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Purification and Characterization of Two Alkaline Proteases Produced by *Pseudomonas* sp. BK7

LEE, EUN GOO, EUN-HEE PARK, AND HYUNG-HWAN HYUN*

Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yongin, Kyonggi-Do 449-791, Korea

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Abstract *Pseudomonas* sp. BK7, an alkalophile, displayed the highest growth and protease activity when grown in a fermenter which was controlled at a pH level of 9.0, and the enzyme production was significantly enhanced by the increase of agitation speed. Two forms of alkaline proteases (BK7-1 and BK7-2) were fractionated and purified to near homogeneity. Protease BK7-1 was purified through CM-Sepharose CL-6B and Sephadex G-75 column chromatographies, and Protease BK7-2 was purified through CM-Sepharose CL-6B, DEAE-Sepharose, and Sephadex G-75 column chromatographies. The molecular weights of proteases BK7-1 and BK7-2 determined by gel filtration chromatography were 20,700 and 40,800, respectively. The K_m value, isoelectric point, and optimum pH of protease BK7-1 were 2.55 mg/ml, 11.0, and 11.0, respectively, whereas those of protease BK7-2 were 1.57 mg/ml, 7.2, and 10.0, respectively. Both proteases were practically stable in the pH range of 5–11. The optimum temperatures for the activities of both protease BK7-1 and BK7-2 were 50°C and 45°C, respectively. About 56% of the original protease BK7-2 activity remained after being treated at 50°C for 30 min but protease BK7-1 was rapidly inactivated at above 25°C. Both proteases were completely inhibited by phenylmethane sulfonyl fluoride, a serine protease inhibitor. Protease BK7-2 was stable against EDTA, EGTA, STP, and detergents such as SDS and LAS, whereas protease BK7-1 was found to be unstable.

Key words: Alkaline protease, *Pseudomonas* sp. BK7, purification, characterization

Alkaline proteases of microbial origin are an important group of industrial enzymes which have been extensively used as the major enzyme constituent of laundry detergents for the hydrolysis of protein stains such as blood, milk, and grime. In order to fulfill their requirements for the use as laundering additives, enzymes such as protease, amylase, and lipase should have a high level of activity in the broad ranges of pH and temperature, along with the fact that they must be active and stable in the presence of oxidizing agents such as surfactant, bleach, or other additives which might be present in the formulation [5, 7, 8, 21].

Among alkaline proteases, subtilisin Carlsberg from *Bacillus licheniformis* and subtilisin BPN from *B. amyloliquefaciens* [20] have been widely used in the laundry industry and have been the most extensively characterized. Recently, however, other alkaline proteases from *Streptomyces, Vibrio, Serratia,* and *Pseudomonas* have also been isolated and characterized [4, 6, 9]. In our previous report, we described that *Pseudomonas* sp. BK7 isolated from soils produced an extracelluar protease which was highly active in the alkaline pH range and it was completely stable in a broad range of pHs, from acidic to alkaline pH [10]. In this report, we describe the purification and characterization of two alkaline proteases from *Pseudomonas sp.* BK7 which might have a potential for industrial application.

Materials and Methods

Microbial Strain

Pseudomonas sp. BK7, isolated from the soil sample as described previously [10], was used.

Medium and Cultivation

Pseudomonas sp. BK7 was cultured aerobically at 30°C in MB medium (Maltose 20 g/l, Beef extract 10 g/l, NaCl 0.24 g/l, Yeast extract 0.75 g/l, K_2HPO_4 1.24 g/l, $MgSO_4$ 80 mg/l, $FeSO_4$ 0.3 mg/l, $ZnSO_4$ 0.7 mg/l, $NaHCO_3$ 10 g/l, pH 10.0). To prepare culture broth for purification of proteases, cells were cultivated in a 5-l jar fermenter (Korea Fermenter Company, Incheon, Korea) containing 2.5 l of MB medium,

*Corresponding author
Phone: 82-31-330-4279; Fax: 82-31-331-1096;
E-mail: khhyun@san.huss.ac.kr

which was agitated at 700 rpm, aerated at 1.0 vvm, and controlled at pH 9.0 with 3 N NaOH. The seed culture to be inoculated into the fermenter was prepared by cultivating cells in a 500-ml Erlenmeyer flask containing 50 ml of MB medium at 30°C for 18 h on a rotary shaker (200 rpm).

Assay of Protease Activity

Protease activity was measured using casein as a substrate according to the method described by Fujiwara *et al.* [5]. The reaction was initiated by adding 0.3 ml of enzyme solution into a reaction tube containing 1.2 ml of 0.5% (w/v) casein solution in a 100 mM sodium bicarbonate buffer (pH 11.0). After incubation for 15 min at 35°C, the reaction was stopped by adding 0.6 ml of 10% (w/v) trichloroacetic acid. The reaction mixture was then kept at 4°C for 10 min and centrifuged at 10,000 ×g for 5 min. One-half milliliter of the supernatant was mixed with 1 ml of three-fold diluted Folin-Ciocalteu phenol reagent (Sigma) and 1.5 ml of 7.5% (w/v) Na_2CO_3 solution, and incubated at 30°C for 30 min. Absorbance of the mixture was measured at 660 nm. One unit of alkaline protease activity was defined as the amount of enzyme liberating one mmole of tyrosine per min. under the conditions described above.

Protein Concentration

The protein concentration was determined by Bradford's method [3]. A dye reagent was purchased from Bio-Rad and bovine serum albumin (Sigma) was used as a protein standard.

Purification Procedure

All the purification procedures were conducted at 4°C unless otherwise indicated. Cells of *Pseudomonas* sp. BK7 were grown in a 5-l-jar fermenter containing 2.5 l of MB medium which was agitated at 700 rpm, controlled at 30°C, aerated at 1.0 vvm, and controlled at pH 9.0 with 3 N NaOH solution. Cell-free culture supernatant was obtained by centrifugation of the culture broth at 4,500 ×g for 30 min and used as a crude enzyme solution. Solid ammonium sulfate was slowly added to the supernatant while stirring to achieve 80% saturation. The precipitate was collected by filtration using diatomaceous earth, dissolved in a minimal volume of 10 mM phosphate buffer (pH 8.0), and then dialyzed by ultrafiltration with a Diaflo membrane PM3 (Amicon, M.W. exclusion limit 3,000 Da). The dialyzed enzyme solution was applied to a column of CM-Sepharose CL-6B (2.5 × 22 cm, Pharmacia) which was equilibrated with 10 mM phosphate buffer (pH 8.0). The column was washed with the above equilibrating buffer and then eluted at a flow rate of 15 ml/min with a linear concentration gradient of 0 to 1.0 M NaCl in the same buffer.

Fractions of each 4.5 ml were collected and the proteolytic activity and absorbance (A_{280}) in each fraction were determined. Protease activities were detected in two distinct peaks (nonabsorbed and absorbed fractions), in which one (BK7-2) was detected in the washed buffer and the other (BK7-1) was detected in the gradient eluent. The active fractions in each peak were combined and concentrated by ultrafiltration using a stirred cell with a Diaflo PM3 membrane (Amicon). The concentrated protease BK7-1 sample was applied to a column of Sephadex G-75 (2.2× 60 cm, Pharmacia), equilibrated with 100 mM sodium bicarbonate buffer (pH 9.0), and elution was carried out at a flow rate of 12 ml/h with the same buffer.

The protease BK7-2 sample was applied to a column of DEAE-Sepharose CL-6B (2.5×22 cm, Pharmacia) equilibrated with 10 mM phosphate buffer (pH 8.0) and the proteins were eluted with a linear concentration gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 16 ml/h. Fractions of 4.0 ml each were collected from the beginning, and the fractions with proteolytic activity were pooled and concentrated using a stirred cell with a Diaflo PM3 membrane (Amicon). The concentrated protease BK7-2 was further purified using a Sephadex G-75 column as described above.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of proteins was performed using a 12% (w/v) acrylamide slab gel with slight modification of the method described by Taber and Sherman [18]. Twenty-five mM of Tris/192 mM glycine buffer (pH 8.3) was used as a running buffer and gels were stained with Coomassie Brilliant Blue R-250 (Sigma). A current of 30 mA was applied across the gel for 4 h at room temperature. To determine the basic isoelectric point of the protein, reverse-polyacrylamide gel electrophoresis was performed using a 10% (w/v) acrylamide slab gel. The reverse gel electrophoresis was carried out at a reverse current of 10 mA across the gel for 12 h at 4°C. For the protease activity staining, the electrophoresed gel was placed on the top of 10% (w/v) polyacrylamide gel containing 0.5% (w/v) gelatin and pressed with a glass plate. After incubating at 37°C for 1 h, the gelatin gel was stained with 0.1% amidoblack in 7.5% acetic acid solution and subsequently rinsed with 7.5% acetic acid solution. The protease band was shown as a clear zone resulting from the hydrolysis of the gelatin.

Determination of Molecular Weight

The molecular weights of the two purified proteases were determined by the method described by Andrews (1964). Gel permeation chromatography was performed on a Sephadex G-75 column (2.2 × 72 cm) at a flow rate of 9 ml/h using 10 mM phosphate buffer (pH 8.0). Bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen (25.0 kDa), and ribonuclease (13.7 kDa) were used as standard protein molecular weight markers (Pharmacia).

Determination of Isoelectric Point

The isoelectric point was determined by measuring the pH of samples recovered from a preparative isoelectric focusing. The purified protease sample was appropriately diluted with distilled water and mixed with 1 ml of pH 3-10 ampholyte or pH 8-10 pharmalyte (Pharmacia). The mixture was then fractionated using a Rotofor preparative isoelectric focusing unit (Bio-Rad) for 5 h at 12 watts. After electrophoresis, fractions were recovered and the pHs of the fractions were measured.

pH Effect on Protease Activity

To determine the optimum pH of the enzyme, protease activities at pHs between 6.0 and 13.0 were measured at 50°C for protease BK7-1 and at 45°C for protease BK7-2. To measure pH stability of the enzyme, the residual protease activities were assayed after incubation for 1 h at various pHs between 4.0 and 13.0. The buffers used were 100 mM acetate in the pH range of 4.0-6.0, 100 mM phosphate in the pH range of 6.0-8.0, 100 mM Tris-HCl in the pH range of 8.0-9.0, 100 mM sodium bicarbonate in the pH range of 9.0-11.0, and 100 mM K_2HPO_4-NaOH in the pH range of 11.0-13.0.

Temperature Effect on Protease Activity

The dependence of protease activity on temperature was studied under the standard assay conditions of 25-75°C. To examine the thermal stability of the enzyme, the residual protease activities were assayed after incubating the enzyme solutions in 10 mM sodium bicarbonate buffer (pH 10.0) at various temperatures for 30 min. To investigate the effect of calcium ion on the thermal stability of the protease, the enzyme in 10 mM sodium bicarbonate buffer (pH 10.0) was incubated in the presence or absence of 10 mM calcium at 40°C for protease BK7-1 and at 50°C for protease BK7-2, respectively, and the residual activities in the mixture were assayed at every 10 min.

Effects of Metal Ions and Inhibitors

Effects of metal ions were determined by adding each metal ion to a 0.5% (w/v) casein solution in 100 mM sodium bicarbonate buffer (pH 11.0 for protease BK7-1 and pH 10.0 for protease BK7-2) to achieve 1 mM or 10 mM, and by measuring the relative proteolytic activity under the standard assay conditions. Effects of enzyme inhibitors or components of laundry detergent on the protease activity were determined by measuring the

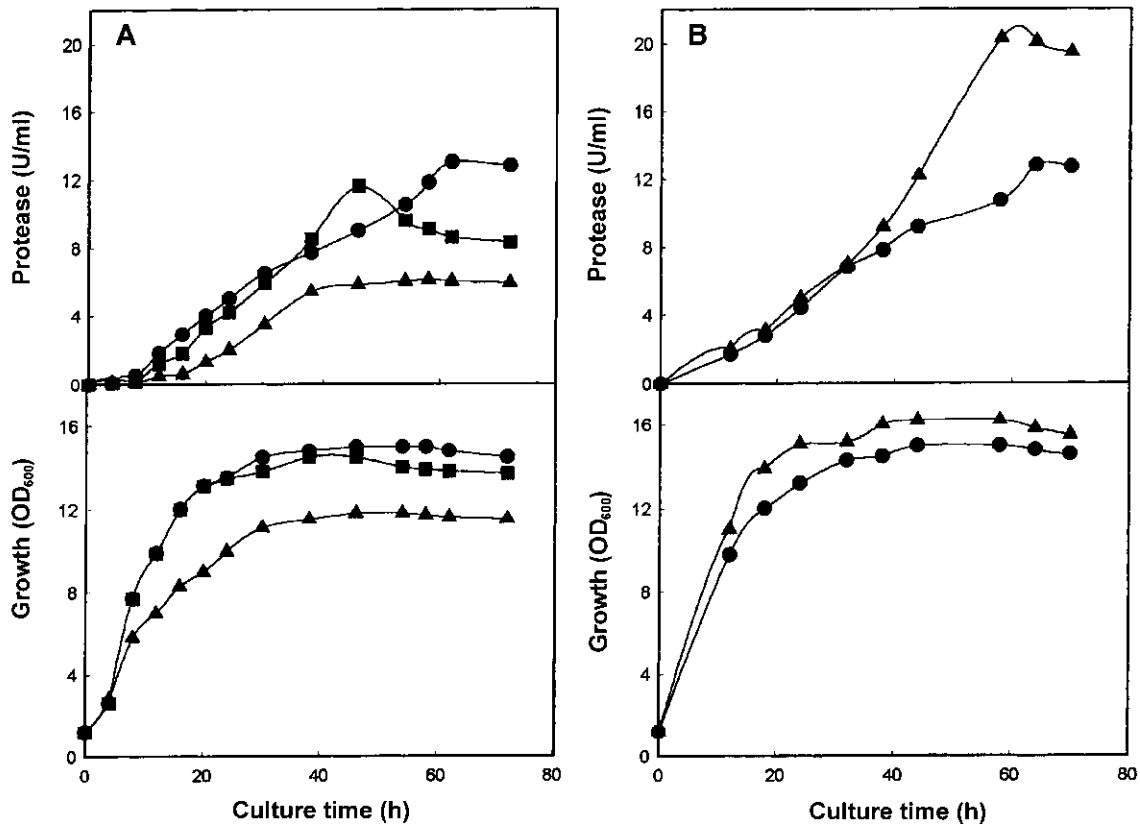

Fig. 1. Effects of pH (A) and agitation speed (B) on the growth and protease production of *Pseudomonas* sp. BK7.
A: Cells were grown in a 5-l-jar fermenter containing 2.5 l of MB medium, agitated at 600 rpm, aerated at 1 vvm, controlled at 30°C, and pH was controlled at 8 (▲), 9 (●), and 10 (■), respectively, with a 3 N NaOH solution. B: The fermenters were controlled at pH 9.0 and agitated at 600 rpm (●) or 700 rpm (▲).

residual activity under the standard assay conditions after incubating the enzyme and inhibitor at room temperature for 1 h. Phenylmethane sulfonyl fluoride (PMSF) solution was prepared by dissolving it in a minimal volume of ethanol and diluting it with distilled water.

RESULTS

Protease Production

The time courses for fermentation of *Pseudomonas* sp. BK7, when grown in the fermenters controlled at different pHs, is shown in Fig. 1A. The strain displayed the highest protease activity and growth when cultured at pH 9.0. As shown in Fig. 1B, the protease production was significantly enhanced by the increase of agitation speed, and the maximum enzyme activity (20.3 U/ml) was obtained when grown in a 5-l jar fermenter agitated at 700 rpm and controlled at pH 9.0.

Purification of Two Alkaline Proteases

The overall purification procedures of two alkaline proteases produced by *Pseudomonas* sp. BK7 are summarized in Table 1. To obtain the culture supernatant, *Pseudomonas* sp. BK7 was cultured in a 5-l jar fermenter containing 2.5 l of MB medium as described in Materials and Methods, and cells were removed by centrifugation at 4,500 ×g for 30 min. The culture supernatant was concentrated by ammonium sulfate precipitation, followed by dialysis using the ultrafiltration process. The concentrated enzyme solution was passed through a CM-Sepharose CL-6B column. The column was washed with 10 mM phosphate buffer (pH 8.0) and then it was eluted with a NaCl gradient (0- 1.0 M) in the same buffer. Two protease activities were detected in this procedure. The protease in the nonabsorbed fraction was labeled as protease BK7-2 and the other found in the eluent was named protease BK7-1. Protease BK7-1 was further purified through Sephadex G-75, while protease BK7-2 was further purified using DEAE Sepharose CL-6B and Sephadex G-75 column chromatographies. The protease BK7-1 and protease BK7-2 were purified 22.1-fold and 9.5-fold over the culture supernatant, respectively. The overall purification yield of protease BK7-1 was 26.8% and it was

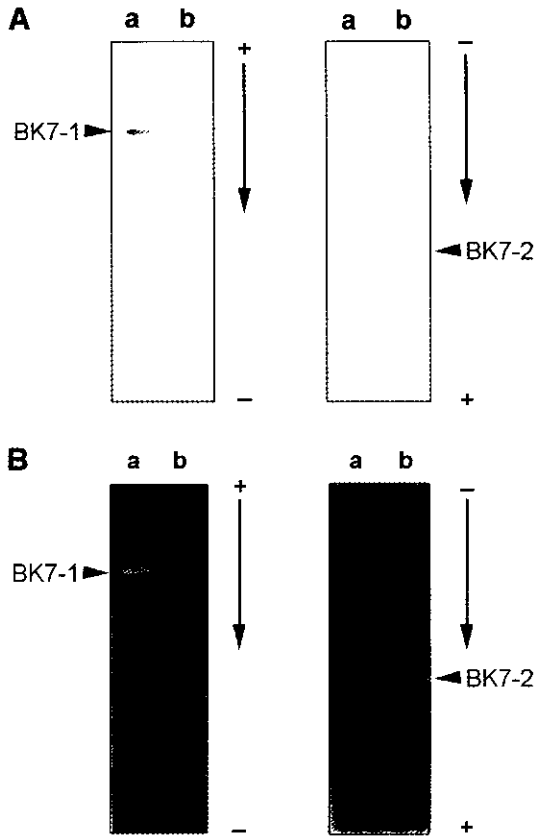

Fig. 2. A: Nondenaturing PAGE (A) and activity staining on gelatin gel (B) of two alkaline proteases purified from *Pseudomonas* sp. BK7.
The polarity of the gel is indicated by long arrows. Lanes a and b represent protease BK7-1 and protease BK7-2, respectively.

Table 1. Summary of the purification of two alkaline proteases produced by *Pseudomonas* sp. BK7.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture fluid	2,567.2	140.3	18.3	1.0	100.0
Ammonium sulfate Precipitation	1,720.8	63.1	27.3	1.5	67.0
Ultrafiltration	1,240.7	38.8	32.0	1.7	48.0
CM-Sepharose CL-6B					
Washing (protease BK7-2)	203.1	2.0	101.6	5.6	7.9
Elution (protease BK7-1)	724.0	2.7	266.2	14.5	28.2
Protease BK7-1					
Sephadex G-75	687.5	1.7	404.4	22.1	26.8
Protease BK7-2					
DEAE-Sepharose CL-6B	164.3	1.4	117.4	6.4	6.4
Sephadex G-75	121.2	0.7	173.1	9.5	4.7

Table 2. Comparison of properties of protease BK7-1 and protease BK7-2.

Properties	Protease BK7-1	Protease BK7-2
Molecular weight (kDa)	40.80	20.70
pI	11.00	7.20
K_m (casein, mg/ml)	2.55	1.57
V_{max} (casein, mg/min)	0.21	0.09
pH optimum	11.00	10.00
Temperature optimum (°C)	50.00	45.00
Specific activity (U/mg)	117.40	173.10

found to be 6-fold higher than that of protease BK7-2. The homogeneity of the purified protease BK7-1 and protease BK7-2 was also examined by reverse-PAGE and PAGE, respectively (Fig. 2). Protease BK7-1 did not move to the cathode during electrophoresis, suggesting that it might have a strong positive charge on its surface. The purified proteases gave a single band on the electrophoretic gels, and each band precisely coincided with the activity staining band on the gelatin gel.

Isoelectric Point, Molecular Weight, and K_m

The properties of the two alkaline proteases purified from *Pseudomonas* sp. BK7 are summarized in Table 2. The isoelectric points of protease BK7-1 and protease BK7-2 were 11.0 and 7.2, respectively. The molecular weights of protease BK7-1 and protease BK7-2, determined by gel filtration chromatography using a Sephadex G-75 column, were estimated to be 40.8 kDa and 20.7 kDa, respectively. The Michaelis-Menten constant (K_m) of protease BK7-1 and protease BK7-2 for casein as a substrate were 2.55 and 1.57 mg/ml, respectively, and V_{max} values of protease BK7-1 and BK7-2 were 0.21 mg/min and 0.09 mg/min, respectively.

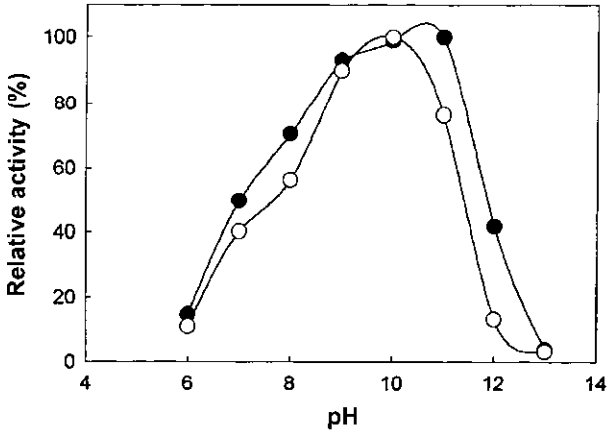

Fig. 3. Dependence of activity of protease BK7-1 (●) and protease BK7-2 (○) from *Pseudomonas* sp. BK7 on pH.
Protease activities were assayed at 50°C for protease BK7-1 and at 45°C for protease BK7-2 in 100 mM of phosphate buffer (pH 6.0–8.0), 100 mM of Tris-HCl buffer (pH 8.0–9.0), 100 mM of sodium bicarbonate buffer (pH 9.0–11.0), and 100 mM K_2HPO_4-NaOH buffer (pH 11.0–13.0)

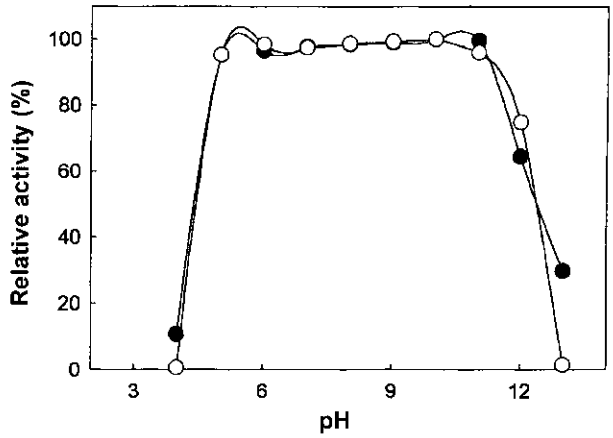

Fig. 4. Effects of pH on the stability of protease BK7-1 (●) and protease BK7-2 (○) from *Pseudomonas* sp. BK7.
After the enzyme solutions were preincubated in 100 mM of acetate buffer in the pH range of 4.0–6.0 and the same buffers as described in Fig. 3 in the pH range of 6.0–13.0, the residual activities of protease BK7-1 and protease BK7-2 were measured at 50°C and at 45°C, respectively.

Effect of pH on the Activity and Stability

The effect of pH on the two proteases was examined in the range of pH 6.0–13.0 at 50°C for protease BK7-1 and at 45°C for protease BK7-2. Both BK7-1 and BK7-2 displayed activity in the broad pH range of 6.0–12.0 (Fig. 3), and the optimum pHs for protease BK7-1 and BK7-2 were 11.0 and 10.0, respectively.

The pH stabilities of the two proteases were examined by incubating the proteases in the pH range of 4.0–13.0 for 1 h at 25°C. Both proteases were completely stable in the pH range of 5.0–11.0 (Fig. 4).

Effects of Temperature on the Activity and Stability

The effect of temperature on the activity of the two purified alkaline proteases was investigated at temperatures from 25°C to 75°C. The optimum temperature of protease BK7-1 and protease BK7-2 was 50°C and 45°C, respectively (Fig. 5). In general, the temperature optima of both proteases were lower than alkaline proteases produced by *Bacillus* sp. and other bacterial strains [5, 13, 19]. Protease BK7-2 was more active than protease BK7-1 at temperatures lower than the optimum and displayed 55% of the maximum activity at 25°C. The thermal stability of the purified proteases was measured by determining the residual activities after incubating the enzyme solutions at various temperatures for 30 min. Protease BK7-2 was almost stable up to 40°C, and retained 56% of the initial activity after being treated at 50°C for 30 min. Protease BK7-1, however, was unstable and rapidly inactivated at above 25°C (Fig. 6). Figure 7 shows the effect of $CaCl_2$ on the thermal stability of the two proteases. The thermal stability of protease BK7-2 was not affected by $CaCl_2$, whereas protease BK7-1 was completely stable for 60 min in the presence of

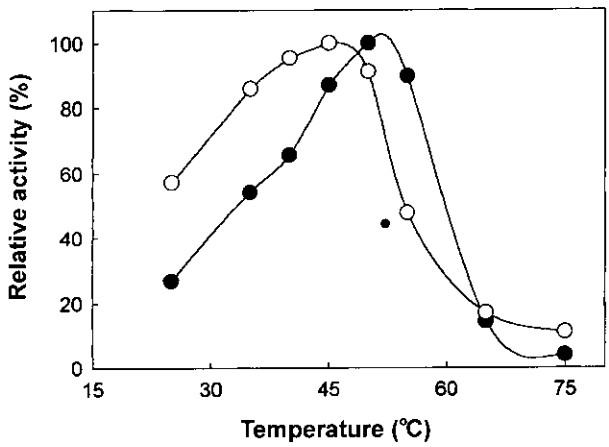

Fig. 5. Dependence of the activity of protease BK7-1 (●) and protease BK7-2 (○) from *Pseudomonas* sp. BK7 on temperature.
Protease BK7-1 and protease BK7-2 were assayed at pH 11.0 and pH 10.0, respectively.

10 mM $CaCl_2$ however, it lost as much as 82% of the original activity after 60 min in the absence of $CaCl_2$.

Effects of Metal Ions and Inhibitors on the Protease Activity

The sensitivity of the two proteases towards metal ions is shown in Table 3. Protease BK7-2 activity was slightly stimulated by Ca^{2+} or Cu^{2+}, but protease BK7-1 was stimulated only by Ca^{2+}. Other metal ions did not significantly affect either protease activities.

Effects of various denaturants and chemicals, used in formulating detergents, on the activity of protease BK7 and commercial alkaline proteases were examined by measuring residual activities after incubating the enzyme with such proteases. As shown in Table 4, the two BK7 proteases as well as subtilisin Carlsberg and BPN were completely inhibited by phenylmethane sulfonyl fluoride (PMSF), a serine protease inhibitor. Protease BK7-2, subtilisin BPN, and subtilisin Carlsberg were slightly inhibited by inhibitors of metallo-protease, such as ethylenediamine-tetraacetic acid (EDTA) and ethyleneglycol-bis-tetraacetic acid (EGTA),

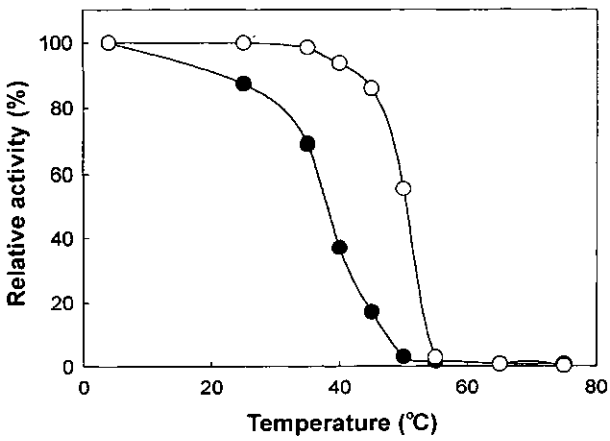

Fig. 6. Thermal stability of protease BK7-1 (●) and protease BK7-2 (○) from *Pseudomonas* sp. BK7.
After the enzyme solutions at various temperatures were incubated for 30 min, the residual activity was measured as described in Materials and Methods.

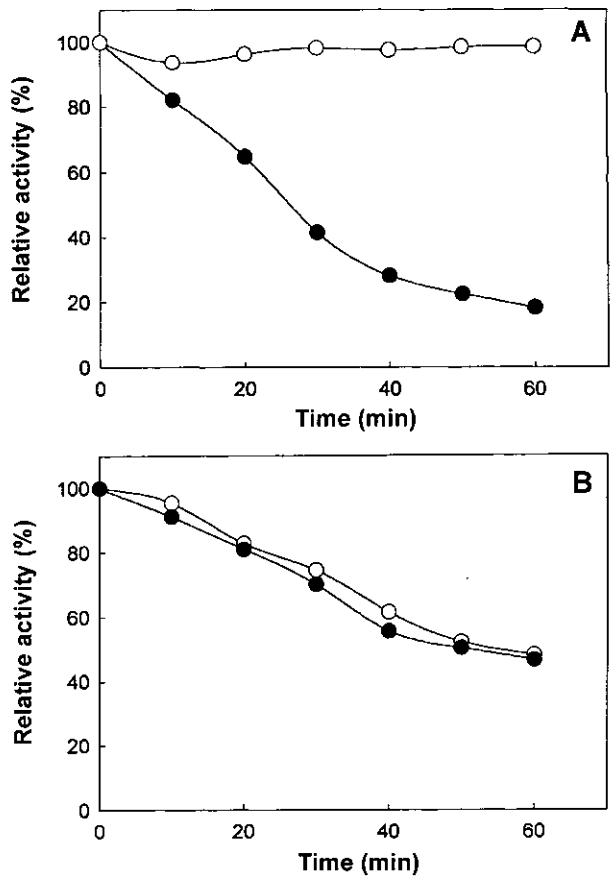

Fig. 7. Effect of Ca^{2+} on the thermal stability of the protease BK7-1 (A) and protease BK7-2 (B).
Protease BK7-1 and protease BK7-2 were incubated at 40°C and 50°C, respectively, in the presence (○) or absence (●) of 10 mM $CaCl_2$. One-hundred μl aliquots were withdrawn at intervals of 10 min and the residual activity was assayed as described in Materials and Methods.

Table 3. Sensitivity of protease BK7-1 and protease BK7-2 toward metal ions.

Metal ion	Concentration (mM)	Relative activity (%)	
		Protease BK7-1	Protease BK7-2
Not added	-	100.0	100.0
$CaCl_2$	10	96.4	123.4
$CoCl_2$	1	90.0	90.8
$CuSO_4$	1	117.5	114.4
$FeSO_4$	1	103.6	104.2
$MgSO_4$	10	99.8	107.1
$MnCl_2$	1	99.4	100.7
KCl	10	99.5	100.9
$ZnSO_4$	1	99.2	90.3

Table 4. Effects of several inhibitors and chemicals used in detergents on the activity of protease BK7-1, protease BK7-2, and commercial proteases.

Inhibitor	*Concentration*	Residual activity (%)			
		Protease BK7-1	Protease BK7-2	Subtilisin BPN	Subtilisin Carlsberg
Not added	-	100.0	100.0	100.0	100.0
EDTA	5 mM	38.7	89.2	88.3	91.7
EGTA	5 mM	42.0	90.1	87.1	92.5
H_2O_2	3% (v/v)	86.4	84.7	84.0	91.5
LAS	0.5% (v/v)	1.4	81.6	<1.0	<1.0
PMSF	1 mM	<1.0	7.4	<1.0	<1.0
SDS	10 mM	3.2	93.8	44.4	53.5
STP	20 mM	51.1	94.4	92.3	97.0

EDTA: Ethylenediamine-tetraacetic acid. EGTA: Ethyleneglycol-bis-tetraacetic acid. LAS: Sodium linear alkyl-benzene sulfonate. PMSF: Phenylmethane sulfonyl fluoride. SDS: Sodium dodecyl sulfate. STP: Tripolyphosphate, pentasodium salt.

H_2O_2 produced from sodium perborate as a bleaching agent, and tripolyphosphate (STP), a sequestering agent used as a constituent of detergent. However, protease BK7-1 was significantly inhibited by EDTA, EGTA, STP and slightly inhibited by H_2O_2. These results are in good agreement with the fact that Ca^{2+} is required for the enhancement of the thermal stability of protease BK7-1 but not for that of protease BK7-2. It is of a great interest to note that protease BK7-2 was slightly inhibited by 0.5% (v/v) sodium linear alkyl-benzene sulfonate (LAS, 18.4% inhibition), surface active agents, and 10 mM sodium dodecyl sulfate (SDS, 6.2% inhibition), but subtilisin BPN and subtilisin Carlsberg were almost completely inhibited by LAS and inhibited by 46.5% and by 55.6%, respectively, by SDS. Protease BK7-1, however, was completely inhibited by both LAS and SDS.

DISCUSSION

Pseudomonas sp. BK7, an alkalophile isolated from soils, produces the extracelluar alkaline proteases which are highly active in the alkaline pH range [10]. During the purification studies to assess their potential for industrial use, two proteases, one of which (BK7-1) was adsorbed on CM-Sepharose and the other (BK7-2) not, were found to be produced by the strain. We concluded that both proteases belong to a class of serine proteases, based on their optimum pH for the activity and complete inactivation by PMSF, a common inhibitor of serine protease [1].

Protease BK7-1, however, is quite different from protease BK7-2 in physico-chemical properties such as isoelectric point, molecular weight, thermal stability, and sensitivity toward inhibitors. First of all, protease BK7-1 has a basic isoelectric point, whereas protease BK7-2 possesses a neutral isoelectric point. The basic pI value of protease BK7-1 explains the reason why the protease did not move to the cathode during nondenaturing PAGE. The pI value of protease BK7-1 was similar to that of protease VapK which was produced from *Vibrio metschinikovii* strain RH530 [9], but slightly higher than pI values of most of alkaline proteases [5, 6, 13]. The alkaline proteases with neutral or acidic isoelectric points like protease BK7-2 also have been reported to be produced by *Serratia marcescens* [16] and by *Bacillus thermoruber* [12]. Secondly, protease BK7-2 has a higher molecular weight than protease BK7-1 or most of bacterial alkaline proteases which fall in the range of 15 to 30 kDa [14]. Thirdly, protease BK7-1 is less thermostable than protease BK7-2 and its thermal stability can be enhanced by Ca^{2+} which is similar to other serine proteases [8, 20], but that of protease BK7-2 cannot be enhanced. Finally, protease BK7-2 is much more resistant than protease BK7-1 against a surface active agent such as LAS or chelators such as EGTA, EDTA, and STP (Table 4).

The optimal pHs for enzyme activity of *Pseudomonas* sp. BK7 proteases are similar to those of Savinase and Esperase which are commercially produced by *Bacillus* sp. [6], and the pH ranges for enzyme stability of the proteases are similar to that of M protease from *Bacillus sp.* KSM-K16 [8], but broader than those of alkaline proteases from *Streptomyces corchorusi* ST36 [4] and *Neurospora crassa* [11]. Notably, protease BK7-2 is outstanding in its resistance to chemical constituents which are contained in laundry detergents: The enzyme BK7-2 is resistant to LAS and SDS, but subtilisin BPN and subtilisin Carlsberg are completely inactivated by LAS and severely inactivated by SDS. The alkaline protease resistant to SDS or LAS has earlier been reported by Shimogaki *et al.* [17], but not yet been applied in industrial production.

In particular, the stability of protease BK7-2 against surfactants or inhibitors frequently used in the laundry industry such as LAS or SDS, is considered to be superior to that of proteases such as subtilisin Carlsberg, subtilisin BPN, and other serine proteases [13, 15]. Therefore, protease BK7-2 produced by *Pseudomonas* sp. BK7 may have a potential to be used as an additive in laundry detergent. Hence, our future effort will hopefully establish better

means of producing this protease by improving enzyme yield via genetic manipulation and optimization of fermentation processes.

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