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NOTE

Multicatalytic Alkaline Serine Protease from the Psychrotrophic *Bacillus amyloliquefaciens* S94

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An extracellular protease of *Bacillus amyloliquefaciens* S94 was purified to apparent homogeneity. The enzyme activity was strongly inhibited by general inhibitor for serine protease, PMSF, suggesting that the enzyme is a serine protease. The purified enzyme activity was inhibited by leucine peptidase inhibitor, bestatin, suggesting that the enzyme is a leucine endopeptidase. The maximum proteolytic activity against different protein substrates occurred at pH 10, 45°C (protein substrate) and pH 8, 45°C (synthetic substrate). The purified enzyme was specific in that it readily hydrolyzed substrates with Leu or Lys residues at P_1 site. The protease had characteristics of a cold-adapted protein, which was more active for the hydrolysis of synthetic substrate in the range of 15°C to 45°C, specially at low temperature.

Key words: *Bacillus amyloliquefaciens,* multifunctional endopeptidase, serine protease, cold active enzyme.

Proteases are degradative enzymes which catalyze the specific and selective modifications of proteins. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990).

Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermostability. Neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity. Bacterial alkaline proteases are characterized by their high activity at alkaline pH and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable use in the detergent industry.

Proteases are grossly subdivided into two major groups, exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond promixal to the amino or carboxy termini of substrate, while endopeptidases cleave the internal peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases can be divided into four groups, serine protease, cysteine protease, aspartic protease and metalloprotease (Hartley, 1960).

The caseinolytic protease, designated as protease SE910, from *Bacillus amyloliquefaciens* S94 isolated from the intestinal flora of rainbow trout, was characterized to be a serine protease (Son and Kim, 2002). In this work endopeptidase activity of protease SE910 was analyzed. The protease was purified as previously described (Son and Kim, 2002). For routine assays, pH optimum determination, temperature effects, and the determination of substrate specificity, the enzyme activity was measured spectrophotometrically with using azocasein or synthetic substrate as substrate. The standard mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8.0), 0.1% azocasein, and enzyme. The reaction mixture incubated at 30°C for 60 min and was stopped by adding equal volume of 10% TCA and stand on ice for 10 min. After removal of the precipitated azocasein by centrifugation (12,000 g, 3 min), the absorbance at 440 nm was measured. Hydrolytic activity toward synthetic substrate was estimated with *p*-nitroanilides of amino acid (0.2 mM). Activity was determined by measuring the release of *p*-nitroanilide at 405 nm with an extinction coefficient of 9,620 $M^{-1}cm^{-1}$.

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For the determination of specificity of protease, P_1 specificity of the purified enzyme was tested with a series of p-nitroanilide substituents of amino acids (Kasche *et al.*, 1989; Rajak *et al.*, 1992). Among the substrates analyzed for susceptibility to protease SE910, the enzyme hydrolyzed N-succinyl-Ala-Ala-Pro-Leu-pNA more rapidly than other aminoacyl *p*-nitroanilides. Synthetic substrate with Leu at P_1 residue was the most effectively hydrolyzed, suggesting that this enzyme could cleave the peptide bond on the carboxyl side of a small hydrophobic amino acid residues. However, protease activity with the other synthetic substrate was negligible. There was no measurable hydrolysis for *N*-succinyl-Ala-Ala-Ala-pNA, *N*-succinyl-Ala-Ala-Val-pNA, *N*-glutaryl-Phe-pNA, and *N*-benzoyl-Tyr-pNA. The purified enzyme also hydrolyzed the substrate with Lys residues at P1 residue. The enzyme was able to release *p*-nitroanilide from *N*-Tosyl-Gly-Pro-Lys-pNA, which contains a modified moiety at free NH_2 group of lysine residue and is a substrate for the protease of trypsin family, at the lower level (8%). But *N*-benzoyl-Arg-pNA, which is a substrate of trypsin, was not hydrolyzed by the enzyme. Protease SE910 was not able to cleave substrates with a single amino acid, leucine linked to para-nitroanilide (Table 1). These results demonstrate that protease SE910 has the endopeptidase activity only toward *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA and *N*-Tosyl-Gly-Pro-Lys-*p*NA, but not Leucine aminopeptidase (exopeptidase) activity.

The proteases of trypsin family are serine protease with endopeptidase activity and hydrolyze peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of polypeptide chain. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. Clostripain, produced by the anaerobic bacteria *Clostridium histolyticum*, exhibits a stringent specificity for arginyl residues at the carboxyl side of the splitting bond (Ullmann and Jakubke, 1994). Some of the serine protease from *Achromobacter* spp. are lysine specific endopeptidase (Masaki *et al.*, 1978) whereas those from *Flavobacterium* spp. are post-proline specific (Yoshimoto *et al.*, 1980). Endopeptidases that are specific to glutamic acid and aspartic acid residues have also been found in *B. licheniformis* and *S. aureus* (Drapeau *et al*, 1972).

Table 1. Substrate Specificity of protease SE910

Substrate	Initial velocity (μM/min)
N-succinyl-Ala-Ala-Ala-*p*-nitroanilide	0.001
N-succinyl-Ala-Ala-Val-*p*-nitroanilide	0.002
N-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide	6.89
L-Leucine-*p*-nitroanilide	0.001
N-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide	0.563
L-Lysine-*p*-nitroanilide	0.001
N-Glutaryl-L-Phe-*p*-nitroanilide	0.001
N-Benzoyl-L-Tyr-*p*-nitroanilide	0.002
L-Methionine *p*-nitroanilide	0.001
L-Glutamic acid-γ-*p*-nitroanilide	0.001
Nα-Benzoyl-DL-Arg-*p*-nitroanilide	0.002

Enzyme assay was carried out at 37°C in the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 0.2 mM each synthetic substrate. Changes in the absorbance at 405 nm were monitored continuously for 60 min to each substrate.

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, or CAPS. The enzyme was active over a pH range of 6 to 11. Protease activity was increased as the increasing pH ranges from 5 to 8. The higher levels of hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA were observed at alkaline pHs rather than at acidic or neutral pH and maximal protease activity occurred at pH 8 against *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA (Fig 1A). At alkaline pH above 8, compared to portein substrate (azocasain) (Fig. 1C), significant decreases of enzyme activity have been observed. At pH 11 the enzyme maintained approximately 50% of maximal activity, but there was no activity at pH≥12. Variation of the buffers caused a slight effect on enzyme activity; at pH 9, Tris buffer yielded 98% of the activity compared with pH 8.0, and the activity in HEPES buffer was 93.5% of that in Tris buffer at the same pH and concentration.

When the enzyme activity was measured with *N*-Tosyl-Gly-Pro-Lys-pNA as substrate over a pH range from 5 to 11 the pH activity curve was quite similiar to the pH profile of the reactions with *N*-succinyl-Ala-Ala-Pro-Leu-pNA as substrate (Fig. 1B). The protease displayed a broad pH activity profile in the neutral to basic range. At pH ranges of 5 to 9, enzymatic activity was significantly increased with increasing pH. Very little activity was seen below pH 6, but a gradual increase was seen above pH 7. The purified protease was maximally active at pH 8 against *N*-Tosyl-Gly-Pro-Lys-pNA. Approximately 43% of maximal activity was observed at pH 11, but there was no activity over pH 12. Both Leu-endopeptidase and Lys-endopeptidase were active at pH 8. It indicates that the lower hydrolytic activity toward *N*-Tosyl-Gly-Pro-Lys-pNA than toward *N*-succinyl-Ala-Ala-Pro-Leu-pNA (Table 1) was the intrinsic catalytic activity of enzyme against substrates, not caused by doing the reaction in suboptimal pH condition.

When the proteolytic activities were examined with protein substrate (azocasein), the pH profile, shown in Fig. 1C, was somewhat different. Protease activity was increased from pH 5 to 9. The maximum proteolysis has occurred at pH 10. At pH 11 the enzyme retained approximately 63% of maximal activity, but there was no activity at pH 12. This could be partly due to the inactivation of enzyme at pH 12. At alkaline pH ranges from 8 to 10,

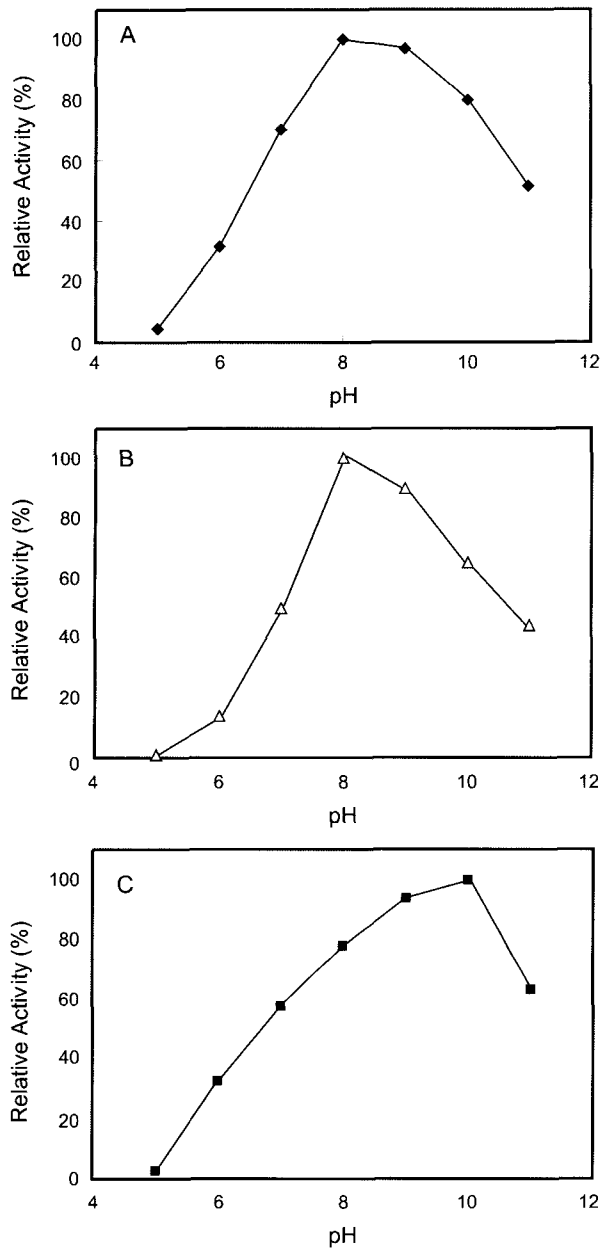

Fig. 1. Effect of pH on enzyme activity. The enzyme activity was measured using (A) *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA (B) *N*-Tosyl-Gly-Pro-Lys-pNA (C) azocasein as substrate in the 50 mM buffer of pH indicated at 30°C.

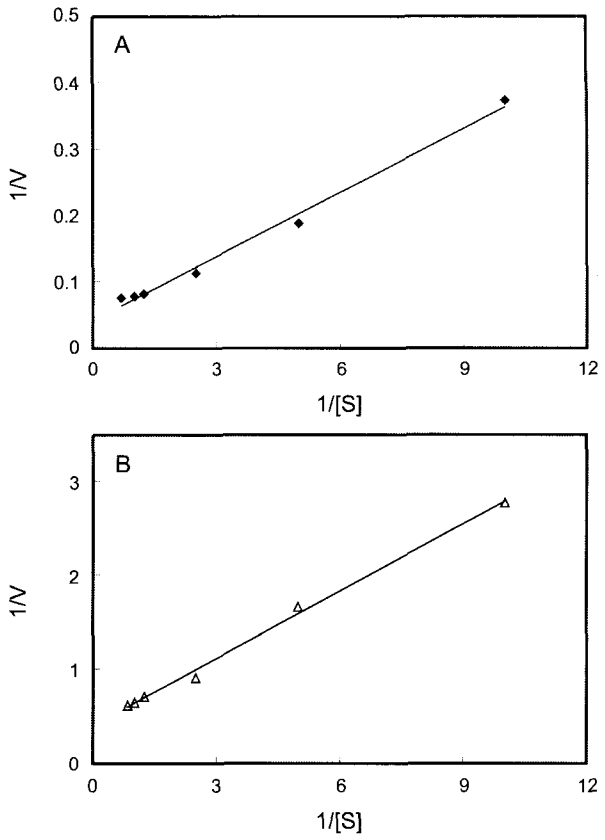

Fig. 2. Lineweaver-Burk plot for the hydrolysis of synthetic substrate by protease SE910. The hydrolytic activity against (A) N-succinyl-Ala-Ala-Pro-Leu-pNA (B) N-Tosyl-Gly-Pro-Lys-pNA was determined in the reaction mixture containing 50 mM buffer (pH 8)

compared to synthetic substrate (*N*-succinyl-Ala-Ala-Pro-Leu-pNA), significant increases of enzyme activity have been observed. This could be partly due to the unstabilization of the protein substrate, which could contribute the easy accessibility of the enzyme to the cleavage site on protein.

The kinetic parameters for the hydrolysis of hydrolyzable substrates by the enzyme were estimated. The Michaelis constant (K_m) and maximum velocity (V_{max}), for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-pNA, determined by the analysis of Lineweaver-Burk plot, were 0.7 mM and 22.5 μM of pNA released/min, respectively (Fig. 2A). Those values are comparable to those of kinetic parameters of other endopeptidases, which ranged between 0.1 and 2 mM (Gonzales and Robert-Baudroy, 1996). The K_m and V_{max} values for hydrolysis of *N*-Tosyl-Gly-Pro-Lys-pNA was 0.64 mM and 2.5 μM/min, respectively (Fig. 2B). In terms of catalytic efficiency (k_{cat}/K_m) the value for *N*-succinyl-Ala-Ala-Pro-Leu-pNA (substrate for leucine endopeptidase) was 2.68×10^3 $M^{-1}s^{-1}$, which was ten-fold higher than that for *N*-Tosyl-Gly-Pro-Lys-pNA (substrate for lysine endopeptidase). This indicates that protease SE 910 could hydrolyze leucyl peptide bond more easily than lysyl peptide peptide bond.

The temperature dependence of purified enzyme was measured. To do this, temperature profile of the protease SE910 with natural substrate azocasein was analyzed, the enzymatic activity of protease SE910 increased with temperature from 15°C to 45°C with maximal activity occurring at 45°C (Fig. 3B adotped from Son and Kim, 2002). With synthetic substrate *N*-succinyl-Ala-Ala-Pro-Leu-pNA, the enzymatic activity of protease SE910 increased with temperature from 15°C to 45°C (Fig. 3A). The enzyme

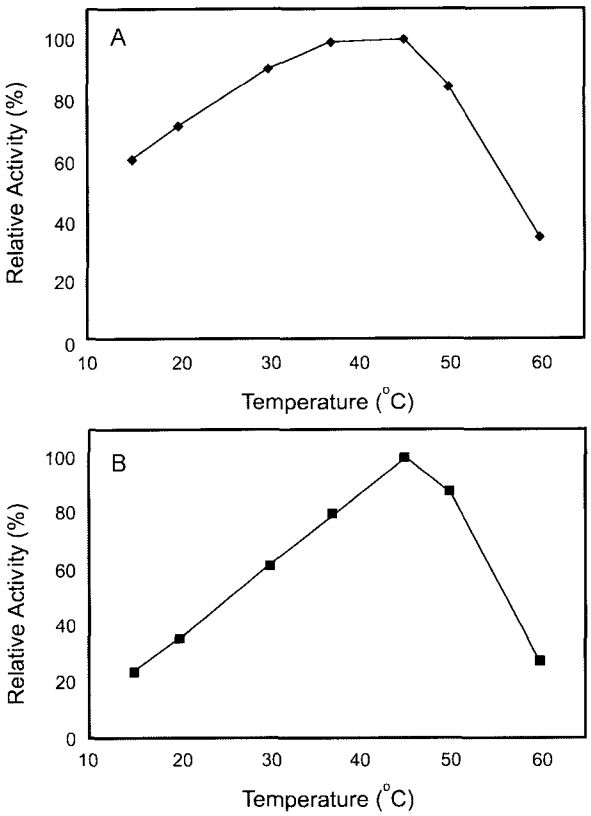

Fig. 3. Effect of temperature on enzyme activity. The enzyme activity was measured using (A) *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA (B) azocasein as substrate in the 50 mM Tris-HCl (pH 8).

exhibited its maximal activity at 45°C but a broad maximum of activity between 37°C and 45°C was observed. This activity was abruptly lost above 50°C because of thermal inactivation. At temperatures below 20°C, the enzyme maintained over 60% of its activity at the optimum temperature. The enzyme retained higher levels of the activity at low temperature. There was a greater increase in protease activity at 37°C than over 50°C, presumably indicating greater stability at low temperatures. Thus, protease SE910 is underestimates of the low-thermophilic, psychrotropic protease. When enzyme activities using synthetic substrate *N*-Tosyl-Gly-Pro-Lys-pNA as substrate were measured, judging from the initial velocity, the enzyme exhibited the same pattern of temperature profiles as those observed with *N*-succinyl-Ala-Ala-Pro-Leu-pNA as substrate (data not shown). Protease SE910 exhibited the highest levels of activity with both azocasein and synthetic substrates at 45°C. No distinct change was observed in the dependence of enzyme activity on temperature or in thermal stability when the hydroytic activities were estimated with both *N*-succinyl-Ala-Ala-Pro-Leu-pNA and *N*-Tosyl-Gly-Pro-Lys-pNA. Protease SE910 retained the higher hydrolytic activity against synthetic substrates than against protein substrate and it was more active for the hydrolysis of synthetic substrate at low temperatures. The higher catalytic activities of protease SE910 with synthetic substrate probably expected to have increased structural flexibility at low temperature. This prominent feature is characteristics of cold-active enzymes, which exhibit high levels of activity at low temperatures. Cold-adapted microorganisms, which include psychrophiles and psychrotrophs, are known to produce cold-active enzymes. Homologous counterparts of the cold-active enzymes are produced by mesophilic or thermophilic microorganisms but are less active at low temperatures (Kulakova *et al.*, 1999). In order to obtain high catalytic efficiency, cold-active enzymes probably have evolved to have high conformational flexibility, although stability has been sacrificed (Davail *et al.*, 1994). Thermal instability of cold-adapted enzymes is therefore regarded as a consequence of their conformational flexibility. When compared to their mesophilic counterparts, cold-active enzymes display a higher catalytic efficiency over a temperature range of roughly 0-30°C and a high thermosensitivity. It appears that the molecular adaptation for the flexible structures resides in a weakening of the intramolecular interactions such as salt bridges, hydrogen bonding, hydrophobic interactions, and aromatic-aromatic interactions (Zavodszky *et al.*, 1998), and in some cases in an increase of the interaction with the solvent, leading to more flexible molecular edifices capable of performing catalysis at a lower energy cost (Gerday *et al.*, 1997).

Ten enzyme inhibitors were tested for their ability to block the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA. The only compound to demonstrate complete inhibition of protease SE910 activity was PMSF. Partial inhibition was observed with the leucine peptidase inhibitor, Bestatin (Son and Kim, 2002). Inhibition studies suggest that protease SE910 is a serine protease.

In conclusion protease SE910 cleaved on the carboxyl side of both leucine and lysine residues as determined by substrate susceptibility studies. The lack of cleavage of the substrate L-Leucine *p*-nitroanilide suggests that protease SE910 requires the presence of more than one amino acid for proteolysis to occur. This demonstrates that protease SE910 is a multifunctional endopeptidase. The enzyme activity was strongly inhibited by serine protease general inhibitor, PMSF, suggesting that the enzyme is a serine protease. The maximum proteolytic activity against different protein substrates occurred at pH 8.0, 45°C (synthetic substrate). The protease had characteristics of a cold-adapted protein, i.e., it was more active for the hydrolysis of synthetic substrate in the range of 15°C to 45°C and had an optimum activity at 45°C. However, the enzyme exhibited decreased activity over 50°C probably because of thermal instability.

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