Jour. Microbiol. June 1995 p. 115-119 Vol. 33, No. 2

Purification and Characterization of an Alkaline Protease Produced by a *Xanthomonas* sp. YL-37

Chang-Ho Lee, Hee-Sik Kim, Gi-Seok Kwon, Hee-Mock Oh, Sang-Mo Kang[1], Tae-Jong Kwon[1], and Byung-Dae Yoon*

Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Taejon 305-600, Korea
[1]Department of Microbial Engineering, Kon-Kuk University, Seoul 133-701, Korea

(Received May 17, 1995/Accepted June 9, 1995)

The alkaline protease of *Xanthomonas* sp. YL-37 has been purified, and the properties of the enzyme investigated. The alkaline protease of *Xanthomonas* sp. YL-37 was purified from crude enzyme by ammonium sulfate fractionation, CM-cellulose ion exchange chromatography, and Sephadex G-100 gel filtration. Through the series of chromatograpies, the enzyme was purified to homogenecity with specific activity of 4.23 fold higher than that of the crude broth. The molecular weight of the purified protease has been estimated to be 62 KDa on SDS-polyacrylamide gel electrophoresis. The optimal pH and temperature for alkaline protease activity were 11.0 and 50°C, respectively. The enzyme was stable between pH 5.0 and 10.0, and up to 50°C. Enzyme activity was lost up to 50% on heating at 70°C for 30 minutes. The activity of alkaline protease was inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, PMSF, and activated by Mn^{2+} and Ca^{2+}. The K_m value for casein as a substrate was 4.0 mg/ml.

Key words: *Xanthomonas* sp. YL-37, alkaline protease

Protease are differentiated by mechanism of catalysis such as serine protease, thiol protease, acid protease, and metallo protease (4). These protease have been widely applied to the food industry, detergent and pharmaceutical industries. The serine protease, alkaline protease, is used in the detergent industry. Alkaline protease exhibit a maximum activity at a pH of about 10.0 to about 11.0. Most of them exhibit a maximum activity at a relatively high temperature, especially around 60℃, but are inactive or less active at a relatively low temperature, especially around a room temperature. This is suggested that the enzyme is not fully utilized as a detergent additive in north-eastern Asia, where clothes are generally washed at 15~25℃. Since the production of alkaline protease from alkalophilic bacteria was first reported by Horikoshi (5), many microbial alkaline proteases have been purified and characterized from actinomycete (18), fungi (15), *Bacillus* sp. (9, 14, 16), and *Streptomyces* (8). Alkaline protease produced by bacteria of the genus *Bacillus* show an optimal activity and a good stability at high alkaline pH values. They are stabilized by calcium ions and inactivated by serine active-site inhibitors (6). Over the past several years, proteolytic enzymes have frequently been used in laundry products, mostly because of decreased use of phosphate in detergents and as a means of compensating for the poorer performance at lower washwater temperatures (7). In this present work, purified alkaline protease was obtained from the culture solution of *Xanthomonas* sp. YL-37 after ammonium sulfate fractionation, CM-cellulose chromatography, and Sephadex G-100 gel filtration. Several enzymatic properties and the effect of several enzyme inhibitors and metal ions have been investigated.

* To whom correspondence should be addressed.

Materials and Methods

Organism and culture medium

Bacteria strain used in this study was *Xanthomonas* sp. YL-37 which discribed previous paper (11). The strain cultivation was carried out at 20℃ for 84 hours with reciprocal shaking (120 strokes/min). *Xanthomonas* sp. YL-37 was grown on a liquid culture medium contained

sucrose 60 g, soybean meal 20 g, tryptone 10 g, K_2HPO_4 2 g, NH_4NO_3 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, and Na_2CO_3 3 g in 1 liter of distilled water. This last salt was sterilized separately (in 10% of total volume) and then added into the medium.

Protease assay

Azocasein hydrolysis was measured by the method discribed by Leighton *et al.* (12) with minor modifications as follows: Commercial azocasein (Sigma) was dissolved as a 5% (w/v) solution in 0.2 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$. The solution was stored at −20℃. A 1 ml reaction mixture contained; 0.2 ml of azocasein solution, 0.2 ml of 1 M Tris-HCl (pH 8.0), 0.1 ml of 10 mM $CaCl_2$, 0.1 ml of protease solution and D.D.W. to make 1 ml. This mixture was incubated for 30 min at 40℃. The reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The reaction tubes were cooled at 0℃ for 15 min and centrifuged to remove the precipitated protein. The supernatant fraction was carefully withdrawn and filtered through a Millipore filter (pore size, 0.45 μm). A 1.2 ml amount of the filtrate was mixed with 0.3 ml of 1.8 N NaOH and the absorbancy read at 420 nm with a Beckman spectrophotometer (model DU-60A). One unit of proteolytic activity was expressed as μg of azocasein hydrolyzed per 30 min, at 40℃.

Protein determination

Protein concentration was measured by the method of Bradford (3) with bovine serum albumin as a standard. During the purification steps, A280 was used to indicate the elution pattern of proteins.

Purification of protease

All purification steps were carried out at 4℃. The culture filtrate was precipitated with ammonium sulfate (30~80% of saturation) and precipitant was dissolved with 10 mM phosphate buffer (pH 6.0). The enzyme solution was dialysed overnight against the same phosphate buffer. The resultant insoluble materials were removed by centrifugation. The dialysed enzyme solution was applied to a CM-cellulose column (3.5×30 cm) which had been previously equilibrated with the same buffer at pH 6.0. After the column was washed with the equilibrating buffer, 0 M to 0.5 M NaCl linear gradient elution was performed in the same buffer at a flow rate of 12 ml/hr. A total of active fractions were collected, and the A_{280} was measured. The fractions containing the enzyme activity were collected. The concentrated fractions by ultrafiltration with a PM 10 membrane (Amicon Corp. Danvers, MA. USA) were put on a Sephadex G-100 column (1×100 cm) equilibrated with 20 mM phosphate buffer (pH 8.0) containing 0.2 M sodium chloride and eluted with the same buffer at a flow rate of 7 ml/hr. The active fractions were collected and concentrated. The concentrated fractions were put on a Sephadex G-100 column (1×100 cm) equilibrated with 20 mM phosphate buffer (pH 8.0) and eluted with the same buffer at a flow rate of 5 ml/hr. The purified enzyme was concentrated by ultrafiltration and stored −20℃.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out by the methods described Laemmli (10) using 10% acrylamide gel containing 0.1% (w/v) SDS under constant power supply of 3 mA per column for 3 hours at room temperature, respectively and the gel were stained with 0.25% Coomasie brilliant blue R-250. The molecular weight of the protease was estimated from the mobility of the protein band by the method of Andrew with marker proteins (1). The molecular weight marker proteins obtained from Bio-rad were myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200) and ovalbumin (45,000).

Effects of pH and temperature on protease activity

Protease activity was measured at different pH values under standard assay conditions with azocasein as a substrate. The activities of enzymes at pH 5.0~13.0 were assayed with citrate buffer (pH 5.0), phosphate buffer (pH 6.0), Tris-HCl buffer (pH 7.0~9.0), sodium borate buffer (pH 10.0~11.0), and NaOH-KCl buffer (pH 12.0~13.0). At various temperatures (10~80℃), at pH 11.0, the protease activity was also assayed to examine the optimum temperature for activity.

Effects of pH and temperature on protease stability

The initial activities of the enzyme were assayed under the standard assay conditions. The enzyme solution was preincubated for 30 min under various conditions without the substrate and the remaining activities were then assayed.

Results and Discussion

Purification of protease from *Xanthomonas* sp. YL-37

The overall purification procedures are summarized in Table 1. The results of the last step in the procedure are shown in Fig. 1. The enzyme was succesively purified enzyme was demonstrated to be homogeneous by

Table 1. Purification procedures of the alkaline protease from *Xanthomonas* sp. YL-37

Procedures	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture broth	10,279,000	1,700	6,064	1	100
Ammonium sulfate (30~80%)	3,211,704	396	8,110	1.34	31.2
CM-cellulose	1,102,572	108	10,209	1.68	10.7
Sephadex G-100 (1st)	377,839	20	18,798	3.10	3.5
Sephadex G-100 (2nd)	235,952	9	25,647	4.23	2.3

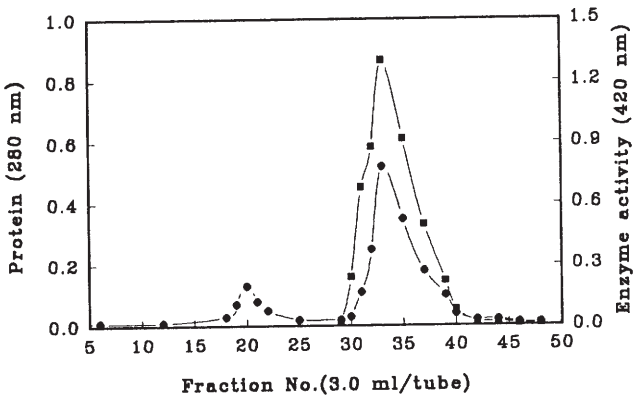

Fig. 1. Gel filtration chromatography of alkaline protease using a column of Sephadex G-100. Sephadex G-100 was equilibrated with 0.02 M phosphate buffer (pH 8.0) The column was eluted with 0.02 M phosphate buffer at a flow rate of 5 ml/hr. ●; Protein, ■; Enzyme activity.

SDS-PAGE as shown in Fig. 2. The overall process achieved about 4.23 fold purification with a yield of about 2.3% as shown in Table 1. The molecular weight of the enzyme was estimated to be about 62 KDa by SDS-PAGE as shown in Fig. 2. Approximately the same value was also obtained by gel filtration with Sephadex G-100 (data not shown). The purified enzyme had a specific activity of 25,647 units/mg protein when assayed at 50℃ with azocasein as a substrate.

Effects of pH and temperature on protease activity

Protease activity was measured at various pH values and temperature under standard assay conditions as described in materials and methods. The effects of pH on the enzyme activity were exhibited in Fig. 3A. The optimum pH of protease was pH 11.0. In particular, protease showed the 90% of the remaining activity even at pH 13.0. The effects of temperature on the enzyme activity were exhibited in Fig. 3B. The optimum temperature for the enzyme activity was 50℃.

Effects of pH and temperature on protease stability

Protease was incubated various pH values for 30 min

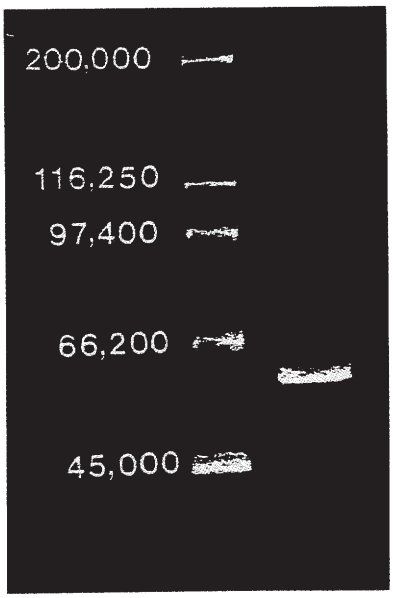

Fig. 2. SDS-PAGE of purified protease from *Xanthomonas* sp. YL-37. Left lane, marker proteins: Myosin (200,000), β-Galactosidase (116,250), Phosphorylase B (97,400), Bovine serum albumin (66,200) and Ovalbumin (45,000). Right lane, purified enzyme after Sephadex G-100 gel filtration.

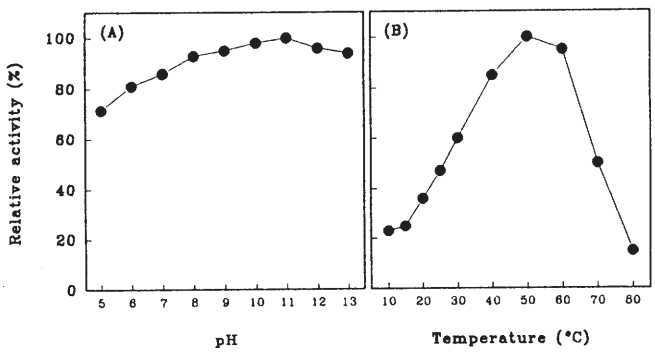

Fig. 3. Effects of pH and temperature on protease activity. (A) The following buffer systems were used: Citrate buffer (pH 5.0) Phosphate buffer (pH 6.0), Tris-HCl buffer (pH 7.0~9.0), Sodium borate buffer (pH 10.0~11.0) and NaOH-KCl buffer (pH 12.0~13.0). (B) The reaction was carried out at the temperature indicated for 30 min; pH, 11.0 (10 mM/l sodium borate buffer).

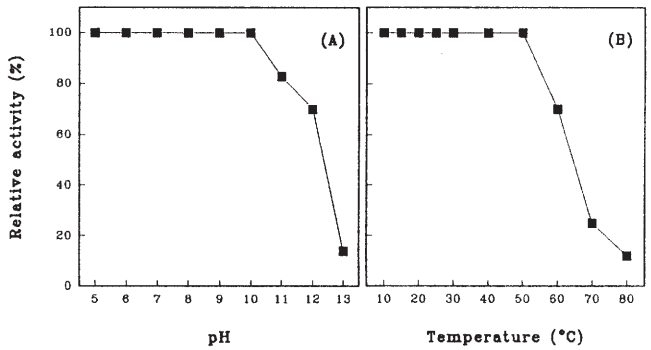

Fig. 4. Effects of pH and temperature on protease stability. (A) The reaction mixtures were incubated at 50℃ for 30 min and the remaining activities were measured under the standard assay condition. (B) Enzyme solution in 10 mM sodium borate buffer (pH 11.0) was preincubated at various temperature for 30 min. After preincubating, the remaining activities were measured under the standard assay condition.

at 50℃ and then the remaining activities were measured. The results are shown in Fig. 4A. As the results of this determination, the enzyme seemed to be relatively stable in the range of pH 5.0~10.0. but the stability was progressively decreased from above 11.0 and completely inactivated at pH 13.0. These results agreed with those of other report from *Bacillus* sp. No. 8-16 (2). The effects of temperature on the stability of the enzyme were provided in Fig. 4B. As the results, the enzyme seemed to be relatively stable below 50℃, but the stability was progressively decreased from above 60℃ and completely vanished around 80℃. These results agreed with those of the other report from *Bacillus* No. 221 (5), but didn't with the other report from *Streptomyces rimosus* (8) and *Bacillus* sp. No. 8-16 (2) Therefore, this enzyme seemed to be generally stable to about 50℃.

Effects of metal ions and inhibitors on protease activity

The results of metal ions on protease activities are shown in Table 2. The reaction mixtures contained various metal ions as indicated in the table. As shown in the table, the presence of 5 mM of K^+, Ca^{2+}, Mn^{2+} and Mg^{2+} in the reaction mixtures increased more or less the enzyme activity. And the presence of Hg^{2+}, Cu^{2+} and Zn^{2+} in the mixtures showed inactivation of the enzyme activity. This result corresponded generally report from *Bacillus* sp. No. 8-16 (2) but didn't with the other report from *Streptomyces rimosus* (8) and from actinomycete (18). It was reported that the enzyme activity from *Beauveria bassiana* (15) was inhibited by the presence of 10 mM of Fe^{2+}, Ca^{2+}, Mg^{2+} and K^+ and also suggested that the enzyme from *Streptomyces rimosus* (8) was strongly inactivated by the presence of 10 mM of Fe^{2+} and Hg^{2+}.

Table 2. Effect of metal ions on protease activity

Metal ions	Relative activity (%)	
	1 mM	5 mM
None	100	100
Ca^{2+}	102	111
Mg^{2+}	96	113
Fe^{2+}	98	103
Ba^{2+}	108	103
K^+	95	112
Na^+	100	99
Hg^{2+}	90	58
Cu^{2+}	102	78
Mn^{2+}	110	124
Zn^{2+}	83	77
Ag^{2+}	104	108
Al	99	101

Metal ions were preincubated with the enzyme solution at 50℃ for 30 min.
Buffer system: 10 mM sodium borate buffer (pH 11.0).

Table 3. Effect of inhibitors on protease activity

Inhibitors	Concentration	Relative activity (%)
Control		100
Serine inhibitor		
PMSF[1]	1 mM	10
	5 mM	1
Cystein inhibitor		
SDS[2]	0.5%	43
Metal chelator		
EDTA[3]	1 mM	89
	5 mM	78
Calcium specific chelator		
EGTA[4]	1 mM	76
	5 mM	63
Zinc specific chelator		
o-Phenanthroline	1 mM	88
	5 mM	85
Reducing agent		
Potassium-cyanide	1 mM	98
	5 mM	96
L-cystein	1 mM	87
	5 mM	66

[1] PMSF: Phenylmethanesulfonyl fluoride
[2] SDS: Sodiumdodecyl sulfate
[3] EDTA: Ethylenediaminetetraacetic acid
[4] EGTA: Ethylene glycol-bis(*B*-aminoethyl ether) N,N,N',N'- tetraacetic acid. All reagents were preincubated with the enzyme solution at 50℃ for 30 min. Buffer system: 10 mM sodium borate buffer (pH 11.0).

The purified enzyme was preincubated with various reagents of 1 mM and 5 mM concentrations for 30 min at 50℃ and the enzyme activities were assayed. As shown in Table 3, the PMSF was completely inactivated which suggested that it is a serine protease. The proteases could be classified by their sensitivity to various inhibitors (17). These properties of their enzyme to inhibitors tested were generally similar with the other reports from *Bacillus* sp. No. 8-16 (2), *Beauveria bassiana* (15) and actinomycete (18).

Kinetics of the purified protease

The Michaelis constant (K_m) was determined graphically by Lineweaver-Burk plot (13). The K_m and V_{max} of the enzyme for casein were estimated to be 4.0 mg/ml and 5,500 units/ml, respectively. This value was somewhat lower than that (10 mg/ml) of *Bacillus* sp. (9).

References

1. **Andrews, P.,** 1964. Estimation of molecular weight of proteins by Sephadex gel filtration. *J. Biochem.* **91**, 222-233.
2. **Bae, M. and P.-Y. Park,** 1989. Purification and characterization of thermotolerable alkaline protease by alkalophilic *Bacillus* sp. No. 8-16. *Kor. J. Appl. Microbiol. Bioeng.* **17**, 545-551.
3. **Bradford, M.M.,** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
4. **Hatley, B.S.,** 1960. Proteolytic enzymes. *Annu. Rev. Biochem.* **29**, 459-473.
5. **Horikoshi, K.,** 1971. Production of alkaline enzymes by alkalophilic microorganisms. Part I. Akaline protease produced by *Bacillus* No. 221. *Agric. Biol. Chem.* **35**, 1407-1414.
6. **Horikoshi, K. and T. Akiba,** 1982. Alkalophilic microorganisms: A new microbial world. Springer-Verlag, New York.
7. **Ito, S., S. Shikata, K. Ozaki, S. Kawai, K. Okamoto, S. Inoue, and T. Satoh,** 1989. Alkaline cellulase for laundry detergents: Production by *Bacillus* sp. KSM-615 and enzymatic properties. *Agric. Biol. Chem.* **53**, 1275-1281.
8. **Kim, K.-M., T.-K. Lee, and H.-C. Yang,** 1989. Purification and properties of extracellular protease from *Streptomyces rimosus. Kor. J. Appl. Microbiol. Biotech.* **17**, 407-411.
9. **Kim, T.-H., S.-H. Park, D.-S. Lee, T.-K. Kwon, J.-K. Kim, and S.-D. Hong,** 1990. Properties of alkaline protease produced by an alkalophilic *Bacillus* sp. *Kor. J. Appl. Microbiol. Biotech.* **18**, 159-164.
10. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685.
11. **Lee, C.-H., T.-J. Kwon, S.-M. Kang, H.-H. Suh, G.-S. Kwon, H.-M. Oh, and B.-D. Yoon,** 1994. Production and characterization of an alkaline protease from an isolate, *Xanthomonas* sp. YL-37. *Kor. J. Appl. Microbiol. Biotech.* **22**, 515-521.
12. **Leighton, T.J., R.H. Doi, R.A.J. Warren, and R.A. Kelln,** 1973. The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis. J. Mol. Biol.* **76**, 103-122.
13. **Lineweaver, J. and D. Burk,** 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-666.
14. **Margesin, R., N. Palma, F. Knauseder, and F. Schinner,** 1992. Purification of an alkaline serine protease produced by a psychrotrophic *Bacillus* sp. *J. Biotech.* **24**, 203-206.
15. **Michael, J.B. and G.G. Khachatourians,** 1987. Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana. Appl. Environ. Microbiol.* **53**, 1679-1684.
16. **Shimogaki, H., K. Takeuchi, T. Nishimo, M. Ohdera, T. Kudo,K. Ohba, M. Iwana and M. Irie,** 1991. Purification and properties of a novel surface-active agent and alkaline-resistant protease from *Bacillus* sp. Y. *Agric. Biol. Chem.* **55**, 2251-2258.
17. **Sigman, D.S. and G. Mooser,** 1975. Chemical studies of enzyme activities. *Annu. Rev. Biochem.* **44**, 889-931.
18. **Tsujibo, H., K. Miyamoto, T. Hesegawa, and Y. Inamori,** 1990. Purification and characterization of two types of alkaline serine proteases produced by an alkalophilic actinomycete. *J. Appl. Bact.* **69**, 520-529.