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Purification and Characterization of an Alkaline Protease Prot 1 From *Botrytis cinerea*

Biodetergent Catalyst Assay

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Abstract

Alkaline thiol protease named Prot 1 was isolated from a culture filtrate of *Botrytis cinerea*. The enzyme was purified by ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography. Thus, the enzyme was purified to homogeneity with specific activity of 30-fold higher than that of the crude broth. The purified alkaline protease has an apparent molecular mass of 43 kDa under denaturing conditions as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The native molecular mass (45 kDa), determined by gel filtration, indicated that the alkaline protease has a monomeric form. The purified protease was biochemically characterized. The enzyme is active at alkaline pH and has a suitable and high thermostability. The optimal pH and temperature for activity were 9.0–10.0 and 60°C, respectively. This protease was stable between pH 5.0 and 12.0. The enzyme retained 85% of its activity by treatment at 50°C over 120 min; it maintained 50% of activity after 60 min of heating at 60°C. Furthermore, the protease retained almost complete activity after 4 wk storage at 25°C. The activity was significantly affected by thiol protease inhibitors, suggesting that the enzyme belongs to the alkaline thiol protease family. With the aim on industrial applications, we focused on studying the stability of the protease in several conditions. Prot 1 activity was not affected by ionic strength and different detergent additives, and, thus, the protease shows remarkable properties as a biodetergent catalyst.

Index Entries: Alkaline protease; *Botrytis cinerea*; purification; characterization; detergent enzyme.

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Introduction

Proteases account for approx 60% of the total sales of enzyme in various industrial market sectors, such as the detergent, food, pharmaceutical, diagnostic, leather, and waste management sectors *(1)*. Proteases are involved in numerous biologic functions, such as septum formation, sporulation, protein turnover, catabolite inactivation, protein secretion, and nutrition *(2,3)*. They are also one of the standard ingredients of all types of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approx 25% of the total worldwide sales of enzymes *(1)*. The wide diversity and specificityof proteases make them advantageous in the development of effective therapeutic agents. Proteases belonging to the subtilisin group are used in the treatment of burns and wounds. Oral administration of protease produces an anti-inflammatory response in burn patients and speeds up the process of healing *(4,5)*. Latha et al. *(6)* reported that trypsin and chymotrypsin preparation acted as anti-inflammatory and antioxidant agents in human burn wounds.

The stability of proteolytic enzymes is a major concern regarding their industrial applications, because biocatalysts with high thermostability have a prolonged viability. For many biocatalysts, activity and stability at high temperature are prerequisites for industrial use. The serine and alkaline proteases are used in the detergent industry. Alkaline proteases exhibit a maximum activity at a pH range of 10.0–11.0, and most of them display a maximum activity at a relatively high temperature, especially at about 60°C, but are inactive or less active at a relatively low temperature, at about room temperature. Since Horikoshi and Akiba *(7)* first reported the production of alkaline protease from alkalophilic bacteria, several microbial alkaline proteases have been purified and characterized from *Actinomyces (8)*, fungi *(9)*, *Bacillus* sp. *(10,11)*, *Bacillus cereus (12)*, and *Streptomyces (1)*. Alkaline protease produced by bacteria of the genus *Bacillus* shows an optimal activity and a good stability at high alkaline pH values. These proteases are stabilized by calcium ions and inactivated by serine active-site inhibitors *(10)*.

Since 1960, proteolytic enzymes have frequently been used in laundry products, mostly because they decreased the need to use phosphate in detergents and they provided a means for compensating poorer detergent performance at lower wash-water temperatures *(13)*. In fact, the high economic value of proteases still provides an impetus to search for new proteases with novel properties.

Herein we report our findings on an alkaline protease (Prot 1) with caseinolytic and gelatinolytic activity produced by a *Botrytis cinerea* strain isolated from cultivated plant. The biochemical characterization and stability against pH, temperature, and some detergent additives of the produced protease were studied. The results indicate that Prot 1 protease is suitable for use as a biodetergent biocatalyst.

Materials and Methods

Organism

B. cinerea strain was isolated from infected cultivated plants. The plant pathogenic fungi were successively cultivated on potato dextrose agar plates in the presence of 0.5% yeast extract.

Media and Culture Conditions

B. cinerea strain was grown in liquid basal medium containing 1 g/L of KCl, 0.5 g/L of $Mg(SO_4)$, and 1 g/L of KH_2PO_4, 5 g/L of glucose, 2 g/L of yeast extract, $NaNO_3$ (70 m*M*), and $(NH_4)_2SO_4$ (70 m*M*) were added. To 1 L of this medium we added 1 mL of oligo-elements containing $CoCl_2$ (2 g/L), $MnSO_4 \cdot H_2O$ (1.6 g/L), $ZnSO_4 \cdot H_2O$ (1.4 g/L), and $FeSO_4 \cdot H_2O$ (5 g/L) and potassium phthalate (5 g/L). The pH was adjusted to 6.5 by the addition of 2 *N* NaOH solution. The culture was grown for 9 d at 25°C with shaking at 150 rpm. The effects of the culture conditions on protease production were assayed by growing the microorganisms in a medium containing different inducer substrates and at different pH values.

Purification of Enzyme

The culture broth was filtered and centrifuged at 8000*g* for 20 min at 4°C, and the supernatant was used for further purification. Proteins from the supernatant were precipitated with 80% ammonium sulfate overnight at 4°C with constant stirring. The precipitate formed was recovered by centrifuging at 25,000*g* and 4°C for 30 min using a Beckman ultracentrifuge and resuspended in a minimum amount of 20 m*M* Tris-HCl buffer, pH 8.0.

Gel Filtration Chromatography with Sephacryl S-200-HR

The dissolved proteins were applied to a Sephacryl S-200-HR column (2.2 H 100 cm) (Pharmacia, Uppsala, Sweden); equilibrated; and eluted with 20 m*M* Tris-HCl buffer, pH 8.0. Elution was performed at a flow rate of 12 mL/h. Fractions of 3 mL were collected and assayed for absorbance at 280 nm and protease activity. Two peaks of protease activity were eluted from the column. The fractions of peak A were polled and loaded onto an ion-exchange column for further purification of the alkaline protease, whereas those of peak B were stored at 4°C.

DEAE Sepharose Chromatography

The enzymatically active protein fractions of peak A from the gel filtration column were loaded at 18 mL/h onto a DEAE Sepharose column (2.2 H 20 cm) equilibrated with 20 m*M* Tris-HCl buffer, pH 8.0. After loading the sample, the column was washed with equilibration buffer, and elution was performed with a linear gradient from 0 to 0.5 *M* NaCl in the same buffer.

DEAE-Progel TSK High-Performance Liquid Chromatography Column

The active fractions, obtained from the first anion-exchange chromatography, were pooled and concentrated approximately fivefold by an Amicon centriprep-10 concentrator. The active sample was applied to a DEAE-Progel TSK high-performance liquid chromatography (HPLC) column equilibrated with 20 m*M* Tris-HCl buffer, pH 8.0. Proteins were eluted at 0.8 mL/min in a salt gradient from 0 to 0.5 *M* NaCl in the same buffer.

Gel Filtration on TSK SW 2000 HPLC Column

One step of the purification and determination of the relative molecular mass of the purified enzyme in native form was assayed by gel filtration on a TSK-SW-2000 column. The column was previously equilibrated with 20 m*M* Tris-HCl buffer, pH 8.0. Elution was performed at a flow rate of 0.8 mL/min, and detection was carried out at 220 nm. The column was calibrated with thyroglobulin (670 kDa), bovine-γ-globulin (158 kDa), ovalbumin (43 kDa), equine myoglobulin (17 kDa), and vitamin B_{12} (1.35 kDa). Protease activity was tested in the column fractions by azocasein assay.

Protease Assay

Azocasein hydrolysis was measured by the method described by Segers et al. *(14)* with modifications as follows: commercial azocasein (Sigma) was dissolved at 5% (w/v) solution in 0.1 *M* Tris-HCl buffer, pH 9.0.

Proteolytic activity was assayed using azocasein (Sigma) as a substrate. Briefly, 50 µL of diluted enzyme was added to 200 µL of reaction buffer (100 m*M* Tris-HCl, pH 9.0) containing 5% (w/v) azocasein, and the mixture was incubated at 60°C for 30 min. The reaction was stopped by adding 600 µL of 10% (w/v) trichloroacetic acid and left for 15 min on ice, followed by centrifugation at 15,000*g* for 10 min to remove the precipitate protein. Six hundred microliters of the supernatant was neutralized by adding 700 µL of 1 *M* NaOH, and the absorbance at 440 nm (A_{440}) was measured using a Shimadzu spectrophotometer (model 1240). One unit of enzyme activity was defined as the amount that yielded an increase in A_{440} of 0.1 in 30 min at 60°C.

Determination of Protein

Protein concentration of the samples was estimated by Bradford's method *(15)* using Bio-Rad protein dye as reagent concentrate and bovine serum albumin (BSA) to prepare the standard solution range or by measuring directly the absorbance at 280 nm.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli *(16)* in a discontinuous system of 5% concentrating gel and 10% separating gel using a dual slab gel

unit, model DGS-125 system. Low-range SDS-PAGE markers (Bio-Rad) containing phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) were used as molecular mass standards. Under reducing conditions, samples were run on polyacrylamide pre-prepared gels (Bio-Rad). Proteins bands were stained by the silver nitrate method *(17)*.

Zymograms with Native PAGE

Zymograms were performed as described by Schmidt et al. *(18)* with minor modifications. Polyacrylamide gels (10%) were copolymerized with 0.05% gelatin. Slab gels consisted of 10% acrylamide/bisacrylamide (29:1) in 400 m*M*

M acetate buffer,

pH 5.0 (10% glycerol). Slab gels 1-mm thick were subjected to electrophoresis at 4°C using glycine (40 m*M*)–acetic acid (3.5 m*M*) buffer, pH 4.0, and run at 50 V. Then the gels were incubated in 50 m*M* Tris-HCl (pH 9.0) for 60 min at 60°C and stained overnight with 0.25% Coomassie brilliant blue R-250 in methanol/acetic acid/water (5:1:5 [v/v/v]) and later decolorized in 7% acetic acid until the zones of gelatin hydrolysis were visible.

Effects of pH and Temperature on Protease Activity

Protease activity was measured at different pH values under standard assay conditions with azocasein as substrate. The activity of enzyme at pH 5.0–12.0 was assayed with 100 m*M* buffer: glycine-HCl buffer (pH 3.0), acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–7.0), Tris-HCl buffer (pH 8.0–9.0), glycine-NaOH buffer (pH 10.0–12.0). Protease activity was assayed at different temperatures (20, 30, 40, 50, 60, 70, and 80°C) in 100 m*M* Tris-HCl buffer (pH 9.0) with the azocasein method as described above.

Effects of pH and Temperature on Protease Stability

The initial activity of the enzyme was assayed under the standard conditions. The enzyme preparation was preincubated for different times (20, 40, 60, 80, 100, and 120 min) under various temperatures (40, 50, and 6°C) and overnight under various pH values without the substrate, and then the remaining activities were assayed.

Effects of Chemical Agents on Enzymatic Activity

The activity of the purified enzyme was tested by the standard procedure in the presence of different chemical agents to identify potential inhibitors or activators. The effects of several divalent cations ($CaCl_2$, $CuCl_2$, $CoCl_2$, $MgSO_4$, $ZnCl_2$, $FeSO_4$, and $MnCl_2$) were determined at 0.5 m*M*. The effects of two salts (NaCl and ammonium sulfate) used during the purification procedure were also determined at 0.1–2.0 *M*. All reagents were purchased from Sigma.

Stability Against Protease Effectors

To determine the specificity of the purified protease active site, various inhibitors such as henylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EDTA, EGTA, 5,5N-dithiobis (2-nitro-6-benzoic acid) (DTNB), and 2-mercaptoethanol were tested. Ten microliters of purified protease was preincubated with effectors for 1 h at room temperature. The remaining proteolytic activity was assayed using the azocasein method.

Stability Against Detergent Additives

Protease was incubated at 60°C in the presence of different detergent additives: Triton X-100, SDS, H_2O_2, Nonidet P40, Tween-20, dim-ethylsulfoxide (DMSO), carboxymethylcellulose (CMC), sodium sulfate, and sodium silicate (Sigma), as well as some industrial detergent additives: sodium tripolyphosphate (STPP), sodium linear alkyl benzene sulfonate (LABS-Na), and perborate (Henkel Alky). After specific intervals and concentrations, samples were removed to determine the residual activ-ity using the azocasein test.

Results

Production and Purification of Prot 1 Enzyme

Extracellular protease activity was produced by a *B. cinerea* culture at the exponential growth phase. Preliminary experiments showed that production of protease by *B. cinerea* culture is subject to glucose repression, pH values during fermentation, and substrate induction (data not shown). Therefore, throughout these studies we used a culture medium (pH 6.5) containing gelatin (2% [w/v]) as inducer. Proteolytic activity was identified during the growth by a routine detection enzyme activity using azocasein as substrate. Figure 1 shows a growth curve and extracellular enzyme production.

Maximum proteolytic activity for *B. cinerea* found approx 9 d after inoculation. The culture was centrifuged (4°C and 12,000*g* for 30 min), and the supernatant (800 mL) was used for further purification. Protease from *B. cinerea* named Prot 1 was purified by selective ammonium sulfate precipitation and four chromatographic steps. Table 1 summarizes the results of the purification procedure.

All purification operations were performed at room temperature. Eight hundred milliliters of crude enzyme preparation was subjected to ammonium sulfate precipitation (80%) by slow continuous stirring. The saturated solution was left overnight at 4°C. After centrifugation, the precipitate was dissolved in a minimum amount of 20 m*M* Tris-HCl buffer (pH 8.0). This step decreased the protein content by about 46% and, consequently, the specific activity increased about twofold. The active sample, concentrated to 15 mL, was applied to a Sephacryl S-200-HR column, equilibrated, and eluted with 20 m*M* Tris-HCl buffer (pH 8.0). Proteins were

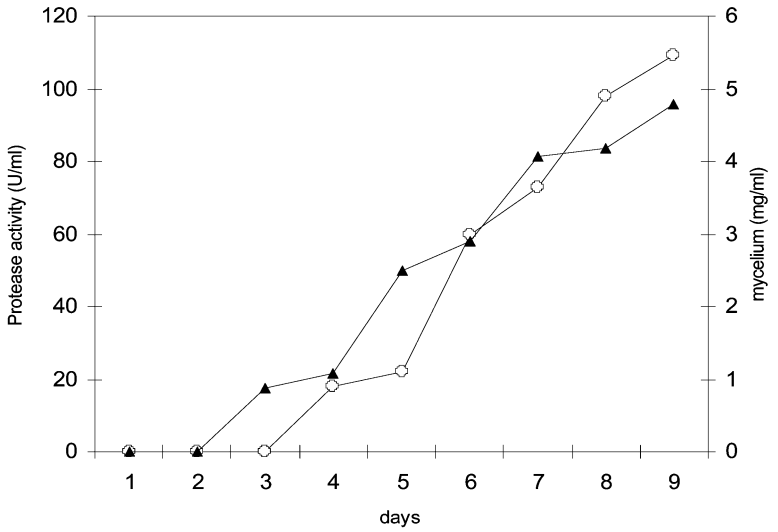

Fig. 1. Mycelium growth and protease synthesis during culture of *B. cinerea* in medium containing gelatin as protease inducer. Culture flasks were incubated at 25°C and pH 6.5 with horizontal shaking for 9 d. (○) Extracellular protease activity; (▲) dry weight.

eluted at 12 mL/h and 3-mL fractions were collected. Two peaks of proteins with protease activity (peaks A and B) were separated by gel filtration chromatography (Fig. 2). The inset in Fig. 2 indicates the gelatin zymogram analysis profile in native cathodic PAGE of the protease (peak A) named Prot 1. The results indicate that Prot 1 is probably an alkaline protease (cathodic migration in acidic conditions). This is why the pooled fractions (20–46) from the previous step were applied to a DEAE Sepharose column (2.2 H 20 cm) equilibrated with 20 m*M* Tris-HCl buffer (pH 8.0). The column was washed with the same buffer, and then elution was performed with a linear gradient from 0 to 0.5 *M* NaCl. The proteolytic activity was concentrated in a single peak that was eluted on the gradient at 300 m*M* NaCl (Fig. 3). In this step, the enzyme was purified 10-fold (Table 1).

The active fractions were pooled, concentrated by an Amicon centriprep-10 concentrator, and applied to a DEAE-Progel TSK column on HPLC previously equilibrated with 20 m*M* Tris-HCl buffer (pH 8.0). Proteins were eluted out from the column at 0.8 mL/min by a continuous NaCl gradient from 0 to 0.5 *M* in the same buffer. The proteolytic activity was tested in eluted fractions (Fig. 4). The pooled Prot 1 enzyme preparation had a 26-fold increase in specific activity. This fraction was used for further characterization.

Determination of Molecular Weight

The protease fraction from the DEAE-Progel TSK HPLC column was chromatographed on a TSK SW 2000 HPLC gel filtration column (Fig. 5). The eluted purified enzyme was enriched 30-fold, with a yield of 6.3% (Table 1).

Table 1
Purification of Alkaline Thiol Protease (Prot 1) from *B. cinerea*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	88	61,866	703	1	100
Ammonium sulfate precipitation	40.25	57,100	1418	2	92
Gel filtration with Sephacryl S-200-HR	3.451	19,200	5563	8	31
DEAE Sepharose chromatography	1.32	9048	6854	10	14
HPLC with DEAE-Progel TSK	0.36	6498	18,050	26	10.6
Gel filtration on TSK SW 2000 HPLC	0.18	3906	21,700	31	6.3

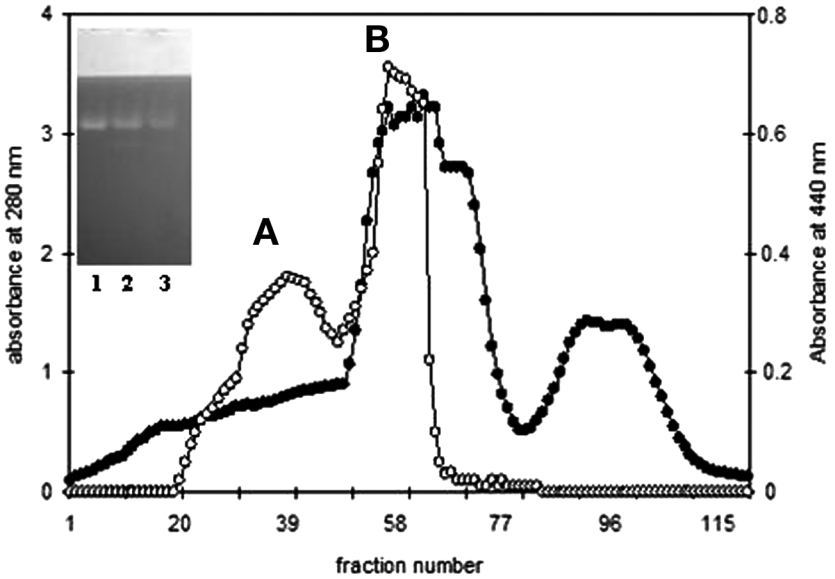

Fig. 2. Elution profile of gel filtration chromatography on Sephacryl S-200-HR column. The column was equilibrated in 25 m*M* Tris-HCl buffer (pH 8.0). The flow rate was 12 mL/h and the collected fraction was 3 mL/tube. Proteins (●) were measured by absorbance at 280 nm. Protease activity (○) was tested by using the azocasein system. The inset indicates gelatin zymography analysis on native cathodic PAGE of the protease activity from the gel filtration column. Incubation and staining of the postrun gels were carried out as described in Materials and Methods. Lanes 1, 2, and 3 represent, respectively, fractions 30, 38, and 46 of peak A.

The native molecular weight of Prot 1 enzyme was determined by comparing the protein elution on the TSK SW 2000 HPLC gel filtration column with the elution of marker proteins under the same experimental conditions. The native molecular weights were calculated from the standard curve, constructed by plotting the log of molecular weight against elution volume on gel filtration. The native molecular weight was found to be about 45 kDa (Fig. 5).

After each purification step, the purity of protease (Prot 1) was analyzed by SDS-PAGE. Protease from the TSK SW 2000 HPLC gel filtration step resulted in a single band of 43 kDa (Fig. 6), suggesting that the purified Prot 1 protein is a monomer.

Biochemical Characterization of Prot 1

Effects of pH and Temperature on Protease Activity

Protease activity was determined at various pH values and temperatures under standard assay conditions as described in Materials and Methods. The results were expressed as a percentage of the activity obtained at optimum pH (Fig. 7A). Prot 1 showed the highest activity at pH 9.0.

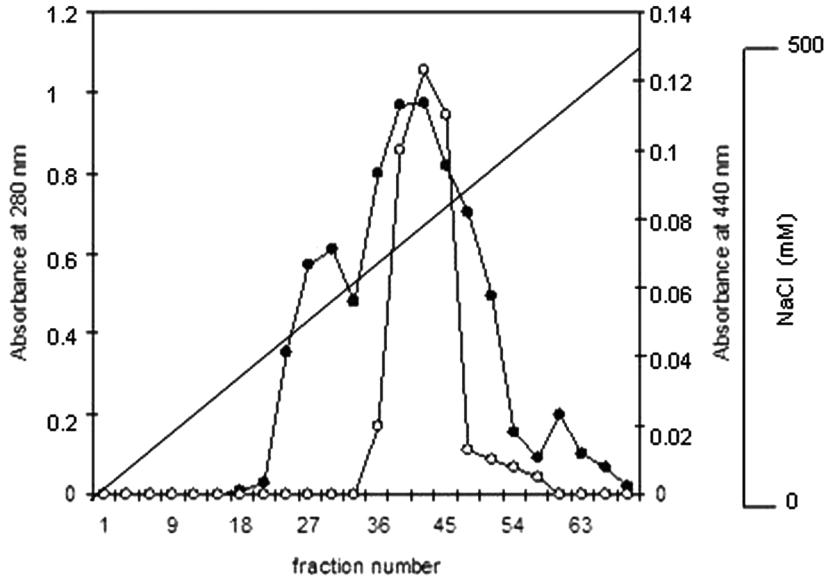

Fig. 3. Chromatography on DEAE Sepharose CL-6B column. Protease activity from peak A after the gel filtration step was fractionated on a DEAE Sepharose column previously equilibrated in 25 m*M* Tris-HCl buffer (pH 8.0). The flow rate was adjusted at 18 mL/h and the collected volume was 3 mL/tube. Elution of proteins was carried out by a linear gradient of 0–500 m*M* NaCl in the same buffer. Protein content (●) was determined for absorbance at 280 nm. Protease activity (○) was assayed by the azocasein system.

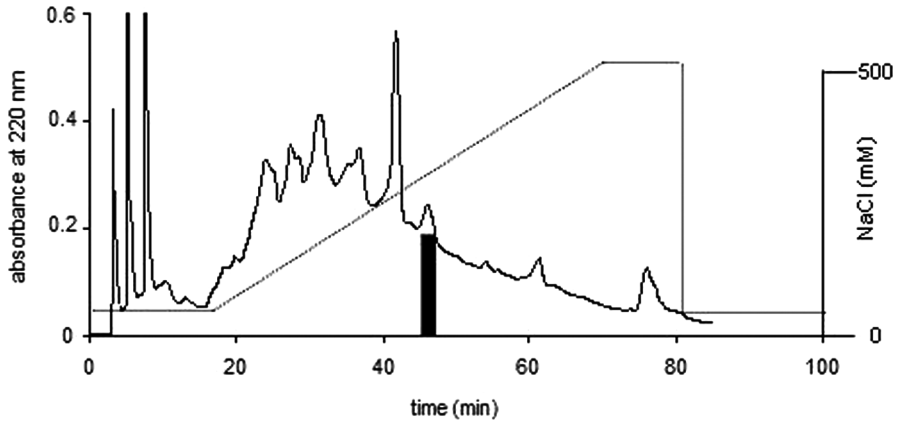

Fig. 4. Anion-exchange HPLC with DEAE-Progel TSK. The sample obtained from the DEAE Sepharose CL-6B column chromatography was concentrated and applied to an HPLC DEAE-Progel TSK column. Then the column was washed with 20 m*M* Tris-HCl buffer (pH 8.0), and elution was performed using a linear gradient of 0–500 m*M* NaCl in the same buffer. Loading, washing, and elution were all performed at 0.8 mL/min. The absorbance at 220 nm was monitored to detect the protein peak. The enzymatic activity (■) was located in the peak corresponding to 300 m*M* NaCl.

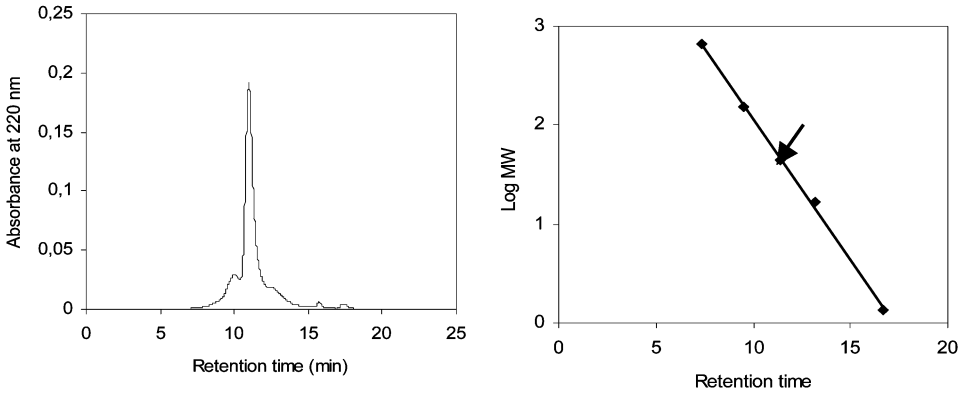

Fig. 5. Elution profile of Prot 1 protease (*Rt* = 11.3 min) on TSK SW 2000 HPLC Gel filtration column. **(A)** The column was previously equilibrated with 20 m*M* Tris-HCl buffer (pH 8.0) and calibrated with thyroglobulin (670 kDa), bovine-γ-globulin (158 kDa), ovalbumin (43 kDa), equine myoglobulin (17 kDa), and vitamin B_{12} (1.35 kDa). **(B)** The native molecular weights were calculated from the standard curve, constructed by plotting the log of molecular weight against elution time on gel filtration. The arrow corresponds to Prot 1 protein.

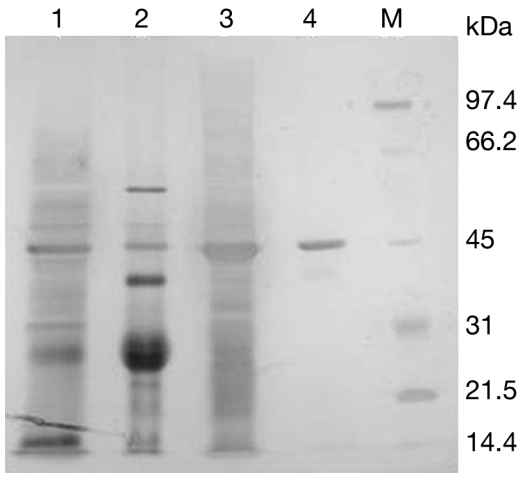

Fig. 6. Homogeneity test of alkaline protease from *B. cinerea* as determined by SDS-PAGE in a 10% slab stained with silver nitrate. Lane 1, crude extracellular protease; lane 2, ammonium sulfate precipitation step; lane 3, purified protease after DEAE Sepharose CL-6B; lane 4, purified protease (Prot 1) from HPLC TSK SW 2000 Gel filtration. M, standard molecular weights markers.

The effect of temperature on enzyme activity was determined in the range of 4–70°C. The substrate solution was previously equilibrated at each temperature, and then the reaction was initiated by the addition of the purified enzyme. The results were expressed as a percentage of the activity obtained at the optimum temperature (Fig. 7B). The optimum temperature for the enzyme activity was 60°C.

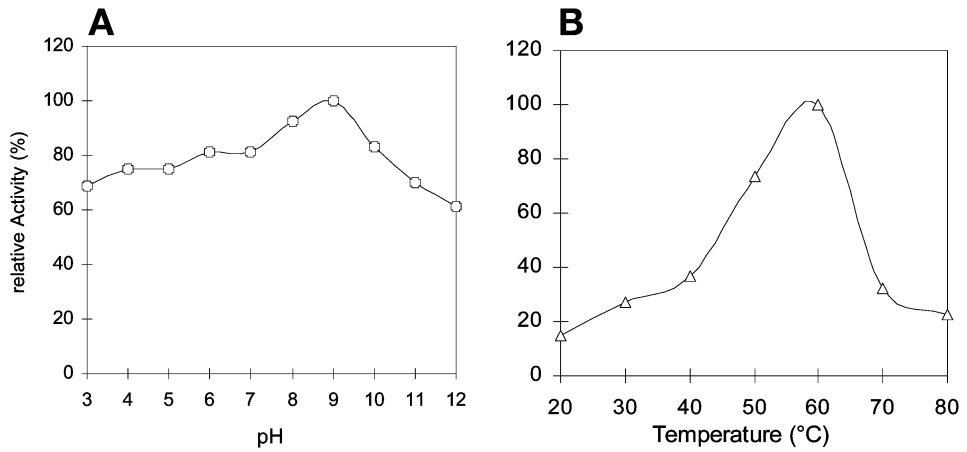

Fig. 7. Effects of pH and temperature on protease activity. **(A)** Enzyme activity was assayed in a pH range of 3.0–12.0 using 100 m*M* buffer: glycine-HCl buffer, pH 3.0; sodium acetate buffer, pH 4.0–5.0; phosphate buffer, pH 6.0–7.0; Tris-HCl buffer, pH 8.0–9.0; and glycine-NaOH buffer, pH 10.0–12.0. The reaction mixtures were incubated for 30 min, and the activity was measured under standard assay conditions. **(B)** The purified enzyme was incubated for 30 min at temperatures from 20 to 80°C in 100 m*M* Tris-HCl buffer, pH 9.0. Protease activity was measured by azocasein assay at 440 nm.

Effects of pH and Temperature on Protease Stability

Prot 1 was incubated at various pH values overnight at various temperatures for different times, and then the remaining activities were measured. The enzyme was found to be significantly stable in the range of pH 5.0–12.0 (Fig. 8A). Regarding the effect of temperature on the stability, the enzyme was stable below 50°C. The enzyme retained 50% of its activity after incubation for 60 min at 60°C (Fig. 8B). These results indicate that Prot 1 has good thermal and pH stabilities.

Enzyme stability during storage was studied at 4 and 25°C. Protease activity was completely retained during 4 wk of storage at 4°C, and the enzyme retained 89% of its activity when stored for the same length of time at 25°C.

Effects of Metal Ions on Protease Activity

The activity of the purified enzyme was assayed in the presence of different chemical agents to identify possible inhibitor or activator by the standard procedure. In this way, the results of the effects of metal ions were also determined. At a concentration of 5.0 m*M* Co^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} at 5.0 m*M* each in the reaction mixture. There was no notable effect on enzyme activity, except for Cu^{2+} showed inactivation of the enzyme. This result corresponded generally to the finding of a study on a protease from *Bacillus* sp. No 8-6 *(19)*.

Influence of Various Effectors on Prot 1 Activity

Table 2 presents the effect of some reagents on protease activity. The presence of a chelating agent such as EDTA and EGTA at higher concentra-

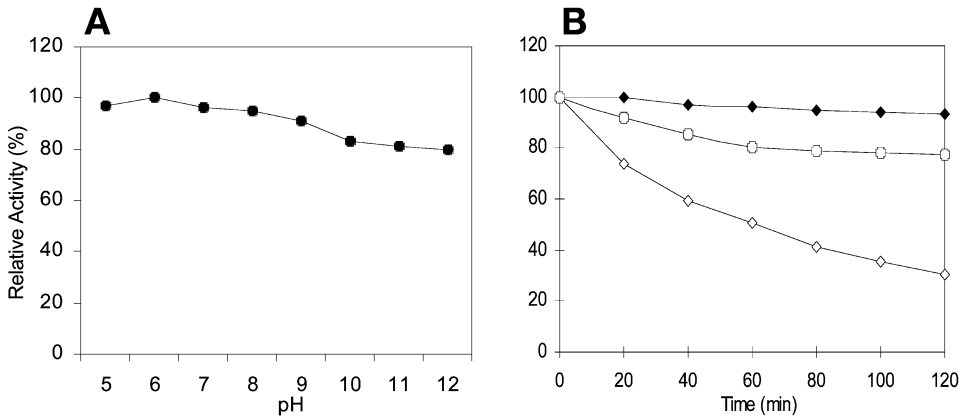

Fig. 8. Effects of pH and temperature on protease stability. **(A)** The pH stability of the enzyme was determined following incubation at various pH values for 24 h. **(B)** To determine the thermal stability of the enzyme, enzyme solution was preincubated in 100 m*M* Tris-HCl buffer at pH 9.0 for different times at various temperatures: (◆) 40°C; (○) 50°C; (◇) 60°C. The residual activity was assayed by the azocasein method.

Table 2
Influence of Various Reagents on Enzyme Activities

Inhibitor	Concentration (m*M*)	Relative activity (%)
Control	None	100
EGTA	10	100
EDTA	10	100
2-Mercaptoethanol	10	112
DTT	10	110
PMSF	1	92
DTNB	3	10

tion (10 m*M*) had no significant effect on activity, indicating that the purified enzyme is not a metalloenzyme. Activity was not affected by the presence of PMSF, which is a specific inhibitor of serine proteases. The activity of purified enzyme was completely inhibited in the presence of DTNB, while the proteolytic activity was slightly stimulated in the presence of 2-mercaptoethanol and DTT, suggesting that the enzyme is a thiol protease.

Compatibility of Prot 1 With Detergents and Surfactants

The purified Prot 1 protease from *B. cinerea* was stable toward nonionic surfactants, such as Triton X-100 and Tween-20, as well as strong anionic surfactants. In particular, it showed high stability against SDS and H_2O_2. The enzyme retained approx 100 and 85% of activity even after incubation with 5% SDS and 5% H_2O_2, respectively. Prot 1 was also stable toward laboratory surfactants, bleaches, and some commercial detergent

Table 3
Effects of Various Surfactants, Bleaches, and Industrial Detergent Additives on Alkaline Protease (Prot 1)

	Concentration	Relative activity (%)
Control	None	100
Triton X-100	5%	98
SDS	5%	100
H_2O_2	5%	87
NaCl	2 *M*	100
Ammonium sulfate	2 *M*	94
Sodium sulfate	50%	77
DMSO	10%	100
Nonidet P40	3%	100
Tween-20	3%	92
CMC	1%	98
STPP	1%	85
LABS-Na	1%	63
Perborate	3%	100
Sodium silicate	2%	87

additives (Table 3). No significant inhibition was detected in the presence of oxidizing agents such as H_2O_2, sodium hypochlorite, and various surfactants. This protease showed its compatibility toward many industrial detergents and bleaches used in laundries such as STPP, LABS-Na, perborate, CMC, and sodium silicate. Furthermore, the protease from *B. cinerea* still retained up to 85% of its initial activity after lyophilization, conservation at room temperature in a high concentration of NaCl or ammonium sulfate.

Discussion

This work describes the purification and some biochemical properties of extracellular alkaline protease Prot 1 isolated from a phytopathogenic *B. cinerea*. Protease activity was tested by azocasein substrate and by gelatin zymography. The protease, with excellent resistance to pH and detergents, was purified by a combination of ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography. The finally purified enzyme showed high specific activity in comparison to other purified alkaline proteases *(12,20,21)*. The enzyme protein is monomeric and has an apparent molecular mass of about 45 kDa, common characteristics with other described proteases *(22)*. The enzyme activity was enhanced by reagents that stabilize sulfhydryl groups such as 2-mercaptoethanol and DTT; it was not affected by EDTA, PMSF, and SDS and was strongly inhibited by thiol-blocking reagent DTNB. Moreover, Prot 1 recovered 50% of its activity when treated by both 3 m*M* DTNB and 5 m*M* 2-mercaptoethanol. Thus, the results suggest that Prot 1 enzyme is a thiol protease. The highest optimal

pH previously reported for alkaline protease was between 10.0 and 10.5 *(12,23,24)*, and only a few reports indi-cate an optimal pH of activity at 11.0 or above from some *Bacillus* sp. *(25,26)*.

The Prot 1 enzyme was active over a wide range of temperatures and maximal activity was recorded at 60°C. A number of alkaline proteases isolated from *Bacillus* sp. have been reported to have a high optimal temperature *(26,27)*, whereas there are only a few reports on the fungal proteases with high optimal temperature *(28,29)*.

The stability of the enzyme over wide pH and temperature ranges suggests that protease may play an important role in the growth and development of *Botrytis*. Consequently, proteases were important determinants of pathogenicity *(30,31)*.

The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains from food and blood. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergent are among the major prerequisites for the use of protease in detergents. In this context, the results obtained in the present study indicate the scope for utilizing *B. cinerea* for extracellular protease production through fermentation.

Conclusion

The use of alkaline protease from *B. cinerea* as an additive in industrial applications such as the detergent industry is desirable. Prot 1 was found to be functionally stable over a wide range of pH values and temperatures and also was compatible with surfactants, oxidants, and some commercial detergent additives. It would be important to test Prot 1 protease in complete biodetergent systems. Our preliminary experiments show some interesting results in the detergent catalyst assay, such as enzyme stability and activity.

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References

1. Mala, B. R., Aparna, M. T., Mohini, S. G., and Vasanti, V. D. (1998), *Microbiol. Mol. Biol. Rev.* **62,** 597–635.
2. Van Tilburg, R., Hounink, E. H., and Vander Meer, R. R. (1984), in *Innovation in Biotechnology*, Elsevier, Amsterdam, pp. 31–51.
3. Godfrey, T. and West, S. (1996), in *Industrial Enzymology,* 2nd ed., Godfrey, T. and West, S., eds., Macmillan, London, pp. 1–8.
4. Litchfield, T. D. and Prescot, J. M. (1970), *Can. J. Microbiol.* **99,** 406–413.
5. Dalhe, H. K. (1971), *Acta Pathol. Microbiol. Scand. Sect.* B **79,** 739–746.
6. Latha, K., Weiguo, Z., Nathalie, C., Heidi, Y. S., and Ming Z. (2005), *Mol. Cell. Biol.* **25,** 1737–1748.

7. Horikoshi, K. and Akiba T. (1982), in *Alkalophilic Microorganisms: A New Microbial World,* Springer-Verlag, New York: pp. 126–129.
8. Hadey, B. S. (1960), *Annu. Rev. Bio-Chem.* **29,** 459–473.
9. Ricardo, A. B., Milton, H. G. A., Roberta, D. R., and Ieso, M. C. (2002), *J. Biosci. Bioeng.* **94 (4),** 304–308.
10. Horikoshi, K. (1971), *Agric. Biol. Chem.* **35,** 1407–1414.
11. Margesin, R., Palma, N., Knauseder, F., and Schinner, F. (1992), *J. Biotechnol.* **24,** 203–206.
12. Monika, P., Rathindra, M. B., and Claudia, K. B. (2005), *Appl. Biochem. Biotechnol.* **127 (3),** 143–155.
13. Schimangaki, H., Takeuchi, K., Nishimo, T., Ohdera, M., Kudo, T., Ohba, K., and Irie, M. (1991), *Agric. Biol. Chem.* **55,** 2251–2258.
14. Segers, M., Butt, T. M., Kerry, B. R., and Peberdy, J. F. (1994), *Microbiology* **140,** 2715–2723.
15. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 241–254.
16. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
17. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995), in *Short Protocols in Molecular Biology,* 3rd ed., John Wiley & Sons, New York, pp. 10–38.
18. Schmidt, T. M., Bleakley, B., and Nealson, K. H. (1988), *Appl. Environ. Microbiol.* **54,** 2793–2797.
19. Bae, M. and Park, P. Y. (1989), *J. Appl. Microbiol. Bioeng.* **17,** 545–551.
20. Beg, Q. K. and Gupta, R. (2003), *Enzyme Microb. Technol.* **3,** 294–304.
21. Barindra, S., Debashish, G., Malay, S., and Joydeep, M. (2006), *Process Biochem.* **41,** 208–215.
22. Alam, S. I., Dube, S., Reddy, G. S. N., Battacharya, B. K., Shivaji, S., and Singh, L. (2005), *Enzyme Microb. Technol.* **36,** 824–831.
23. Monica, F., Miguel, M., and Fidel, T. (2000), *Meat Sci.* **56,** 247–254.
24. Rashbehari, T., Binita, S., and Rinutu, B. (2003), *Process Biochem.* **38,** 1553–1558.
25. Horikoshi, K. (1983), in *Microbial Enzymes and Biotechnology,* 2nd ed., Elsevier Applied Science, St. Louis, MO, pp. 275–294.
26. Kumar, C. G., Tiwari, M. P., and Jany, R. D. (1999), *Process Biochem.* **34,** 441–449.
27. Banerjee, U. C., Rajesh, K. S., Azmi, W., and Raman S. (1999), *Process Biochem.* **35,** 213–219.
28. Li, D., Yang, Y., and Shen, C. (1997), *Mycol. Res.* **101,** 18–22.
29. Dobinson, K. F., Lecomte, N., and Lazarovits, G. (1997), *Can. J. Microbiol.* **43,** 227–233.
30. Tunlid, A. and Jansson, S. (1991), *Appl. Environ. Microbiol.* **57,** 2868–2872.
31. Yang, J. K., Huang, X. W., Tian, B. Y., Wang, M., Niu, Q. H., and Zhang, K. Q. (2005), *Biotechnol. Lett.* **27,** 1123–1128.