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Purification and Characterization of an Extracellular Alkaline Protease from *Aspergillus niger* C-15

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An alkaline protease produced by *Aspergillus niger* C-15 was purified and characterized. The enzyme was purified 19.41-fold with a specific activity of 74150 U/mg and a recovery of 34.4% by gel filtration and ion exchange chromatography. The molecular weight of the enzyme was estimated to be 34 kDa. The optimum pH and temperature for the protease activity were pH 8.0 and 60°C, respectively. The enzyme activity inhibited by EDTA suggests that the preparation contains a metalloprotease. The enzyme activity of the metalloprotease was completely inhibited by 5 mM $HgCl_2$ and $FeCl_3$, while partially inhibited by $CuSO_4$ and $MnCl_2$. When polyols such as glycerol, mannitol, sorbitol and xylitol, were added to the reaction medium, most polyols tested enhanced protease activity. Especially, glycerol showed the highest effect. The alkaline metalloprotease was stable at high temperature and retained more than 90% of the initial activity at 60°C and 86.4% under addition of glycerol.

KEYWORDS: Alkaline protease, *Aspergillus niger*, Metalloprotease, Protease

Alkaline proteases are one of the most important groups of industrial enzymes, which are now used in a variety of considerable application in the detergent, leather tanning, food and pharmaceutical industries (Kembhavi *et al.*, 1993; Gessesse and Gashe, 1997; Manachini and Fortina, 1998). Several alkaline proteases have been obtained and purified from different sources such as bacteria, fungi and certain insects (Anwar and Saleemuddinm, 1998). Alkaline proteases active in a wide range of pH and temperature range are suitable for application in biotechnology (Anwar and Saleemuddinm, 1998). It is well known the ability of many different species of the genus *Aspergillus* to produce alkaline protease (Katz *et al.*, 1994; Reichard *et al.*, 1995). In some cases, these enzymes have been associated with allergenic of inflammation response (Hanzi *et al.*, 1993; Monod *et al.*, 1995). In recent, more attention has been paid to species of the genus *Aspergillus*, since it is recognized as an opportunistic fungal pathogen responsible for an increasing number of serious infections in immunocompromised individuals and an abundant producer of proteases.

This article reported the purification and characterization of an alkaline protease produced by *Aspergillus niger* strain C-15 isolated from poultry farming soil in Korea (Kim, 2003).

Materials and Methods

Microorganism. The *Aspergillus niger* strain C-15 was isolated from poultry soil samples in Korea (Kim, 2003). The fungal strain was maintained at 28°C on potato dextrose agar (PDA; Difco). The conidia were harvested from 7-day-old PDA culture and suspended in sterile distilled water by shaking with glass beads. The suspension was filtered through cotton wool, counted a hemocytometer and diluted to 10^5-10^6 spores per *ml*.

Media and cultivation. The glucose peptone medium containing 0.6% glucose and 0.4% peptone was used to induce a high protease activity (Beauvais *et al.*, 1997). The collagen medium includes 0.6% glucose and 15 mg/*ml* powdered native collagen type I (Sigma, St. Louis, MO) in a solution of salts (400 mg of KH_2PO_4, 50 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 3 mg of $FeCl_3$, 3 mg of $ZnSO_4 \cdot 7H_2O$ and 3 mg of $MnCl_2 \cdot 4H_2O$ in 1 litter). The medium was prepared by autoclaving collagen at 115°C for 20 min and adding sterile solution of glucose and salts before inoculation. The casein medium contains 0.6% glucose and 0.4% casein in the solution of salts. Solution of glucose and casein were autoclaved in separate. The pH of all the media was adjusted to 7.0±0.2 before inoculation.

A 250 *ml* of the medium in 500-*ml* Erlenmeyer flasks was inoculated with 2.5 *ml* spore suspension and incubated at 28±2°C and 120 rpm for 21 days. After 3 weeks of incubation, the culture filtrate was passed through a filter paper and centrifuged at 20,000×g for 30 min. The supernatant was used for further experiments on the extracellular protease. The biomass concentration was determined as mycelia dry weight after centrifugation (5,000×g,

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5 min) of 10 *ml* samples of culture broth. Samples were dried at 105°C.

Protease assays. Total extracellular protease activity was estimated by a modified method after Kunitz (1947). To 500 *μl* of 0.25% (w/v) casein in 50 mM Tris-HCl buffer with pH 8.0, a volume of centrifuged culture broth containing 5 *μg* of protein sample was added. The reaction was carried out at 28±2°C for 45 min and proteins were precipitated with 500 *μl* of 0.5% (w/v) trichloroacetic acid (TCA). After 15 min on ice bath, the supernatant was separated by centrifugation at 20,000×g for 10 min and examined by absorbance at 280 nm (Mode HP8453B, Hewlett Packard, Waldbronn, Germany). The blank was prepared by adding 2.5 *ml* of TCA solution before adding the sample. One unit of protease activity was defined as *μ*mol tryptophan released per hour. Protein content was determined using the method described by Bradford (1976), with bovine serum albumin as a standard.

Purification of protease. The culture broth supernatant containing the extracellular enzyme was first submitted to ammonium sulfate precipitation procedures as described by Green and Hughens (1955). Ammonium sulfate was added to the solution to 70% saturation which was then kept overnight. The precipitate collected by centrifugation was dissolved in a small volume (1/50) of 20 mM Tris-HCl buffer with pH 8.0 and dialyzed against the same buffer. After exclusion of ammonium sulfate, protease activity in each dialyzed solution was assayed as described above.

The dialyzed enzyme preparation was applied on a Sephadex G-100 column (2×85 cm; Pharmachia, Sweden) pre-equilibrated with 20 mM Tris-HCl (pH 8.0). Fractions (3 *ml*) were collected and those showing protease activity were pooled. The active fractions pooled from Sephadex G-100 were resolved on a DEAE-cellulose column (2×9.5 cm) equilibrated with 20 mM Tris-HCl (pH 8.0). The unabsorbed protein fraction was eluted with the same buffer (150 *ml*). The enzyme was eluted with a linear gradient of 0 up to 0.75 M NaCl in the same buffer at a flow rate of 0.66 *ml*/min. Fractions (2 *ml*) were collected. Active protease fractions containing 80% of the total enzyme activity were pooled, concentrated by lyophilization and subsequently used for characterization. All steps were carried out at 4°C.

Temperature and pH effects on protease activity and thermal stability. The optimal temperature was tested by assaying the protease activity at different temperatures from 20 to 80°C in 100 mM Tris-HCl buffer (pH 8.0). To determine the optimal pH, protease activity was measured using different buffers as the following 20 mM buffers such as citric acid/Na_2HPO_4 for pH 4~6, $NaHPO_4$/Na_2HPO_4 for pH 6~8, Tris-HCl for pH 7~9, glycine/NaOH for pH 9~11 and $NaHCO_3$/NaOH for pH 11~12. Thermal stability of the protease was investigated by heating the purified enzyme for 15 min at different temperatures in 100 mM Tris-HCl (pH 8.0) in the absence and presence of various polyols. Aliquots were withdrawn at desired time interval to test the remaining activity at standard conditions.

Determination of protease type. Phenylmethanesulphonyl fluoride (PMSF), ethylenediaminetetraacetate (EDTA), iodoacetate and different metal ions were tested for ability to inhibit protease activity. Enzyme was preincubated with each inhibitor in 20 mM Tris-HCl buffer with pH 8.0 for 1 h at 25°C. Protease activity was then determined and residual activity was expressed as percentage activity of the uninhibited enzyme.

Results and Discussion

Enzyme production. A fermentation profile in shaking culture of the *A. niger* strain C-15 is shown in Fig. 1. Maximum biomass production occurred after 13 days of growth, while enzyme production was observed around 17 days-incubation when the medium pH was 8.0. However, after 17 days the biomass started to reduce, which could be related to the diminishing nutrient into the medium (Hasbay and Ögel, 2002). It is demonstrating that protease formation occurs with the decline of available nutrients after nutritional shift-down.

Protease purification. The initial step of purification was the concentration of protein by precipitation using ammonium sulfate and organic solvents. Precipitation by 70% ammonium sulfate yielded a recovery of more than 82.1% and this treatment did not increase specific activity (Table 1). The chromatographic profiles ob- tained on

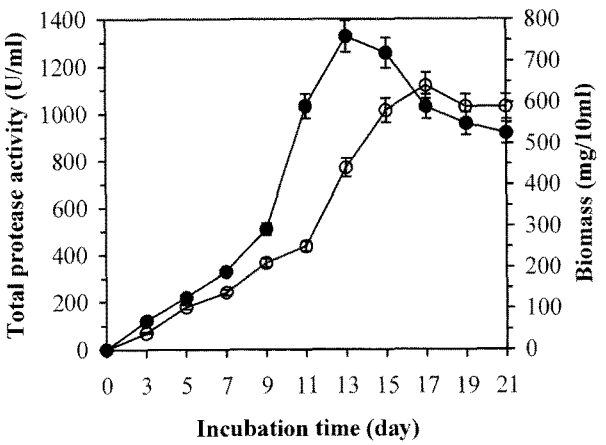

Fig. 1. Protease production (○) and growth (●) of *Aspergillus niger* strain C-15.

Table 1. A summary of the purification of extracellular alkaline protease from *Aspergillus niger* C-15

Purification steps	Total activity (U×10^3)	Total protein (mg)	Yield (%)	Specific activity (×10^3 U/mg)	Purification factor (fold)
Cell-free culture supernatant	893.80	234.01	100.0	3.82	1.00
Ammonium sulfate fraction (70%)	733.81	184.90	82.1	3.96	1.04
Sephadex G-100	459.89	17.41	51.5	26.34	6.88
DEAE-cellulose chromatography	310.71	4.19	34.4	74.15	19.41

All purification steps were carried out at 4°C.

Sephadex G-100 are shown in Fig. 2, after the centrifuged precipitate was dissolved in 20 mM Tris-HCl (pH 8.0). There was a simple peak of protease activity, but three peaks of protein were present. This step resulted in 51.5% yield and 6.88-fold purification of the enzyme. All protein present in fractions, 28 up to 39, was reapplied to the ion exchange chromatography. The final step was purification by ion exchange chromatography on DEAE-cellulose using a linear sodium gradient. The procedure yielded a single peak of protease activity. The chromatogram obtained is given in Fig. 3. The protease activity was eluted from the column with 0.20 M NaCl. The active fraction assayed for activity was analyzed on the 15% SDS-polyacrylamide gel. The molecular weight (MW) was estimated to be 34 kDa by SDS-PAGE (Fig. 4). The overall purification scheme is summarized in Table 1. After the final step, the enzyme was purified 19.41-fold with a specific activity of 74150 U/mg and a yield of 34.4%.

Effect of pH and temperature on protease activity. The optimal pH for protease activity was 8.0. Activities declined rapidly above pH 8.0; 70% and 45% of the maximal activity were observed at pH 9.0 and 10.0, respectively. The enzyme retained the activity even at pH 12.0. The optimal temperature for protease activity was determined by varying the reaction temperatures at pH 8.0. Enzyme activity was estimated between 30°C and 80°C. The optimal temperature was 60°C for protease activity, but above 65°C the activity sharply decreased. The enzyme exhibited 60% and 15% of the maximum activity at 70 and 80°C, respectively (Fig. 5). Thermal stability of the protease was investigated by heating the purified enzyme for 15 min at different temperatures in 100 mM Tris-HCl (pH 8.0). The enzyme was very stable up to 50°C and followed by a rapid loss of activity above 70°C. The enzyme retained more than 90% and 15% of the activity at 60 and 70°C, respectively. However, the enzyme was completely inactivated at 80°C. The activity and stability of the enzyme were higher than those reported for salt-tolerant protease from *Aspergillus* sp. FC-10 (Su and Lee, 2001). Su and Lee (2001) reported 80% inactivation of alkaline protease activity during incubation at 60°C. Nevertheless, alkaline serine protease from *A. terreus* (Syamal *et al.*, 2000) showed maximum activity of pro-

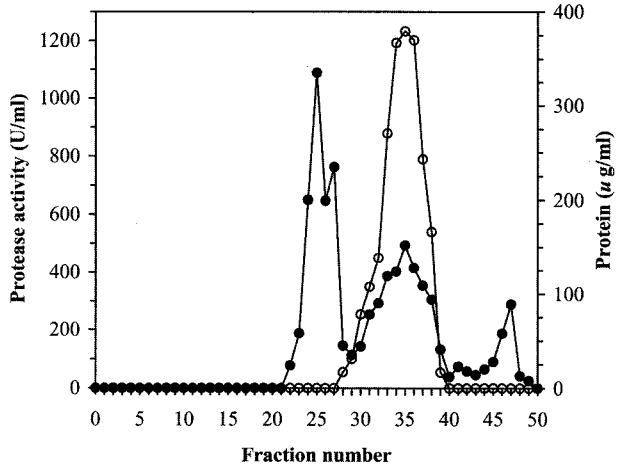

Fig. 2. Purification of *A. niger* protease by gel filtration on Sephadex G-100. The enzyme preparation was applied to a 2×85 cm column equilibrated and eluted with 20 mM Tris-HCl with pH 8.0. Fraction (3 *ml*) collected from the column were assayed fro protease activity. Flow rate 0.23 *ml*/min.

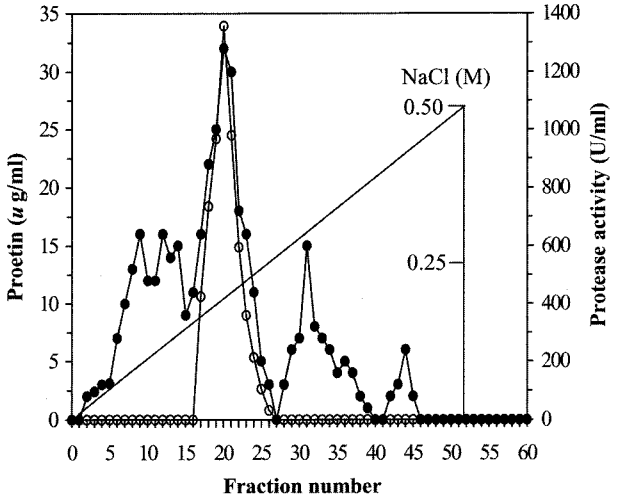

Fig. 3. Chromatography of *A. niger* protease previously purified by gel filtration on DEAE-cellulose. The column (2×9.5 cm) was equilibrated with 20 mM Tris-HCl (pH 8.0) loaded with enzyme preparation and eluted with a linear gradient (0 up to 0.75 M NaCl) at a flow rate 0.66 *ml*/min. No proteins were eluted after 0.5 M NaCl.

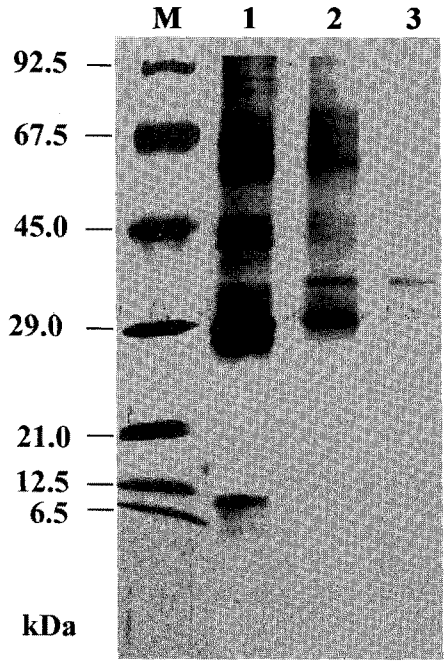

Fig. 4. Silver-staining SDS-PAGE profiles of molecular weight makers (M), crude enzyme extract (lane 1), Sphadex-100 bound enzyme fraction (lane 2), and the purified protease (lane 3) on a 15% polyacrylamide gel.

tease at pH 8.5 and 60°C, and the enzyme retained about 60% at 70°C; at 80°C only 20% of the enzyme activity was retained and at 90°C it was inactive.

Effect of metal and reagent on enzyme activity. The effects of various metals and reagents on the activity of the purified protease from *A. niger* are summarized in Table 2. The protease activity was almost completely inhibited by 5 mM of either $HgCl_2$, or $FeCl_3$, partially (31.1% or 32.2%) inhibited by $CuSO_4$ or $MnCl_2$, and 100% inhibited by 5 mM of EDTA. Other metals or reagents were judged to have no effect on the enzymatic activity. These results, taken together, suggest that the purified extracellular alkaline protease is a metalloprotease. *A. fumigatus* also produces extracellular Zn-metalloprotease, representing up to 30% of total protease activity (Markaryan *et al.*, 1994; Monod *et al.*, 1993; Jaton-Ogay, 1994). Although the isoelectoric point of the enzyme purified by Monod *et al.* (1993) is 5.5, the metalloenzyme was inhibited by EDTA and not affected by PMSF. Therefore it is similar to the protease purified in this study. However, the alkaline protease produced by *A. niger* strain C-15 was completely distinguished from the protease of *A. flavus* having characteristics of serine type protease (Kim, 2003).

Effect of polyols on protease thermostability. Polyols such as glycerol, mannitol, sorbitol and xylitol, were added to the reaction medium (final concentration 5%, w/v). Most polyols tested enhanced protease activity. Glycerol induced the best outcome (data not shown). The influence of polyols on thermostability of the protease was studied. Enzyme was first incubated at 60°C in 50 mM Tris-HCl buffer (pH 8.0) in the absence or the presence of polyols (final concentration 10%, w/v). After 150 min incubation, the remaining protease activity was investigated. As shown in Fig. 5, thermostabilization was the most effective with glycerol. After incubation at 60°C in the presence of glycerol or in the absence of any additive, 86.5% and 55.0% of the initial activity remained, respectively. No protective effect was observed with other polyols.

In conclusion, the *A. niger* strain C-15 produced a ther-

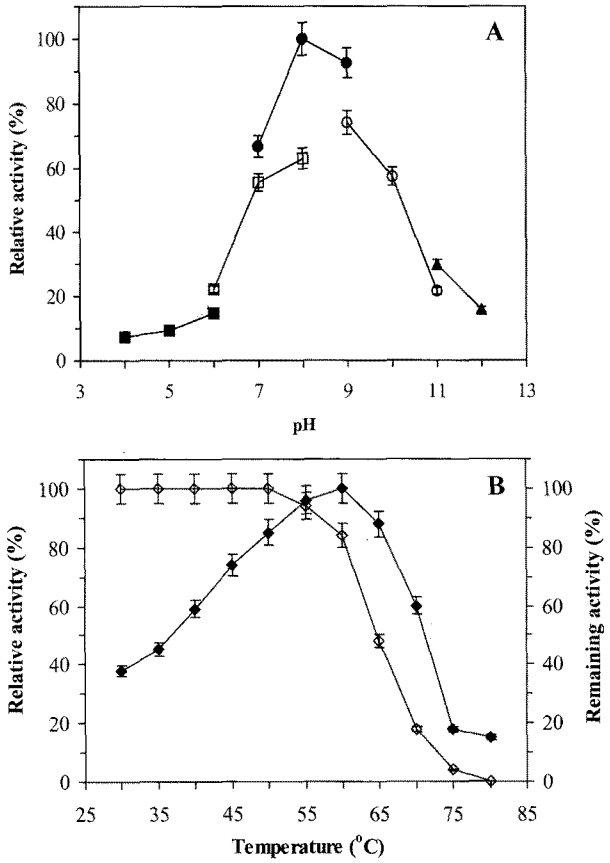

Fig. 5. Effect of pH and temperature on protease activity and effect of temperature on thermal stability of protease. (A) Effect of the pH protease activity. The pH profiles were determined in different buffers by varying pH values at 60°C. (■), citrate buffer (pH 4~6); (□), $NaHPO_4/Na_2HPO_4$ buffer (pH 6~8); (●), Tris-HCl buffer (pH 7~9); (○), glycine/NaOH buffer (pH 9~11); (▲), $NaHCO_3$/NaOH buffer (pH 11~12). (B) Effect of temperature on protease activity (◆) and on thermal stability (◇) of protease. The enzyme was incubated in 100 mM Tris-HCl buffer (pH 8.0) at different temperatures for 15 min and the residual activity was determined as described in materials and methods.

Table 2. Effect of metal ions and reagent on the activity of alkaline protease from *A. niger* C-15

Reagent	Concentration (mM)	Residual activity (%)
None		100.0
$CaCl_2$	5	89.6
$MgCl_2$	5	96.2
$MnCl_2$	5	68.9
$CuSO_4$	5	67.8
$FeCl_3$	5	1.3
$HgCl_2$	5	0.0
$ZnSO_4$	5	105.2
PMSF[a]	5	100.0
Idoacetate	5	100.0
EDTA[b]	5	0.0

The protease was incubated with 5 mM of metal ions or reagent at room temperature for 30 min and the enzyme activity measured in the absence of any inhibitors was taken as 100%.
[a]PMSF; Phenylmethanesulphonyl fluoride.
[b]EDTA; Ethylenediaminetetraacetate.

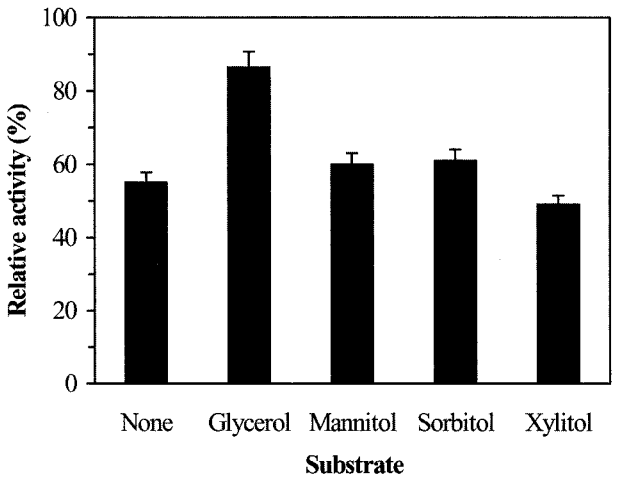

Fig. 6. Effect of polyols on protease thermostability. A protease preparation was heated at 60°C in the absence or presence of polyols. After 150 min, the remaining activity was measured.

mostable alkaline metalloprotease. The optimum temperature and pH a for protease activity were 60°C and pH 8.0. The metalloenzyme retained more than 70% and 45% of the maximal activity at pH 9.0 and 10.0, respectively. The protease had thermostability with 100%, 90% and 15% activity retained at 50°C, 60°C and 70°C, in that order, after 15 min-incubation. For these reasons, the protease of *A. niger* C-15 may have a potential use in the detergent industry.

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