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Purification and characterization of a novel serine protease from the mushroom *Pholiota nameko*

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A novel serine protease, with a molecular mass of 19 kDa and the N-terminal sequence of ARTPEAPAEV, was isolated from dried fruiting bodies of the mushroom *Pholiota nameko*. The purification protocol comprised ion exchange chromatography on DEAE-cellulose, Q-Sepharose and SP-Sepharose, and gel filtration on Superdex 75. It was unadsorbed on DEAE-cellulose and Q-Sepharose but adsorbed on SP-Sepharose. It exhibited an optimum temperature at 50°C, an optimum pH at pH 8.8, a Km of 5.64 mg/mL and a Vmax of 0.98 μmol/min/mL against substrate casein. A number of metal ions inhibited the enzyme including Pb^{2+}, Mn^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+} and Al^{3+}, with the inhibition of the last two cations being the most potent. K^{+} and Mg^{2+} slightly enhanced, while Li^{+} moderately potentiated the activity of the protease. The protease was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), suggesting that it is a serine protease.

[**Key words:** Protease; Fruiting bodies; Mushroom; *Pholiota nameko*]

Protease represents one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (1). They are the proteolytic enzymes which participate in many pathological processes such as protein turnover, sporulation and conidial discharge, germination, enzyme modification, nutrition, regulation of gene expression, and part of them also are considered as important virulence factors of many pathogens, including viruses, bacteria, fungi and parasites (2,3). As well as their metabolic functions, proteases play a vital role in commercial fields, i.e., in the food, leather, detergent, pharmaceutical industries and in ecological bioremediation processes. Proteases have been isolated from different organisms including animals (4,5), plants (6–8), and microorganisms, e.g., mushrooms such as *Agaricus bisporus* (9,10), *Armillariella mellea* (11,12), *Flammulina velutipes* (13), *Grifola frondosa* (14), *Helvella lacunose* (15), *Lyophyllum cinerascens* (16), *Pleurotus eryngii* (17), *Pleurotus ostreatus* (18), *Pleurotus citrinopileatus* (2), and *Tricholoma saponaceum* (19). *A. bisporus* protease is a serine protease, proteases from *Irpex lacteus* and *P. eryngii* are aspartic proteinases, *L. cinerascens* protease is an aminopeptidase (20), *P. citrinopileatus* protease is an alkaline protease, while the rest are metalloproteases. They exhibit a range of molecular masses and different thermostability.

Regarding the mushroom *Pholiota nameko*, only information on polysaccharides and several proteins including a tyrosinase, ribonucleases, an acid phosphatase, a glucose-1-phosphatase, and a hydrophobin are available (21–24). In view of the importance of proteases and the differences in characteristics of proteases from different sources, the present investigation was undertaken to isolate and characterize a protease from the edible mushroom *P. nameko*.

MATERIALS AND METHODS

Materials Dried fruiting bodies of the edible mushroom *P. nameko* were purchased from a local supermarket. The sources of other materials and chemicals used in this work are as follows: DEAE-cellulose was from Sigma. Q-Sepharose, SP-Sepharose and Superdex 75 were obtained from GE Healthcare. Substrate casein, protease inhibitors, agarose, glycine, ammonium sulfate, metal ions, buffers and all other chemicals were from Sigma.

Isolation of protease Dried fruiting bodies of *P. nameko* (20 g) were homogenized in 0.15 M NaCl (10 mL/g) at 4°C followed by centrifugation at 8000×*g* for 25 min. Proteins in the supernatant were precipitated by 80% $(NH_4)_2SO_4$. Centrifugation at 8000×*g* for 25 min was carried out. The precipitate was collected, dissolved in water, and dialyzed extensively against distilled water to remove $(NH_4)_2SO_4$. NH_4HCO_3 buffer (pH 9.4) was added until the concentration of NH_4HCO_3 reached 10 mM. Ion exchange chromatography on a column of DEAE-cellulose (Sigma) (2.5×20 cm) in 10 mM NH_4HCO_3 buffer (pH 9.4) was carried out. After removal of unadsorbed proteins in fraction D1, adsorbed proteins were desorbed and eluted into three fractions D2, D3 and D4, by addition of 50 mM NaCl, 150 mM NaCl and 1 M NaCl, respectively, to the NH_4HCO_3 buffer. Ion exchange chromatography of fraction D1 on a column of Q-Sepharose (GE Healthcare) (1.5×20 cm) in 10 mM NH_4HCO_3, (pH 9.4), was carried out. After removal of unadsorbed proteins Q1, adsorbed proteins were desorbed and eluted into three fractions Q2, Q3 and Q4 by addition of 50 mM NaCl, 150 mM NaCl, and 1 M NaCl to the NH_4HCO_3 buffer. Fraction Q1 was next subjected to ion exchange chromatography on a column of SP-Sepharose (GE Healthcare) (1.5×15 cm) in 10 mM phosphate buffer (pH 6.2). Unadsorbed proteins were eluted in fraction SP1. Adsorbed proteins were eluted into fractions SP2 and SP3 using a gradient of 0–0.5 M NaCl. FPLC of fraction SP2 on Superdex 75 (GE Healthcare) in 0.2 M NH_4HCO_3 (pH 8.5), at a flow rate of 0.4 mL/min and with a fraction size of 0.8 mL, was used as the final purification step. The first fraction SU1 represented the purified enzyme.

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Determination of molecular mass and N-terminal sequence The active peak (SU1) was subsequently analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (25). The molecular mass of the purified protein was determined in SDS–PAGE as well as in FPLC-gel filtration as described above. N-terminal sequencing of the protein was carried out using an HP G-1000A Edman degradation unit and an HP 1000 HPLC system (2).

Assay for protease activity Proteolytic activity was assayed according to the method of Satake et al. (26). A solution of casein, which could be used in the protease assay, was freshly prepared as follows. To 0.1 g casein, 10 mL 50 mM phosphate buffer (pH 7.5) were added. Subsequently, the solution was incubated at 4°C for overnight. The precipitate was removed and the resulting solution could be used. The test sample (25 μL) was mixed with 140 μL of the above casein solution, and the reaction mixture was incubated at 37°C for 15 min. Subsequently, 600 μL 5% trichloroacetic acid (TCA) was added. The reaction mixture was allowed to stand at room temperature for 30 min, before centrifugation at 8000×*g* for 15 min. The absorbance of the supernatant was read at 280 nm against water as blank using a UV–spectrophotometer. Protease activity was expressed in units, where 1 U represented a 0.001 absorbance increase per minute in the supernatant per milliliter of reaction mixture under specified conditions (15).

Determination of optimum pH and temperature In the determination for optimum pH and temperature, a solution of casein, which was used as substrate, was freshly prepared as described above (15). The assay buffers were prepared in 50 mM NaH_2PO_4–citric acid (pH 4.0–7.0), 50 mM Tris–HCl (pH 7.0–8.6), 50 mM glycine–NaOH (pH 8.8–9.8), 50 mM $NaHCO_3$–NaOH (pH 9.8–11.0) (Buffer A–D, respectively). The purified protease (10 μL) was incubated at 37°C for 15 min with 90 μL 1% casein solution (pH 7.5) and 100 μL assay buffer as described above. The reaction was subsequently ended by addition of 600 μL 5% trichloroacetic acid (TCA). The reaction mixture was allowed to stand at room temperature for 30 min before centrifugation at 8000×*g* for 15 min. The absorbance of the supernatant was read at 280 nm against water as blank. The protease activity tested at pH 7.5 (50 mM Tris–HCl) was regarded as 100%. To determine the optimum temperature, the reaction mixture was incubated at 20–85°C for 15 min. The assay buffer was 50 mM phosphate buffer (pH 7.5). The protease activity tested at 37°C was regarded as 100%.

Assay of the enzyme thermostability In order to check thermal stability, the purified protease was incubated at 37°C and 50°C for 0, 30, 60, 90 and 120 min, respectively, cooled to 37°C and then assayed using the standard protease assay above. The protease activity tested at 37°C was regarded as 100%.

Assay of 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 substrate. The Km and Vmax of the enzyme were calculated based on the Lineweaver–Burk plot constructed by plotting the reciprocal of substrate concentration on the *x*-axis, and reciprocal of the enzyme reaction velocity on the *y*-axis (27).

Assay of mechanistic class In the determination of mechanistic class for the purified protease, the protease was exposed to protease inhibitors including ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethylether)-tetraacetic acid (EGTA), lima bean trypsin inhibitor, Pepstatin A, and phenyl methyl sulfonyl fluoride (PMSF) with different concentrations of 0.04 mM–1.0 mM for 30 min. The residual enzyme activity was measured (28).

Assay of metal ions and effects of chemical reagents The enzyme solution (10 μL) was preincubated, at 37°C for 30 min at pH 7.5, with 10 μL of different metal ions and chemical reagents at concentrations ranging from 5 mM to 200 mM (15). The protease activity was assayed as described above.

RESULTS

Isolation of *P. nameko* protease and determination of molecular mass and N-terminal sequence The unadsorbed fraction D1 from DEAE-cellulose, but not the adsorbed fractions, exhibited protease activity (Table 1). Similarly, the unadsorbed fraction Q1 was the only fraction with protease activity from the Q-Sepharose column (Table 1). Fraction Q1 was fractionated on SP-Sepharose into a large inactive unadsorbed fraction SP1, a large adsorbed fraction SP2 exhibiting protease activity eluted by 0.2 M NaCl, and a very small inactive adsorbed fraction SP3 (Fig. 1A). Fraction SP2 was resolved on Superdex 75 into a main fraction SU1 in which protease activity resided and a very small fraction SU2 (Fig. 1B). Fraction SU1, which represents purified protease, appeared as a single band with a molecular mass of 19 kDa (Fig. 2). The enzyme was purified 37.9-fold from ammonium sulfate precipitate with 31.2% yield, and 10,679 U/mg of the purified protease (Table 1). The N-terminal sequence of the purified protease was ARTPEAPAEV, which showed no similarity to purified fungal protease sequences previously reported (Table 2).

TABLE 1. Yields and protease activities of various chromatographic fractions derived from *Pholiota nameko* dried fruiting body extract (20 g).

Fraction	Yield (mg)	Specific activity (U/mg)	Total activity ($U/10^4$)	Recovery of activity (%)	Purification fold
Ammonium sulfate precipitate	1386.6	282	39.1	100	1
D1	124.1	1862	23.1	59.1	6.6
Q1	51.7	4028	20.8	53.2	14.3
SP2	16.6	8754	14.5	37.1	31.0
SU1	11.4	10679	12.2	31.2	37.9

Enzyme assay conditions: 37°C/15 min, in 0.1 M Tris–HCl, pH 7.5.

Physiochemical properties of *P. nameko* protease The purified protease demonstrated an optimum pH of 8.8 (Fig. 3A) and an optimum temperature of 50°C (Fig. 3B). Maximal activity was detected at pH 8–9. There was an abrupt fall in activity (about 60% reduction) when the pH was lowered to 7 or increased to 11. The protease activity continued to decline as the pH was further lowered until negligible activity remained at pH 4 (Fig. 3A). Maximal activity of the protease depended on a temperature of 50°C. A slight decrement in activity was observed at 55°C and 60°C. About 60% of the activity remained when the temperature was reduced to 20°C or elevated to 70°C. A further fall in activity was seen at or above 70°C (Fig. 3B). The isolated enzyme showed strong thermal stability at 37°C and its optimal temperature (50°C). When the enzyme was incubated at 37 or 50°C for 2 h, the protease activity almost maintained its primal levels (Fig. 4). From the Lineweaver–Birk-plot, Vmax and Km were estimated to be 0.98 $\mu mol \cdot min^{-1} \cdot ml^{-1}$, and 5.64 $mg \cdot mL^{-1}$, respectively (Fig. 5). The Km of *P. nameko* protease is lower than that of proteinase K, which is 29.6 $mg \cdot mL^{-1}$ in our experiment. The Vmax of proteinase K is 5.01 $\mu mol \cdot min^{-1} \cdot mL^{-1}$ higher than that of *P. nameko* protease (0.98 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) (28). The effects of various

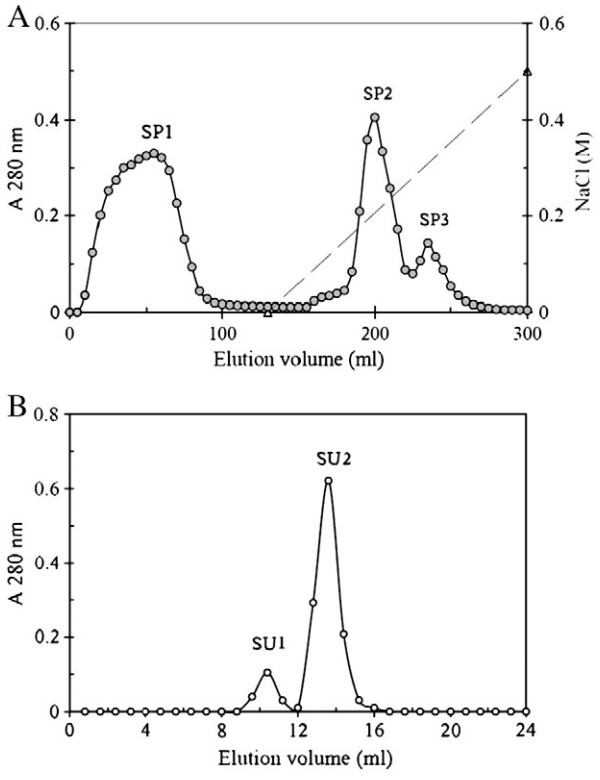

FIG. 1. Elution profiles of protease from *Pholiota nameko*. (A) Ion exchange chromatography on SP-Sepharose. Sample: Fraction of *P. nameko* fruiting body extract unadsorbed on DEAE-cellulose and Q-Sepharose. Buffer: 10 mM phosphate buffer (pH 6.2). Broken line across right half of chromatogram indicates linear NaCl concentration gradient used to desorb adsorbed proteins. (B) FPLC-gel filtration on Superdex 75.