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Purification and Characterization of Two Thermostable Proteases from the Thermophilic Fungus *Chaetomium thermophilum*

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Abstract Thermostable protease is very effective to improve the industrial processes in many fields. Two thermostable extracellular proteases from the culture supernatant of the thermophilic fungus *Chaetomium thermophilum* were purified to homogeneity by fractional ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose, and Phenyl-Sepharose hydrophobic interaction chromatography. By SDS-PAGE, the molecular mass of the two purified enzymes was estimated to be 33 kDa and 63 kDa, respectively. The two proteases were found to be inhibited by PMSF, but not by iodoacetamide and EDTA. The 33 kDa protease (PRO33) exhibited maximal activity at pH 10.0 and the 63 kDa protease (PRO63) at pH 5.0. The optimum temperature for the two proteases was 65°C. The PRO33 had a K_m value of 6.6 mM and a V_{max} value of 10.31 μmol/l/min, and PRO63 17.6 mM and 9.08 μmol/l/min, with casein as substrate. They were thermostable at 60°C. The protease activity of PRO33 and PRO63 remained at 67.2% and 17.31%, respectively, after incubation at 70°C for 1 h. The thermal stability of the two enzymes was significantly enhanced by Ca^{2+}. The residual activity of PRO33 and PRO63 at 70°C after 60 min was approximately 88.59% and 39.2%, respectively, when kept in the buffer containing Ca^{2+}. These properties make them applicable for many biotechnological purposes.

Keywords: Thermophile *Chaetomium thermophilum*, characterization, purification, thermostable protease

Protease is an important group of enzymes in both the physiological and commercial fields, which has found a wide range of industrial applications in the food processing, tannery, laundry detergent, feather digestion, and other chemical or pharmaceutical industries [4, 22]. Microbial proteases dominate commercial applications nowadays [37, 42]. A major requirement for commercialization of enzymes is thermal stability, because thermal denaturation is a common cause of enzyme inactivation [23].

In recent years, there has been increasing interest in proteases from thermophiles, which were expected to produce thermostable proteases. Most of the researches have been on purification and characterization of proteases from thermophilic bacteria of the genera *Bacillus* [34], *Sulfolobus* [3], *Pyrococcus* [6], *Thermoanaerobacter* [39], *Fervidobacterium* [32], and *Geobacillus* [5], and thermophilic fungi of the genera *Thermomyces* [20], *Scytalidium* [15], *Paecelomyces*, and *Aspergillus* [2]. In addition, some protease genes have been cloned from thermophilic bacteria such as *Fervidobacterium pennivorans* [26] and *Thermoanaerobacter yonseiensis* [18].

Although the thermophilic *Chaetomium thermophilum* has shown the ability to produce proteases [35], little work has been carried out on the purification and characterization of proteases from *C. thermophilum*. In this paper, we report the purification and partial characterization of two thermostable extracellular proteases from the thermophilic fungus *C. thermophilum*.

Materials and Methods

Chemicals

DEAE-Sepharose and Phenyl-Sepharose were from Pharmacia (Uppsala, Sweden). Gelatin, iodoacetamide, acrylamide, bisacrylamide, bromophenol blue, Tris, glycine, and tyrosine were from Sigma. PMSF, EDTA, Coomassie brilliant R_{250}, SDS, TEMED, Triton X-100, and ammonium persulfate were from Serva. Yeast extract was from Difco. Casein was from Merck. Other reagents of analytic grade were obtained from Beijing Chemical Reagent Corporation (Beijing, China).

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Organism and Culture Conditions

Chaetomium thermophilum was isolated from compost, dung, and soil collected in Beijing, China, and identified [28]. For the enzyme production, actively growing fungal mycelium was transferred from a potato dextrose agar (PDA) plate to a 250-ml Erlenmeyer flask containing 50 ml of growth medium which was modified from Li *et al.* [27] containing the following (g/l): casein, 40.0 g; glucose, 4.0 g; yeast extract, 4.0 g; $K_2HPO_4 \cdot 3H_2O$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; dissolved in distilled and tap water (3:1). The pH was adjusted to 7.4 with 1 mol/l NaOH. The incubation was carried out at 50°C for 7 days and 120 rpm in a rotary shaker. The culture fluid was filtered and centrifuged at 8,000 ×*g* for 15 min at 4°C, and the supernatant was used as a crude enzyme preparation.

Enzyme Assays

The protease activity was determined by Rick's method with some modifications [38]. The enzyme (0.1 ml, 1 μg of protein) was added to 0.2 ml of 0.5% casein in 0.2 M Tris-HCl (pH 9.0) or 0.2 M CH_3COOH/CH_3COONa (pH 5.0) buffer and incubated at 60°C for 30 min. Then, 0.2 ml of 10% TCA (trichloroacetic acid) was added, and after staying at room temperature for 30 min, the solution was filtered. To 0.5 ml of filtrate, 2.5 ml of water was added, and the optical density (OD) at 280 nm was determined spectrophotometrically in UV-160A (Shimadzu). The blank used was prepared by adding 10% TCA to the enzyme before the addition of casein. One unit of protease activity was defined as the amount of enzyme producing 1 μg tyrosine per min under assay conditions.

Purification

All the procedures of the protease purification were carried out at 4°C. The following buffers were used: buffer A, 50 mM Tris-HCl (pH 8.0); buffer B, buffer A containing 50% saturated ammonium sulfate. (1) Fractional ammonium sulfate precipitation: solid ammonium sulfate was added to the culture filtrate to 90% saturation. After 12 h, the resulting precipitate was collected by centrifugation at 8,000 ×*g* for 15 min, dissolved in buffer A, and dialyzed in a dialysis sack overnight against three changes of the same buffer. Insoluble material was removed by centrifugation at 10,000 ×*g* for 10 min. (2) Ion-exchange chromatography on a DEAE-Sepharose column (1×20 cm) equilibrated with buffer A. After the column had been washed with five column volumes of buffer A, a 200-ml linear gradient of NaCl (0–0.3 M in buffer A) was applied at a flow rate of 40 ml/h. The volume of the fractions was 4 ml. Fractions with protease activity were pooled. (3) Phenyl-Sepharose hydrophobic interaction chromatography: the sample from the DEAE column with 50% saturated ammonium sulfate added was applied to a Phenyl-Sepharose column (1×20 cm) previously equilibrated with buffer B. After the column had been washed with five column volumes of buffer B, protease was eluted with a 120-ml linear gradient of ammonium sulfate from 50% to 0% saturation at a flow rate of 40 ml/h. The volume of the fractions was 3 ml. Fractions with protease activity were pooled for determination of purity and properties.

Electrophoretic Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the purity of the enzyme, on a vertical slab gel according to Laemmli [24]. The gel system included a resolving gel (12% acrylamide) and a stacking gel (3%). The protein was stained with 0.2% Coomassie brilliant blue R250 in 7% (v/v) acetic acid in 50% (v/v) methanol solution. Destaining was carried out with 7% acetic acid in 50% methanol. The protease activity was detected by the method of active staining described by Li *et al.* [27]. The slab gel with 1% gelatin after electrophoresis was washed in 50 mM Tris-HCl (pH 8.0) or CH_3COOH/CH_3COONa (pH 5.0), containing 5% (v/v) Triton X-100, twice for 15 min at 4°C, followed by washing in the same buffer without Triton X-100 for 15 min to remove SDS. The gel was then incubated in 50 mM Tris-HCl (pH 8.0) or CH_3COOH/CH_3COONa (pH 5.0) for 12 h at 60°C to allow the degradation of the gelatin, and stained and destained in the same solution with SDS-PAGE. The activity band was observed as a clear colorless area depleted of gelatin from the gel against the blue background.

Determination of Molecular Weight

The molecular weight of protease was determined by SDS-PAGE, which was carried out as mentioned above; the standard protein markers used were rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa), and hen egg-white lysozyme (14.4 kDa).

Enzyme Inhibition Studies

The protease inhibitors iodoacetamide, ethylenediaminetetraacetic acid (EDTA), and phenylmethanesulfonyl fluoride (PMSF) were used to determine the effects on protease activity [27].

Optimum pH and pH Stability

The optimum reaction pH of purified protease was measured under different pH conditions. The buffers of HCl-KCl (pH 3.0–4.0), CH_3COOH-CH_3COONa (pH 4.0–6.0), NaH_2PO_4-Na_2HPO_4 (pH 6.0–7.0), Tris-HCl (pH 7.0–10.0), and Na_2HPO_4-NaOH (pH 10.0–13.0) at 0.2 M were used. Activity was estimated as a percentage of the maximum. pH stability was investigated by measuring the residual activity of the enzyme after it had been kept for 1 h in various pH conditions at room temperature, then adjusting

the pH to 5.0 or 9.0, and the residual protease activities were tested under standard conditions. The experiment was conducted in triplicate.

Optimum Temperature and Thermal Stability

The optimum temperature was measured in 50 mM Tris-HCl (pH 8.0) or CH_3COOH-CH_3COONa (pH 5.0) at various temperatures from 20–90°C. The activity was estimated as a percentage of the maximum. The thermal stability of protease was examined in the range of 50–90°C, where the protease was incubated in 0.2 M Tris-HCl (pH 9.0) or 0.2 M CH_3COOH-CH_3COONa (pH 5.0) buffer with or without the presence of 1 mM Ca^{2+}, and samples were removed at fixed time intervals and allowed to cool on ice before residual activities were determined under standard conditions. The experiment was conducted in triplicate.

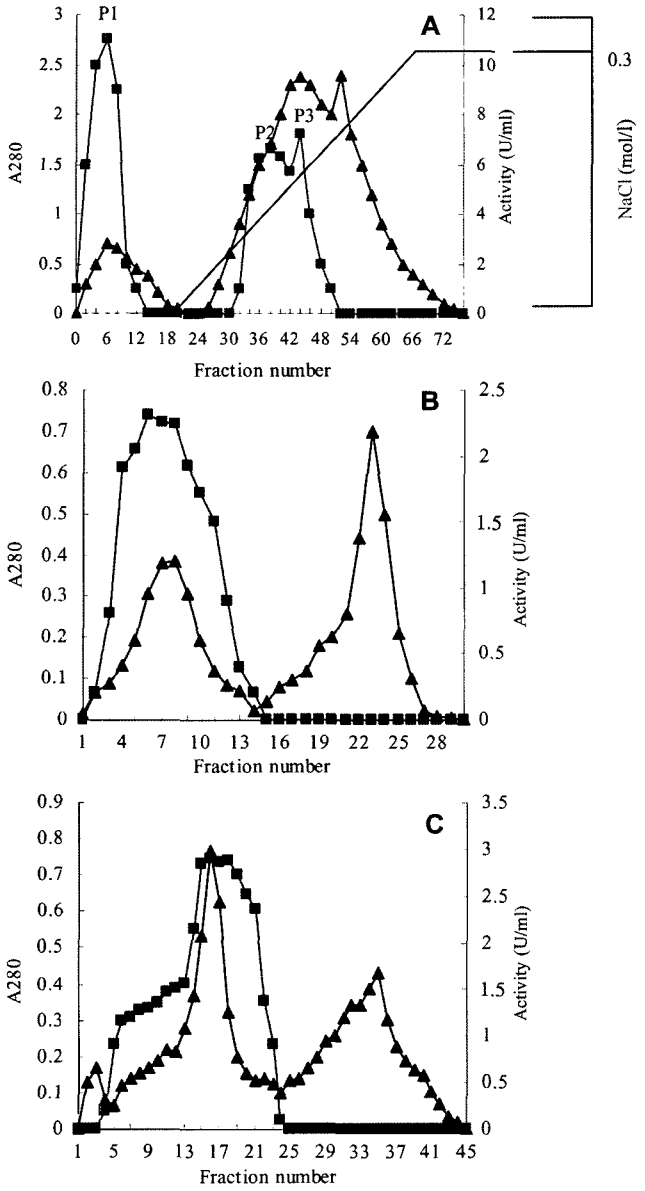

Fig. 1. Purification of the serine protease from *C. thermophilum*. **A.** Chromatography of the protease fraction obtained by ammonium sulfate precipitation on a DEAE-Sepharose column. The Peak 1 (P1) fraction (fraction nos. 2–12), Peak 2 (P2) fraction (nos. 31–42), and Peak 3 (P3) fraction (nos. 43–50) were collected separately. **B.** P1 on Phenyl Sepharose column; fraction nos. 6–9 had been purified as a 32 kDa protein. **C.** P2 on Phenyl Sepharose column; fraction nos. 19–22 had been purified as a 63 kDa protein. Symbols: (▲) protein, (■) protease activity.

Kinetic Parameters

K_m and V_{max} values of the purified enzyme were estimated with 0.15 to 2.00 mM casin as substrate, and were calculated graphically from Lineweaver-Burk plots.

Results

Purification of the Enzymes

The proteases from the culture fluid of *C. thermophilum* after fractional ammonium sulfate precipitation were mainly separated into three fractions by the DEAE-Sepharose column (Figs. 1A–C); one was a large peak of protease activity (Peak 1) (the non-adsorbed fraction) and the other two were smaller peasks (Peak 2 and Peak 3) (the adsorbed fractions), then Peak 1 and Peak 2 were applied to Phenyl-Sepharose hydrophobic interaction chromatography respectively and purified 20 and 19.3-fold with a yield of 3.4% and 4.0%, respectively. A summary of the purification results was given in Table 1. Both of the two purified enzyme showed marked protease activity.

Molecular Weight

Electrophoresis of the two enzymes on SDS-PAGE gave a single band with a molecular weight of 33 kDa (named PRO33) and 63 kDa (named PRO63), respectively (Fig. 2). The mobility of the active protease band determined by activity staining coincided with the single protein band stained by Coomassie brilliant blue. The molecular weight of the purified enzyme was also estimated to be 32.2 kDa and 62.3 kDa by gel filtration on Sephacryl S-100 (data not shown), with standard protein markers from Pharmacia, which suggested that the enzyme might be a monomeric protein.

Effects of Inhibitors

The enzyme activity of PRO33 and PRO63 towards casein was inhibited by PMSF, a well-known inhibitor of serine protease, where almost 25% and 20% of the activity was lost at 2 mM, respectively, but not inhibited by iodoacetamide and EDTA (Table 2). Hence, the proteases examined from *C. thermophilum* may be classified as serine protease [25].

Effects and Stability of pH

The optimum pH was 10.0 and 5.0 for purified PRO33 and PRO63, respectively (Fig. 3). The two proteases

Table 1. Purification of protease from *C. thermophilum.*

Purification steps	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	970	577	19,748	34.2	100	1.00
90% $(NH_4)_2SO_4$	42.0	68.0	11,016	162	55.8	4.74
DEAE-Sepharose						
Peak 1	44.0	7.04	2,875	408.4	14.6	11.9
Peak 2	50.0	9.18	3,350	364.9	17.0	10.7
Peak 1 Phenyl-Sepharose (PRO33)	12.0	0.98	667	680.6	3.4	20
Peak 2 Phenyl-Sepharose (PRO63)	10.5	1.20	795	662.5	4.0	19.3

were relatively stable in a wide range of pHs between 5.0 and 12.0 (Fig. 4), and were able to retain 70% of the full activity, after incubation in the pH 5.0 buffer for 1 h.

Effect and Stability of Temperature

The two proteases from *C. thermophilum* had their maximal activity at 65°C (Fig. 5). Both of the enzymes had high thermal stability (Figs. 6A–6D). At 50 and 60°C the two proteases PRO33 and PRO63 were stable. The protease activity of PRO33 and PRO63 remained at 67.2% and 17.31%, respectively, after 1 h incubation at 70°C. The thermal stability of purified PRO33 and PRO63 was significantly increased by the addition of 1 mM Ca^{2+}. The residual activity of PRO33 and PRO63 after incubation at 70°C for 1 h was approximately 88.59% and 39.2%, respectively, when kept in the buffer containing 1 mM Ca^{2+}.

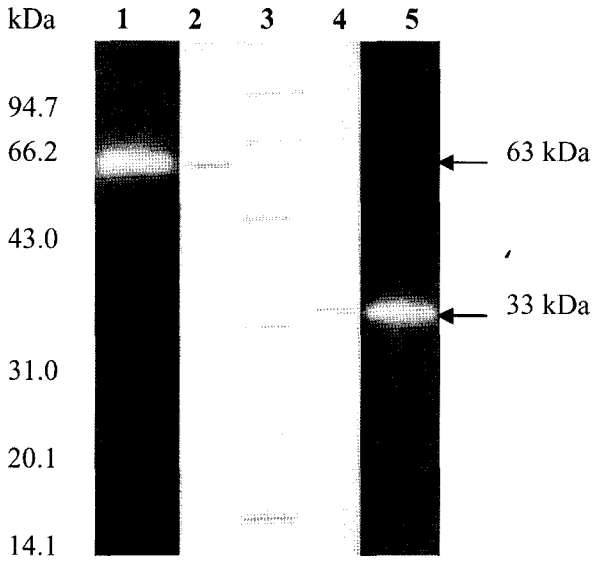

Fig. 2. SDS-PAGE pattern of the purified protease from *C. thermophilum.*
The proteases were visualized by Coomassie brilliant blue staining (lanes 2, 4) and activity staining (lanes 1, 5). Standard proteins were visualized by Coomassie brilliant blue staining (lane 3).

Kinetic Measurements

Using casein as substrate, the PRO32 had a K_m value of 6.6 mM and a V_{max} value of 10.31 μmol/l/min, and PRO63 had a K_m value of 17.6 mM and a V_{max} value of 9.08 μmol/l/min, similar to the pH and temperature values of proteases from other thermophilic fungi [5, 14, 20].

DISCUSSION

In this paper, two serine proteases from *C. thermophilum* were purified to homogeneity. The two enzymes exhibited a molecular mass of 33 kDa and 63 kDa, respectivily. Because of the difference in molecular mass of the two proteases from *C. thermophilum*, they may be encoded by different genes. As far as we know, proteases of at least five species of thermophilic fungi, *M. pulchella*, *T. thermophila*, *P. duponti*, *H. lanuginose*, and *C. thermophilum*, were purified [16, 17, 21, 35, 40]. Shenolikar and Stevenson [40] purified a thiol protease from *T. lanuginosus.* Its molecular mass is 23.7 kDa. Hasnain *et al.* [17] purified a protease from *T. lanuginosus.* The proteinase is a thiol-containing serine proteinase with a molecular mass of 38 kDa. Partial characteristics of proteases from these fungi are listed in Table 3.

Proteases are a complex group of enzymes varying greatly in their physicochemical and catalytic properties. On the basis of catalytic mechanism, proteases are generally classified into serine proteases, cysteine protease, aspartic proteases, and metalloproteases, determined indirectly through reactivity towards inhibitors of particular amino acid residues

Table 2. Effect of inhibitors and chemical reagents on the activity of PRO33 and PRO63.

Reagent	Concentration (mM)	Residual activity (%)	
		PRO33	PRO63
No inhibitor	0	100	100
EDTA	30	101±0.13	94±2.45
PMSF	0.5	87±0.64	92±0.73
	2	76±1.83	82±0.94
Iodoacetamide	0.5	104±2.66	103±0.56

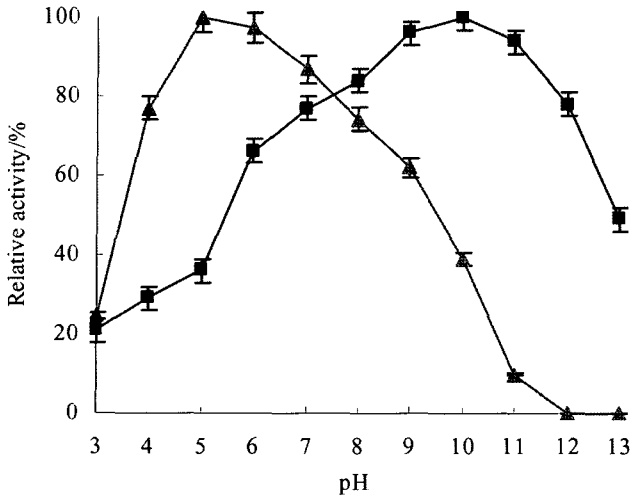

Fig. 3. Effect of pH on the activity of the purified protease from *C. thermophilum*.
PRO33 exhibited maximal activity at pH 10.0 and the PRO63 at pH 5.0. (■) PRO33, (▲) PRO63.

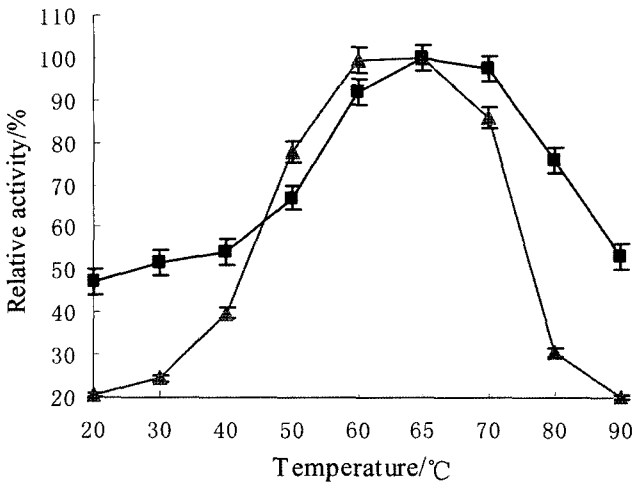

Fig. 5. Effect of temperature on the activity of the purified protease from *C. thermophilum*.
The optimum temperature for the two proteases was 65°C. (■) PRO33, (▲) PRO63.

in the active site region [33]. *C. thermophilum* proteases PRO33 and PRO63 were both inhibited by the serine inhibitor PMSF, but not by EDTA and thiol reagents such as iodoacetamide, which was specific for true cysteine proteases. Therefore, the two proteases were classified as serine proteases. Proteases are also classified on the basis of their optimum pH of activity (*e.g.*, acidic, neutral, or alkaline protease). The two proteases from *C. thermophilum*, PRO33 and PRO63, should belong to alkaline and neutral proteases, respectively.

The two proteases from *C. thermophilum* described in this paper displayed a high thermal stability as compared with those of other proteases in fungi. The two proteases were proven to be some of the most thermostable proteases having been isolated so far from fungi [14, 27, 30], though not as much as those exhibited by bacteria such as *Bacillus amyloliquefaciens* [1], *Desulfurococcus mucosus* [8, 9], *Sulfolobus acidocaldarius* [29], *Pyrococcus furiosus* [6], and *Sulfolobus solfataricus* [3]. On the one hand, the resistance of *C. thermophilum* protease to heat inactivation has probably evolved in response to high temperature environment; on the other hand, the thermal stability characteristic of the protease from *C. thermophilum* makes the enzyme suitable for use in industry because of its tolerance to high temperature.

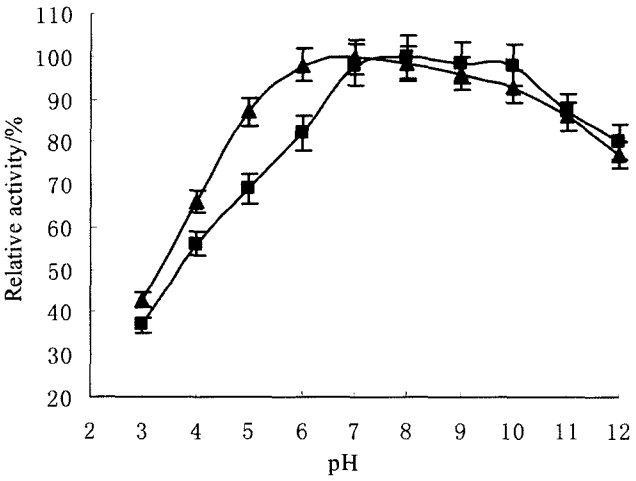

Fig. 4. pH stability of the purified proteases from *C. thermophilum*.
The two enzymes were relatively stable between pH 5.0 and 12.0. (■) PRO33, (▲) PRO63.

The thermal stability of the proteases from *C. thermophilum* was significantly increased by Ca^{2+}, similar to results reported for the purified protease from *M. pulchella* and the amylase from *T. lanuginosus* [31, 36], but the mechanism is unclear until now. It is reported that the neutral proteases from *Bacillus subtilis* and *B. amyloliquefaciens* require Ca^{2+}-cations, which are bound by four residues: Asp138, Asp185, Glu190, and Asp197. These sites are conserved in the thermolabile as well as the thermostable proteases [19]. We suggest that Ca^{2+} is required to stabilize the protein structure and prevent unfolding at high temperatures.

More important and challenging industrial applications of proteases from thermophilic fungi are in many fields. One most important acid protease, named rennin, from *Mucor miehei* is commonly used as a chymosin substitute in cheese making. This enzyme has a high ratio of MCA/PA (milk clotting activity/proteolytic activity) [12, 41]. The acid protease from *P. duponti* K1014 was capable of hydrolyzing various proteins at high temperature and converting trypsinogen into trypsin at an acidic pH range,

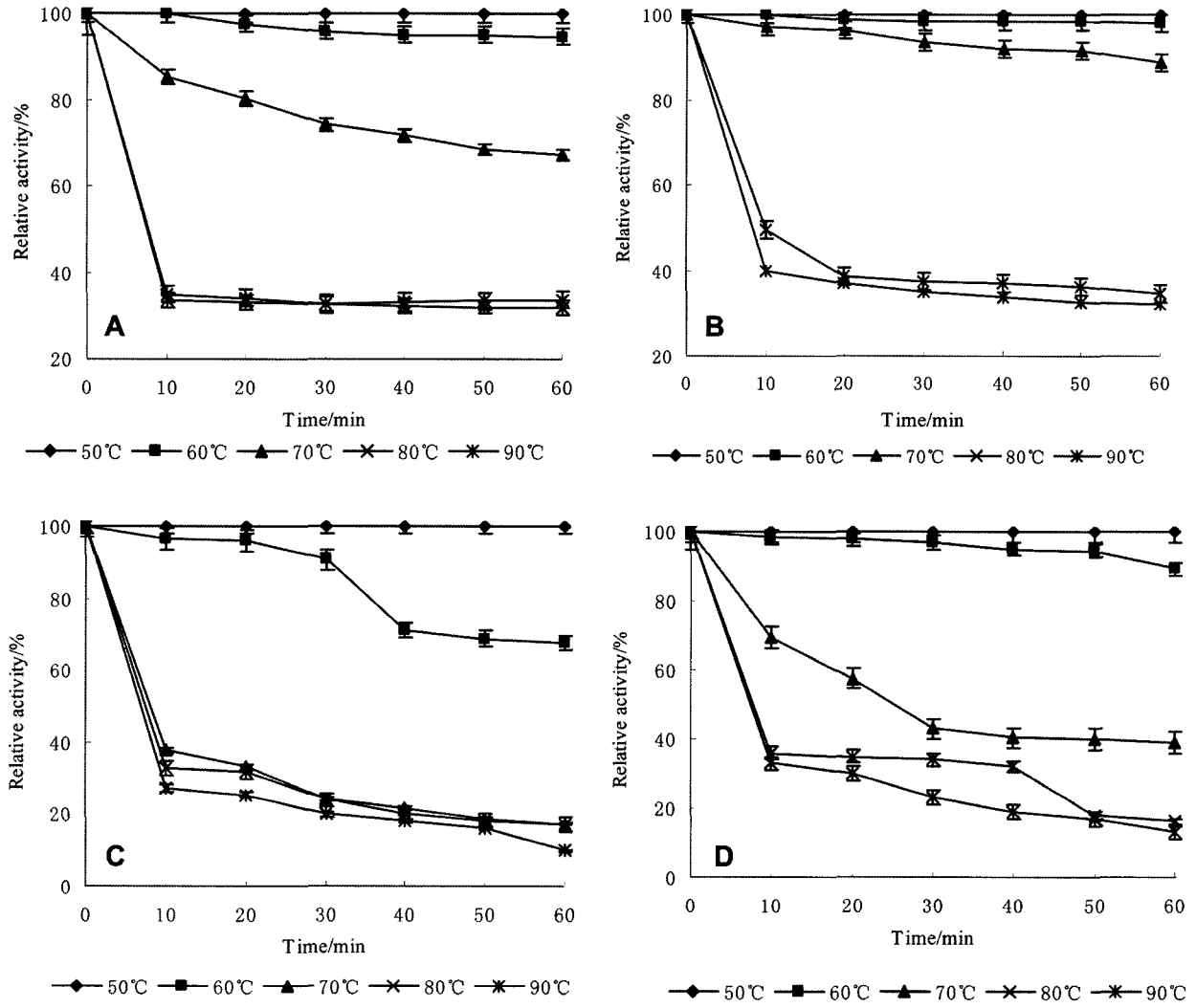

Fig. 6. Kinetics of thermal stability of the purified PRO33 from *C. thermophilum* without (**A**) and with the presence of Ca^{2+} (**B**), and of PRO63 without (**C**) and with the presence of Ca^{2+} (**D**).
From the comparison, we can see that the thermal stability of the two enzymes could be enhanced by the addition of 1 mM Ca^{2+}.

Table 3. Partial characteristics of proteases from some thermophilic fungi.

Organism	Name	Optimum pH for activity	Optimum temperature for activity	pH stability	Thermostability	Inhibitor sensitivity	Molecular mass	Reference
Malbranchea pulchella var. *sulfurea*	Alkaline protease	8.5	45°C	6.0–9.5	Stable at 50°C for 1 h	DFP PMSF	33 kDa	[35]
Torula thermophila	Alkaline protease	8.0	70°C	6.0–8.0	60°C	DFP	-	[21]
Penicillium duponti K-1014	Acid protease	2.5	30°C	2.2–6.0	60°C	Sodium lauryl sulfate $KMnO_4$ N-bromosuccinate	41 kDa	[16]
Humicola lanuginosa	Thiol protease	8.0	30°C	-	-	pClHg-Bzo Hg^+	23.7 kDa	[40]
C. thermophilum PRO33	Serine protease	10.0	65°C	7.0–10.0	60°C	PMSF	33 kDa	
C. thermophilum PRO63	Serine protease	5.0	65°C	5.0–10.0	60°C	PMSF	63 kDa	

which could reduce the probability of putrefaction during incubation. In the detergents trade, if the proteinases included in the detergent are thermostable and alkaline, just like those from thermophilic fungi, the washing can be performed at high temperature and high pH. Another example is meat tenderization with protease, which is done primarily to improve its quality by degrading the connective tissue collagen and elastin. Sine the major enzymic action occurs at 40–60°C, thermostable proteases obtained from thermophilic fungi may be best suited for this purpose. The enzyme from thermophilic *Rhizomucor miehei* has proven to be useful when limited proteolytic activity of myosin is desired, and it can also be used in the process of cheese manufacture [10, 13]. According to the characteristics of *C. thermophilum* proteases, it appears that the proteases may be of potential value in industry. It is necessary to make further studies on the proteases of *C. thermophilum*. Cloning and sequencing of the protease genes are in progress in our laboratory.

Acknowledgments

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