, but there was no activity over pH 12.

(2) Temperature profile. When the proteolytic activities

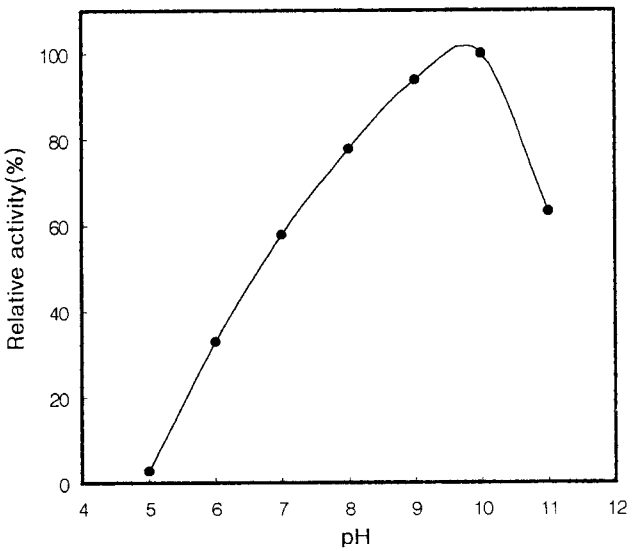

Fig. 6. Activity of the protease at different pH values. The enzyme activity was determined using azocasein (0.2%) as substrate in the reaction buffers. The reaction mixture was incubated at 30°C for 60 min.

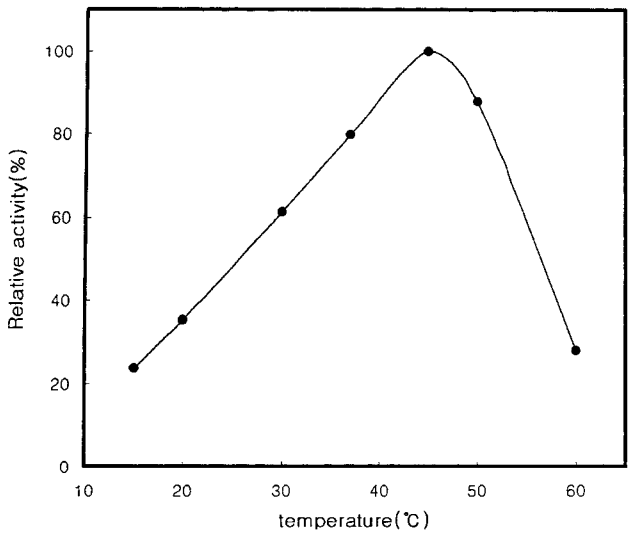

Fig. 7. Effect of temperature on enzyme activity. The enzyme activity was determined using azocasein (0.2%) as substrate in the reaction buffer of 50 mM Tris-HCl (pH7.0). The reaction mixture was incubated for 60 min.

were measured at various temperatures ranging from 15°C to 60°C with natural substrate azocasein, the hydrolysis of azocasein was also influenced by temperature (Fig. 7). Enzymatic activity of the protease increased with temperature from 15°C to 45°C. The enzyme exhibited its maximal activity at 45°C and this activity was abruptly lost above 50°C. At low temperatures below 20°C, the enzyme maintained over 20% of its optimum temperature activity.

(3) Effects of detergents, reducing agents, salts on the protease activity. The purified enzyme was preincubated with various reagents for 10 min at 37°C and the enzyme

Table 2. Effect of detergent, reducing agents, and salts on the protease activity

Substance group	Substance	Conc	Relative activity (%)
None			100
Detergent	SDS	0.01%	86.31
		0.1%	52.09
	Triton X-100	0.01%	74.14
		0.1%	55.13
Reducing agents	DTT	1 mM	101.28
		10 mM	73.82
	β-mercaptoethanol	1 mM	101.72
		10 mM	99.57
Salts	NaCl	0.1 M	93.78
		0.5 M	92.04
	KCl	0.1 M	94.43
		0.5 M	93.78
	NH_4Cl	0.1 M	93.30
		0.5 M	78.86
	LiCl	0.1 M	98.08
		0.5 M	85.65

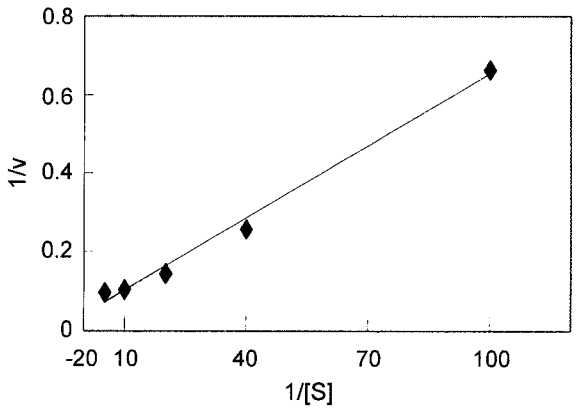

Fig. 8. Lineweaver-Burk plot for the hydrolysis of azocasein by the protease. The protease activity was measured in 50 mM Tris-HCl (pH8.0) at 37°C.

activities were measured. As shown in Table 2, neither ionic (SDS) nor non-ionic (Triton X-100) detergent strongly affected the enzyme activity. In the presence of a relatively high concentration (0.1%) of detergent, the enzyme maintained over 50% of its activity. Reducing agents such as DTT and β-mercaptoethanol had partial or little effect on the enzyme activity. In the presence of salts (KCl, NaCl, NH_4Cl or LiCl) up to 500 mM, the protease activity was not severely inhibited.

(4) Enzyme kinetics. Protease activity obeyed Michae-

Table 3. Effect of inhibitors on the protease activity

Inhibitor	Concentration [mM]	Relative Activity (%)
None		100
PMSF	0.001	68
	0.01	4
	0.1	0
TPCK	0.1	92
	1	90
TLCK	0.1	95
	1	92
Leupeptin	0.1	97
	1	95
Iodoacetamide	0.1	97
	1	90
E-64	0.1	90
	1	86
Pepstatin A	0.1	95
	1	90
EDTA	0.1	86
	1	82
Bestatin	0.001	86
	0.01	40
	0.1	11

Protease (0.2 μM) was incubated for 10 min at 37°C with the inhibitors prior to the addition of substrate. Activity was then assayed by further incubation of the mixtures for 30 min as described under "Experimental Procedures".

lis-Menten type kinetics when azocasein was used as substrate. Kinetic parameter (V_{max}, and K_m) values for the hydrolysis of azocasein by the protease were determined by plotting substrate concentration versus velocity. The K_m and V_{max} for azocasein were 10.1 units and 6.4 units/min, respectively (Fig. 8).

(5) Effects of inhibitors on the activity. The inhibitor studies (Table 3) showed that the protease was insensitive to a number of inhibitors, including the broad-spectrum inhibitor leupeptin. Its insensitivty to metal chelator (EDTA) indicated that it is not a metalloprotease. In addition, iodoacetamide and E-64 had little effect on its activity. Pepstatin A, an acid protease inhibitor, also did not significantly affect its acitivity. The activity of the protease was completely abolished by phenylmethylsulfonyl fluoride (PMSF), but not by TPCK and TLCK which are irreversible inhibitors for serine protease.

Discussion

The enzyme activity in this study was strongly inhibited by serine protease inhibitor PMSF, suggesting that the enzyme is a serine protease. And, the purified enzyme activity was inhibited by leucine peptidase inhibitor, bestatin. The purified protease cleaved on the carboxyl side of leucine residues as determined by substrate susceptibility studies (data not shown). The maximum proteolytic activity against different protein substrates (synthetic substrate) occurred at pH 8.0, 45°C (data not shown). The partial inhibition of the protease by the reducing agents suggest the possible involvement of a cysteine residue close to the active site, or the presence of an essential thiol group in the protein structure (Barrett and Kirschke, 1981). The protease had characteristics of a cold-adapted protein (Sai Ram *et al.*, 1994), i.e., it was more active in the hydrolysis of synthetic substrate in the range of 15 to 45°C and had an optimum activity at 37°C.

Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. Their substrate specificity is similar to but less stringent than that of chymotrypsin (Fiechter, 1986). They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9 (Kang *et al.*, 1995). Their molecular mass is in the range of 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter, Streptomyces* (Bockle *et al.*, 1995), and *Flavobacterium* spp. (Boguslawski *et al.*, 1983), subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *S. cerevisiae* and filamentous fungi such as *Conidiobolus* spp (Phadatare *et al.*, 1992) and *Aspergillus* and *Neurospora* spp. (Rawlings and Barrett, 1993).

For the evaluation of a biotechnological application of the proteinase of *Bacillus* sp, a more detailed understanding of the factors that enable this enzyme to act on compact substrates better than comparable enzymes of the same type would be helpful. Therefore, more research on the specific molecular characteristics of this interesting enzyme will be done.

Acknowledgment

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