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A novel alkaline protease from wild edible mushroom *Termitomyces albuminosus*

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A protease with a molecular mass of 30 kDa and the N-terminal sequence of GLQTNAPWGLARSS, was isolated from fresh fruiting bodies of the wild edible mushroom *Termitomyces albuminosus*. The purification protocol included ion exchange chromatography on DEAE-cellulose, Q-Sepharose, SP-Sepharose and FPLC-gel filtration on Superdex 75. The protein was unadsorbed on DEAE-cellulose and Q-Sepharose, but adsorbed on SP-Sepharose. The optimal pH and temperature of the purified enzyme were 10.6 and 60°C, respectively. The enzyme was stable in the presence of 2% (v/v) Tween 80 and 4 M urea. More than 80% of the enzyme activity was retained in 2% (v/v) Triton X 100, 54% in 10 mM EDTA and 31% in 2% (w/v) SDS. The enzyme was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), but not inhibited by dithiothreitol (DTT), pepstatin or lima bean trypsin inhibitor suggesting that it was a serine protease but not a trypsin-like one. The protease was inhibited by Hg^{2+}, Cu^{2+}, and Fe^{3+} ions. The K_m and V_{max} values of the purified enzyme for casein were 8.26 mg·ml^{-1} and 0.668 mg·ml^{-1}·min^{-1}, respectively.

Keywords: alkaline protease, mushroom, *Termitomyces albuminosus*, purification

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INTRODUCTION

Proteases are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. They are degradative enzymes which catalyze the hydrolysis of proteins. Proteases not only play an important role in the cellular metabolic processes (Tremacoldi *et al.*, 2007), but also have many applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, the chemical industry, as well as waste treatment (Ma *et al.*, 2007). The serine proteases are characterized by the presence of a serine group in their active site. They are vital to the organisms and the most important group of commercial enzymes. Alkaline serine proteases are active and stable in the alkaline pH and represent the largest subgroup of serine proteases. The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9 (Rao *et al.*, 1998). The properties of alkaline serine proteases make them suitable for use in the detergent industry. Presently, some proteases from mushroom have been purified (Terashita *et al.*, 1985; Yoshimoto *et al.*, 1988; Kobayashi *et al.*, 1989; Sattar *et al.*, 1990; Shaginian *et al.*, 1990; Burton *et al.*, 1994; Nonaka *et al.*, 1995; 1997; Shin & Choi, 1998; Healy *et al.*, 1999; Kim & Kim, 1999, 2001; Wang & Ng, 2001; Cui *et al.*, 2007; Zhang *et al.*, 2010a, 2010b).

Termitomyces albuminosus, a well-known symbiotic wild mushroom occurred on the surface of termite nests, also called chicken julienne mushroom or termite mushroom, is a long-stemmed mushroom grown in Yunnan province (China). The termites cultivate this fungus in nests as their food. Enzymes as peroxidase (Johjima *et al.*, 2003; Liers *et al.*, 2010) and laccase (Bose *et al.*, 2007) have been isolated from *T. albuminosus*. In this work, we describe the isolation and general properties of a novel alkaline serine protease from *T. albuminous*, and compared its characteristics to other mushroom serine protease.

MATERIALS AND METHODS

Materials. Fresh fruiting bodies of the mushroom *T. albuminosus* were purchased from Kunming City, Yunnan Province (China). Superdex 75 HR 10/30, Q-Sepharose, SP-Sepharose and AKTA Purifier were from GE Healthcare. All other chemicals were obtained from Sigma Chemical Co., USA.

Isolation procedure. Fresh fruiting bodies of the mushroom were homogenized in 0.15 M NaCl at 4°C overnight. The supernatant was centrifuged at 8 000 × *g* for 15 min before $(NH_4)_2SO_4$ was added to 80% saturation. The precipitate was collected by centrifugation at 8 000 × *g* for 15 min again. Then the precipitate was dissolved and dialyzed to remove $(NH_4)_2SO_4$ before applying to a column (2.5 cm × 20 cm) of DEAE-cellulose, which had previously been equilibrated and then eluted with 10 mM NH_4HCO_3 buffer (pH 9.4). After collecting the unadsorbed peak (D1) containing strong protease activity, two adsorbed peaks, D2 and D3, were eluted with 100 mM and 1 M NaCl in the starting buffer, respectively. Fraction D1 with protease activity was applied directly on a column (1.5 cm × 20 cm) of Q-Sepharose in 10 mM NH_4HCO_3 buffer (pH 9.4). Unbound proteins (fraction Q1) were eluted with the starting buffer while bound proteins (fraction Q2) were desorbed with 1 M NaCl in the starting buffer. Following dialysis, the active fraction Q1 was subject onto

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride

a SP-Sepharose chromatography column (1.5 cm × 10 cm) in pH 7.5 phosphate buffer. Unadsorbed proteins (fraction S1) were eluted with the same buffer while adsorbed proteins were eluted with a linear concentration gradient of NaCl (0–0.3 M) in the phosphate buffer to obtain adsorbed fractions S2, S3, respectively. Protease activity was detected in S2. The active fraction (S2) was subsequently chromatographed on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.15 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier. Two peaks (SU1 and SU2) were obtained. All the above purification procedures were conducted at 4 °C. The second peak (SU2) with protease activity was then analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (Laemmli & Favre, 1973). The N-terminal sequence was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP1000 HPLC system (Lam *et al.*, 1998.

Assay for protease activity. The protease activity was determined at 37 °C in phosphate buffer (pH 7.0, 50 mM) by a method of Cui *et al.* (2007). One unit of protease activity is defined as an absorbance increase of 0.001 at 280 nm in the sample per ml reaction mixture per minute under the experimental conditions. Protein concentrations were determined according to the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Effect of pH and temperature on enzyme activity. In order to determine the effect of pH values on the purified enzyme, the protease activity was measured at different pH values. The pH values of the reaction mixture were adjusted using one of the following buffers: citric acid/sodium citrate (pH 4.0–7.0, 50 mM), barbital sodium/HCl (pH 7.0–9.6, 50 mM), $NaHCO_3$/NaOH (pH 9.6–11.0, 50 mM), KCl/NaOH (pH 12.0–13.0, 50 mM). The purified protease (10 µl) was incubated at 37 °C for 15 min with 90 µl 1% casein solution and 100 µl assay buffer as described above. In the temperature effect assay, the activity of the protease was determined by incubating the reaction mixture at different temperature ranging from 20 °C to 80 °C for 15 min with phosphate buffer (pH 7.0, 50 mM).

Effect of metal ions on protease activity. Purified protease solutions were pre-incubated at 4 °C for 1 h with different metal ion solutions (phosphate buffer, pH 7.0, 50 mM) including Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+} and Fe^{3+}, at a final concentration of 1 mM or 5 mM. The residual activity was assayed as described above.

Enzyme kinetics. Casein at different concentrations (2%, 1%, 0.5%, 0.2%, 0.1%, 0.08%, 0.06%, 0.04%, 0.02%) was used as a substrate. The K_m and V_{max} of the enzyme were calculated based on a Lineweaver-Burk plot constructed by plotting the reciprocal of substrate concentration on the X-axis, and the reciprocal of the enzyme reaction velocity on the Y-axis. The enzyme activity was measured at 37 °C with $NaHCO_3$/NaOH buffer (pH 10.6, 50 mM) for 15 min. The experiment was repeated twice and results were reproducible.

Effect of inhibitors and other chemical reagents on protease activity. The effect of inhibitors on the enzyme activity was determined by its pre-incubation at room temperature (about 30 °C) for 30 min, in the presence of 5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM or 10 mM EDTA, 0.2 mM pepstatin, 0.25 mM lima bean trypsin inhibitor, 1 mM dithiothreitol (DTT), 1% or 2% 2-mercaptoethanol. All the solutions were in phosphate buffer (pH 7.0, 50 mM). The enzyme activity was also measured in the presence of 1% or 2% Triton X 100, SDS and Tween 80, 4 M urea, 25 mM HCl and 25 mM NaOH. After pre-incubation, the residual enzyme activity was measured. The enzyme activity of a control sample (without any inhibitors and other chemicals) was taken as 100.

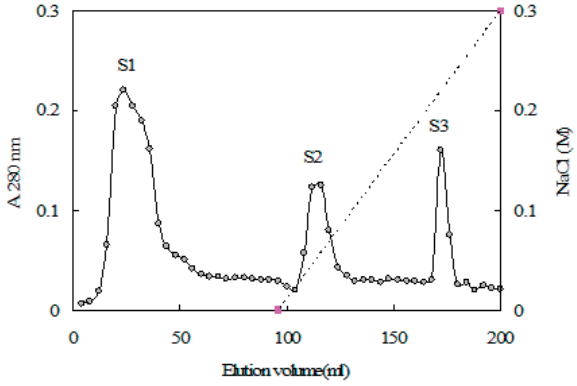

Figure 1. Anion exchange chromography of fraction Q1 on a SP-Sepharose.
Starting buffer: 10 mM phosphate buffer (pH 7.5).

RESULTS

Purification of the protease

Protease activity resided in fraction D1 of the crude extract unadsorbed on DEAE-cellulose. The adsorbed fractions D2 and D3 had weak protease activity (Table 1). Fraction D1 was resolved into an unadsorbed fraction Q1 and adsorbed fractions Q2 upon ion exchange chromatography on Q-Sepharose. The protease activity was detected mainly in the unadsorbed fraction Q1 (Table 1). Fraction Q1 was collected for further purification on a SP-Sepharose column. The activity was eluted with a linear gradient of 0–0.3 M NaCl in 10 mM phosphate buffer (pH 7.5). Three peaks (S1, S2, and S3) were obtained, but only

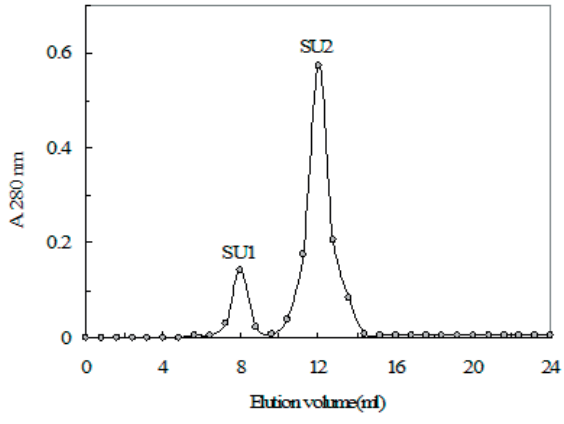

Figure 2. Gel filtration of fraction S2 on Superdex 75 HR 10/30 column

Table 1. Yields and protease activities of chromatographic fractions toward casein
Purification was from 60 g of fresh *T. albuminosus* fruiting bodies

Fraction	Total protein (mg)	Specific activity (U/mg)	Total activity ($U/10^5$)	Activity recovery (%)	Fold purification
Ammonium sulfate precipitate	**443.2**	**6293.0**	**27.9**	**100.0**	**1.0**
D1	**40.3**	**43634.5**	**17.6**	**63.1**	**6.9**
D2	18.7	9126.3	1.7	6.1	1.5
D3	93.9	3420.8	3.2	11.5	0.5
Q1	**27.3**	**53688.5**	**14.6**	**52.5**	**8.5**
Q2	10.0	3716.0	0.4	1.3	0.6
S1	7.5	0	0	0	0
S2	**6.4**	**184919.8**	**11.8**	**42.2**	**29.4**
S3	3.8	0	0	0	0
SU1	0.72	0	0	0	0
SU2	**3.45**	198260.9	**6.84**	**24.52**	**31.5**

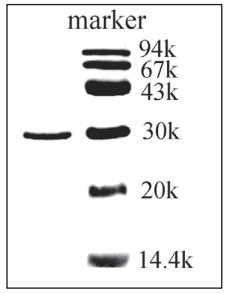

Figure 3. SDS/PAGE of *T. albuminosus* protease
Left lane: *T. albuminosus* serine protease. Right lane: Relative molecular mass standards.

S2 possessed high protease activity (Fig. 1, Table 1). Fraction S2 was fractionated into a small inactive fraction SU1 and a large active fraction SU2 upon FPLC-gel filtration on Superdex 75 (Fig. 2, Table 1). The protease appeared as a single band with a molecular mass of 30 kDa in SDS/PAGE (Fig. 3). The yields and specific protease activities at the various stages of purification are shown in Table 1. The N-terminal amino-acid sequence was GLQTNAPWGLARLSS, which manifested considerable similarity with previously isolated fungal serine proteases, especially those from *Agaricus bisporus*, *Grifola frondosa* and *Hypsizigus marmoreus* (Table 4).

Table 2. Effects of metal ions on *T. albuminosus* protease activity

Metal ions	Relative activity remaining (%)	
	1 mM	5 mM
Control	100	100
Ca^{2+}	81.9	76.3
Mg^{2+}	93.5	72.7
Mn^{2+}	86.2	78.8
Cu^{2+}	69.9	16.7
Pb^{2+}	104.7	56
Co^{2+}	85.3	56.8
Zn^{2+}	82.2	50.1
Hg^{2+}	5	0
Fe^{3+}	65.3	17.4

Biological activities of the protease

The effect of temperature and pH on the activity of the protease is illustrated in Figs. 4 and 5. The optimum pH was around 10.6, and the optimum temperature was around 60 °C. Its K_m was 8.26 mg/ml, and its V_{max} was 0.668 $mg \cdot ml^{-1} \cdot min^{1-}$. The effect of metal ions on the activity of the protease is shown in Table 2. The protease activity was inhibited strongly by Hg^{2+}, Cu^{2+}, and Fe^{3+}. Among the protease inhibitors tested, only PMSF almost completely inhibited the protease activity. The activity of the protease is unaffected by pepstatin, lima bean trypsin inhibitor, DTT and 2-mercaptoethanol. The enzyme activity was enhanced by 25 mM NaOH, and inhibited completely by 25 mM HCl. Protease activity was not affected by Tween 80 (2%, v/v) or 4 M urea under the assay

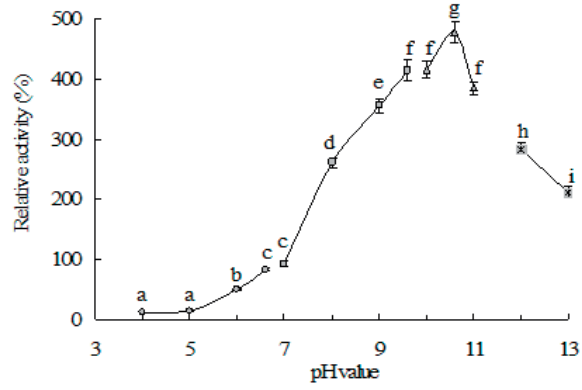

Figure 4. Effect of pH on activity of *T. albuminosus* protease
Citric acid/sodium citrate buffer (pH 4.00–7.0), barbital sodium/HCl buffer (pH 7.0–9.6), $NaHCO_3$-NaOH buffer (pH 9.6–11.0), KCl/NaOH (pH 12.0–13.0). Results represent mean ± S.D. (n = 3). Different letters (a, b, c…) next to the data points indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test. The activity at pH7.0 was taken as 100%.

Table 3. Influence of inhibitors and other chemicals on *T. albuminosus* protease activity

Chemical	Concentration	Residual activity (%)
None		100
Pepstatin A	0.2 mM	100
PMSF	1 mM	2
Lima bean trypsin inhibitor	0.25 mM	100
EDTA	1 mM	88
	5 mM	62
	10 mM	54
DTT	1 mM	96
2–Mercaptoethanol	1%	100
	2%	100
Triton X 100	1%	84
	2%	81
SDS	1%	37
	2%	31
Tween 80	1%	100
	2%	100
Urea	4 M	140
HCl	25 mM	0
NaOH	25 mM	301

condition. More than 80% of the activity was retained in 2% (v/v) Triton X 100, 54% in 10 mM EDTA and 31% in 2% (w/v) SDS (Table 3).

DISCUSSION

In this report, a protease with a molecular mass of 30 kDa was isolated from fresh fruiting bodies of the mushroom *T. albuminosus*. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11, and molecular masses between 18 and 35 kDa (Rao *et al.*, 1998). The apparent molecular mass of 30 kDa for the protease is similar to those of mushroom serine proteases from *A. bisporus* (27 kDa) (Burton *et al.*, 1993), *P. citrinopileatus* (28 kDa) (Cui *et al.*, 2007), *H. marmoreus* (28 kDa) (Zhang *et al.*, 2010b) and *Helvella lacunose* (33.5 kDa) (Zhang *et al.*, 2010a). Its N-terminal sequence is considerably homologous to the previously isolated mushroom serine proteases from *A. bisporus*, *G. frondosa*, *H. marmoreus*, and *P. citrinopileatus*, but different from that of *H. lacunosa* (Table 4).

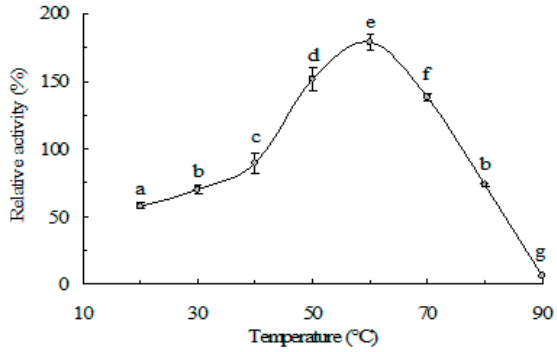

Figure 5. Effect of temperature on activity of *T. albuminosus* protease
Results represent mean ±S.D. (n = 3). Different letters (a, b, c…) next to the data points indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test. The activity at pH 7.0 was taken as 100%.

The protease showed a broad pH profile (pH 7.0–13.0) for casein hydrolysis. The highest activity appeared at pH 10.6. It is suggested that *T. albuminosus* protease might be classified into the family of alkaline proteases, which are represented mostly by serine proteases. Another prominent property of the purified protease is its relative thermostability. The optimal enzymatic activity of *T. albuminosus* protease is obtained at 60 °C, and 73% residual activity was measured at 80 °C. The optimum temperature is higher than for other fungal proteases from *A. bisporus* (35 °C) (Burton *et al.*, 1997a; 1997b), *P. citrinopileatus* (50 °C) (Cui *et al.*, 2007) and *H. marmoreus* (50 °C) (Zhang *et al.*, 2010b).

The activity of purified protease was inhibited by PMSF, but not by other inhibitors, e.g., pepstatin, lima bean trypsin inhibitor, DTT or 2-mercaptoethanol. The strong inhibition by 1 mM PMSF may be an indication that the enzyme belongs to the serine protease family. The enzyme was resistant to thiol reducing agents such as DTT (1 mM) and 2-mercaptoethanol (2%), suggesting that disulfide bonding was not involved in preserving proteolytic activity. EDTA had an inhibitory effect on the enzyme activity at 10 mM concentration, indicating that the enzyme is sensitive to EDTA. The protease activity was not affected by Tween 80 (2%, v/v) or 4 M urea under the assay condition. More than 80% of the activity was retained in 2% (v/v) Triton X 100. The enzyme was insensitive to Triton X 100, Tween 80 and the denaturing agent urea, rather stable in 50–70 °C, and had high activity at high pH. This suggested that the enzyme could have potential applications in detergent uses, such as brewing and leather industries (Dayanandan *et al.*, 2003; Ganesh *et al.*, 2008).

In summary, we have reported the purification and characterization of a novel protease from the wild mushroom *T. albuminosus*. The purified enzyme manifested a molecular mass of 30 kDa, N-terminal amino-acid se-

Table 4. Comparison of N-terminal sequence of *T. albuminosus* serine protease with those of known mushroom serine proteases

Proteinase	N-terminal sequence	Reference
Serine protease [*Termitomyces albuminosus*]	GLQTN APWGL ARLSS	This study
Serine protease [*Agaricus bisporus*]	TGQTN APWGL ARLXS	(Burton *et al.*, 1993)
Serine protease [*Grifola frondosa*]	AQTN APWGL ARISS	BLAST search
Serine protease [*Hypsizigus marmoreus*]	VTQTN APWGL ARLSQ	(Zhang *et al.*, 2010b)
Serine protease [*Helvella lacunosa*]	ANVVQ WPVPC	(Zhang *et al.*, 2010a)
Serine protease [*Pleurotus citrinopileatus*]	VCQCN APWGL	(Cui *et al.*, 2007)

quence of GLQTNAPWGLARLSS, a pH optimum of 10.6 and a temperature optimum of 60 °C. The protease was inhibited by Hg^{2+}, Cu^{2+}, and Fe^{3+} ions. The K_m and V_{max} values of the purified enzyme for casein were 8.26 $mg \cdot ml^{-1}$ and 0.668 $mg \cdot ml^{-1} \cdot min^{-1}$, respectively. Its alkaline pH optimum and sensitivity to PMSF suggested that the purified enzyme was an alkaline serine protease.

Acknowledgements

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