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Helvellisin, a novel alkaline protease from the wild ascomycete mushroom *Helvella lacunosa*

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A 33.5-kDa serine protease designated as helvellisin was isolated from dried fruiting bodies of the wild ascomycete mushroom *Helvella lacunosa*. It was purified by using a procedure which entailed ion exchange chromatography on DEAE-cellulose, CM-Sepharose, Q-Sepharose, and FPLC-gel filtration on Superdex 75. The protease was characterized by unique N-terminal amino acid sequence, thermostability and pH stability. The protease exhibited a pH optimum of 11.0 and a temperature optimum of 65 °C, with about 40% activity remaining at 87 °C and pH 5 and 13. Helvellisin demonstrated a protease activity of 14600 U/mg toward casein. The K_m of the purified protease for casein was 3.81 mg/ml at pH 11.0 and 37 °C. The V_{max} was 5.35×10^{-2} mg ml^{-1} min^{-1}. It was adversely affected by phenylmethylsulfonyl fluoride, suggesting that it is serine protease. The activity of the protease was enhanced by Mg^{2+}, Fe^{2+} and Mn^{2+}, but was curtailed by Cu^{2+}, Hg^{2+} and Fe^{3+}. It was devoid of antifungal and ribonuclease activities.

[**Key words:** Mushroom; Alkaline protease; *Helvella lacunosa*; Purification; Characterization; Fruiting bodies]

Proteases (1–5), lectins (6–11), nucleases (12, 13), ribosome inactivating proteins (14–16), polysaccharides (17–20), polysaccharide–peptides and polysaccharide–protein complexes (21–23) have been isolated from a number of mushrooms which are a component of the diet in many countries. Mushrooms are well liked for their various medicinal functions as well as their delicious taste and nutritive value. Some mushroom lectins and polysaccharides have potentially curative or health-promoting activities for human beings or are under clinical trial (19, 24–26).

Alkaline proteases are a very important group of enzymes widely used in both physiological and commercial settings. They play a specific catalytic role in the hydrolysis of proteins with a high degree of substrate specificity and can be produced in large amounts by microbial approaches (27–29). Proteases are by far the most important group of enzymes produced commercially and are used in the detergent, protein, brewing, meat, photographic, leather and dairy industries (30). In 1994, the total sale of industrial enzymes accounted for approximately $400 million, of which $112 million were used for detergent purposes (31).

Helvella lacunosa, a wild mushroom growing in the western part of China, has considerable protease activity in its extract. Nothing is known about the proteinaceous constituents of ascomycete mushroom especially those of the *Helvella* genus. The intent of the present investigation was to isolate and characterize a protease from its fruiting bodies. The protease isolated exhibits an N-terminal sequence, thermostability and pH stability different from those of previously published mushroom proteases.

MATERIALS AND METHODS

Materials Dried fruiting bodies of the mushroom *H. lacunosa* were purchased from ShanXi Province of China. DEAE-cellulose, EDTA, lima bean trypsin inhibitor, pepstatin A, PMSF, and yeast tRNA were obtained from Sigma Chemical CO., USA. CM-Sepharose CL-6B, Superdex 75 HR 10/30, Q-Sepharose and AKTA Purifier were from GE Healthcare. Casein sodium salt was from Sigma. All other chemicals were of reagent grade.

Isolation of protease Dried fruiting bodies of the mushroom (20 g) were homogenized in 0.15 M NaCl at 4 °C. The homogenate was stirred overnight at 4 °C before centrifugation. After the saline extraction, the mixture was centrifuged at 8000 *g* for 25 min. Then $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation. The mixture was centrifuged at 8000 *g* for 25 min. And then the precipitate was dissolved and dialyzed to remove $(NH_4)_2SO_4$ before applying to a column (1.5 × 20 cm) of DEAE-cellulose, which had previously been equilibrated with and was then eluted with 10 mM NH_4HCO_3 buffer (pH 9.4). After removal of the unadsorbed peak (D1) containing strong protease activity, three adsorbed peaks, D2, D3 and D4, were eluted with 50 mM NaCl, 150 mM NaCl and 1 M NaCl in the starting buffer, respectively. Protease activity toward casein in the chromatographic fractions was monitored as described below. The active fraction (D1) was applied to a column (1.5 × 20 cm) of CM-Sepharose CL-6B, which had been equilibrated and then eluted with 10 mM phosphate buffer (pH 5.8). Unbound material was eluted with the starting buffer while bound material was desorbed by addition of 50 mM NaCl, 150 mM NaCl and 1 M NaCl in the starting buffer. The active peak (D1C3) was then subjected to a column (1 × 10 cm) of Q-Sepharose, which had been equilibrated and was then eluted with NH_4HCO_3 buffer (pH 9.1). After removal of unbound material, the adsorbed material was fractionated using a linear concentration gradient of 0–500 mM NaCl in 10 mM NH_4HCO_3 buffer (pH 9.1). The active fraction (Q2) 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 (GE Healthcare). Only one peak (SU1) was obtained. It represented purified protease.

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TABLE 1. Yields and protease activities of *Helvella lacunosa* chromatographic fractions toward casein (from 20 g dried fruiting bodies).

Chromatographic fraction	Protease activity toward casein (U/mg)	Yield (mg)
D1	**3039**	**159.4**
D2	884	
D3	542	
D4	478	
D1C1	641	
D1C2	**10056**	**24.2**
D1C3	306	
D1C4	1097	
Q1	319	
Q2	**12232**	**15.2**
SU1	**14592**	**9.3**
SU2	<5	

20 g dried fruiting bodies were used as starting material. The protease activities of all preceding chromatographic fractions were calculated based on the activity of trypsin (7900 BAEE units/mg as supplied by Sigma) in the casein assay. D1–D4: DEAE-cellulose, D1C1–D1C4: CM-Sepharose, Q: Q-Sepharose, and SU: Superdex-75. Protease-enriched fractions are highlighted in boldface.

Determination of molecular mass and N-terminal sequence The active peak (SU1) was subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32, 33). 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 (34).

Assay for protease activity A solution of casein, which was used as substrate in the protease assay, was freshly prepared as follows. To 0.1 g casein 10 ml 200 mM phosphate buffer (pH 7.5) was added. Subsequently, the solution was heated at 60 °C for 30 min. The precipitate was removed and the resulting solution could be used (34–36). The test sample or trypsin solution (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. Protease activity was calculated based on the activity of trypsin (7900 BAEE units/mg according to Sigma) in the protease assay using casein as substrate.

Determination of optimum pH and temperature of protease In the assay for optimum pH and temperature, a solution of casein, which was used as substrate, was freshly prepared as described above (5, 13). The assay buffers were prepared in 0.2 M acetic acid (pH 4.0 to 5.0), 0.2 M phosphate (pH 6.0 to 8.0), 0.2 M boric acid (pH 8.0 to 9.0), 0.1 M glycine-NaOH (pH 9.0 to 10.0), 0.05 M sodium bicarbonate-NaOH (pH 10.0 to 11.0), and 0.05 M KCl-NaOH (pH 12.0 to 13.0). 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. To determine the optimum temperature, the reaction mixture was incubated at 27 °C to 87 °C for 15 min. The assay buffer was 200 mM phosphate buffer (pH 7.5).

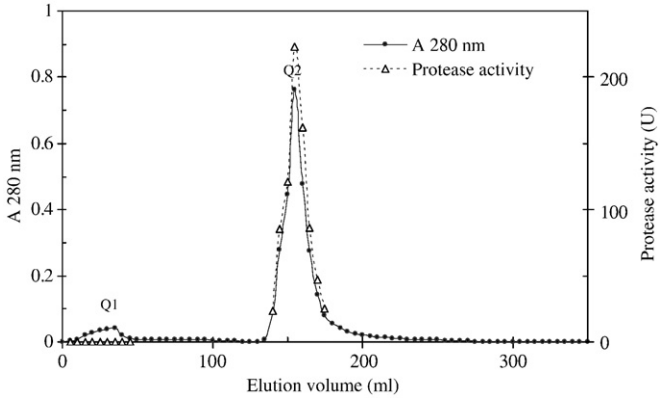

FIG. 1. Anion exchange chromatography of fraction D1C2 from CM-cellulose column on a Q-Sepharose column (1 × 10 cm). The column was first equilibrated and eluted with 10 mM NH_4HCO_3 buffer (pH 9.1) and then with a linear gradient of 0–500 mM NaCl in the same buffer as indicated by the slanting rotted line across the right half of the chromatogram.

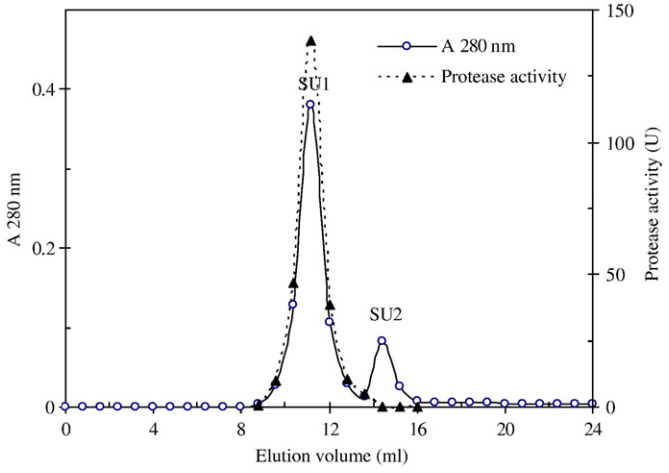

FIG. 2. Gel filtration of Q2 on a Superdex 75 HR10/30 column. The molecular mass of SU1 was 33.5 kDa.

Assay of enzyme kinetics In the assay of helvellisin for enzyme kinetics, the protease solution (10 μl) was incubated at the optimum pH and 37 °C for 15 min with 90 μl 1% casein solution in 100 μl buffer at optimum. The reaction was terminated by addition of 600 μl 5% trichloroacetic acid (TCA). The reaction mixture was then 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 (37).

Assay of mechanistic class In the assay of determination of purified protease mechanistic class, the protease was exposed to the following inhibitors: pepstatin A, PMSF, EDTA and lima bean trypsin inhibitor (5).

Assay of metal ions and chemical reagents effects 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 (8, 36). The protease activity was assayed as described above.

Assay of antifungal activity The assay for antifungal activity toward *Sclerotinia sclerotiorum* and other fungal species was carried out in 100 × 15 mm Petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (10 μl) of the test sample solution was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (38).

Assay of ribonuclease activity Helvellisin was assayed for RNase activity toward tRNA by determining the generation of acid-soluble, UV-absorbing species (12). Yeast tRNA was used as substrate. The RNase was incubated with 200 μg of tRNA in 150 μg 100 mM MES buffer (pH 6.0) at 37 °C for 1 h. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% perchloric acid. After leaving on ice for 15 min, the

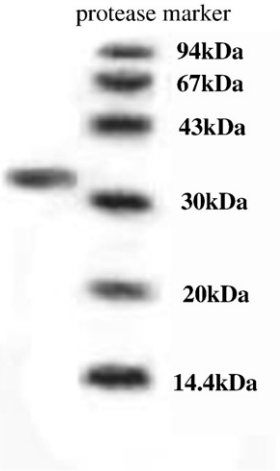

FIG. 3. SDS-PAGE of SU1 (*Helvella lacunosa* protease designated as helvellisin). The molecular mass of SU1 was 33.5 kDa.