ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 3/2017
477–483
https://doi.org/10.18388/abp.2016_1431

Regular paper

Purification and characterization of a novel metalloprotease from fruiting bodies of *Oudemansiella radicata*

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In this study, a 39-kDa metalloprotease was purified from a rare edible mushroom with health-promoting activities, *Oudemansiella radicata*, using a purification protocol which entailed anion exchange chromatography on DEAE-cellulose and Q-Sepharose columns and gel filtration by fast protein liquid chromatography on a Superdex 75 column. Some peptide sequences were obtained by LC-MS/MS analysis and one of the sequences, DAWIQADVNR, manifested 90% identity to *Coprinopsis cinerea* metalloprotease. The optimal reaction pH and temperature for *Oudemansiella radicata* protease were pH 7.0 and 50°C, respectively. The protease was purified 79-fold and demonstrated a specific protease activity of 2.42 U/mg. The K_m of the purified protease for the casein substrate was 0.65 mg/mL at pH 7.0 and 50°C. The activity of the protease was inhibited by Cd^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+} and Fe^{3+} ions, but was enhanced by K^+, Mn^{2+} and Fe^{2+} ions. The marked suppression of the protease activity by EDTA indicates that the protease is a metalloprotease.

Key words: edible mushroom, *Oudemansiella radicata*, protease, purification

Received: 26 September, 2016; revised: 10 March, 2017; accepted: 08 May, 2017; available on-line: 08 September, 2017

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Abbreviations: EDTA, ethylene diamine tetraacetic acid; FPLC, fast protein liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NaOAc-HOAc, sodium acetate-acetic acid; NCBI, national center for biotechnology information; PMSF, phenylmethylsufonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

INTRODUCTION

Mushrooms are abundant in bioactive compounds comprising proteases (Hu *et al*., 2012; Moon *et al*., 2014; Zheng *et al*., 2011), lectins (Wang *et al*., 2013; Xu *et al*., 2014; Zhang *et al*., 2014b), angiotensin I-converting enzyme inhibitory peptides (Geng *et al*., 2015; Kang *et al*., 2013; Lau *et al*., 2014; Mohamad Ansor *et al*., 2013), ribonucleases (Xu *et al*., 2013; Zhang *et al*., 2014a), acid phosphatases (Wannet *et al*., 2000; Zhang *et al*., 2013), laccases (Tian *et al*., 2012; Xu *et al*., 2015a; Xu *et al*., 2015b), antifungal proteins(Suzuki *et al*., 2011; Wong *et al*., 2010), and polysaccharides(Liu *et al*., 2014a; Ma *et al*., 2014; Mao *et al*., 2014; Zhu *et al*., 2014). Many of these mushroom proteins have potential applications or health-promoting activities in human (Liu *et al*., 2014b; Wu *et al*., 2014). There are also many papers documenting that extracts and purified compounds from various edible mushrooms have significant anti-cancer properties, for example, *Antrodia cinnamomea* sulfated polysaccharide exhibited activity in suppressing growth and migration of lung cancer cells (Lu *et al*., 2016), Pro4X, an extract of the edible *Grifola frondosa* mushroom, displayed anti-cancer activity and prevented oncogenesis in BABLc mice (Roldan-Deamicis *et al*., 2016). Recently, a serine protease (AkP) from the *Termitomyces clypeatus* mushroom was effective in killing cancer cells by restoration of the p53 level and cleavage of surface proteoglycans (Majumder *et al*., 2016). Several mushroom proteases, including proteases from *Amanita farinosa* (Sun *et al*., 2011) and *Lepista nuda* (Wu *et al*., 2011), had also shown an antiproliferative activity on human hepatoma HepG2 cells. Proteases catalyze hydrolysis of the amide bond and regulate a myriad of physiological processes (Rao *et al*., 1998; Wolfe, 2009). Apart from physiological functions, proteases occupy a vital position with respect to their diverse applications in a variety of industries, encompassing the brewing, dairy, meat, detergent, leather, and photographic industries (Kalisz, 1988).

Oudemansiella radicata, an edible medicinal mushroom belonging to the *Tricholomataceae* family, inhabits the soil surface or rotten woods of the broad-leaved trees from summer to autumn (Lee, 1988). The literature on *Oudemansiella radicata* in general is not abundant. It has been reported that *Oudemansiella radicata* exhibited an outstanding therapeutic and inhibitory effect on sarcoma 180 and Erhrlich carcinoma, which is attributed to the important chemical compound, i.e. oudenone (Anke *et al*., 1979). In our preliminary experiment, the extract of *Oudemansiella radicata* fruiting bodies displayed a strong protease activity indicating the presence of a metalloprotease.

In order to obtain a valuable new protease, new naturally occurring species should be screened. Hence, in this investigation, a novel metalloprotease was purified from the *Oudemansiella radicata* mushroom.

MATERIALS AND METHODS

Materials. Fresh fruiting bodies of the *Oudemansiella radicata* mushroom were purchased from a market named Shuimuhua in the city of Kunming, Yunnan Province in southwest China. The fruiting bodies were then dried at room temperature. Q-Sepharose, Superdex 75 10/300 column and AKTA Purifier were all acquired from GE

Healthcare (Sweden). DEAE-cellulose, casein sodium salt and protease inhibitors (Pepstatin A, Leupeptin and PMSF) were obtained from Sigma (USA). All other chemicals used were of analytical grade.

Purification of the protease. Dried fruiting bodies of the *Oudemansiella radicata* mushroom were soaked in distilled water (w:v=1:10) at 4°C for 3 hours, and then homogenized in a Waring blender. The homogenate was extracted at 4°C for 1 hour before centrifugation for 20 min at 9000 rpm and 4°C. After the pH of the supernatant had been adjusted, the supernatant was applied to a DEAE-cellulose column, which had previously been equilibrated with 10 mM NaOAc-HOAc buffer (pH 5.6). After removal of the unadsorbed fraction D1, the adsorbed proteins were desorbed stepwise with 50 mM NaCl in the same buffer to yield fraction D2, and then with 100 mM NaCl, 300 mM NaCl and 1M NaCl sequentially to yield fractions D3, D4 and D5, respectively. The highest protease activity was present in fraction D4. Fraction D4 was then subjected to ion exchange chromatography on a column of Q-Sepharose in 10 mM NaOAc-HOAc buffer (pH 5.2). Unadsorbed proteins were eluted with the starting buffer, and the adsorbed material was fractionated using a linear concentration gradient of 0-1 M NaCl in the same buffer. The active fraction Q1 was subsequently purified by fast protein liquid chromatography (FPLC) on a Superdex 75 HR 10/300 gel filtration column. Seven peaks, F1 to F7, were eluted with 150 mM NH_4HCO_3 buffer (pH 8.5). The active fraction F3 was ultimately re-chromatographed on the Superdex 75 HR 10/300 gel filtration column. Only one peak (FP1), which represented purified protease, was obtained.

Assay for protease activity. Protease activity was determined using casein as the substrate following the method of Wang and Ng (2001) with slight modification. In brief, 20 μL sample solution was incubated with 180 μL 1 mg/mL casein (in distilled water) at 50°C for 15 min. Subsequently, the reaction was ended by adding 400 μL 5% (w/v) trichloroacetic acid (TCA). Then, the reaction mixture was centrifuged at 12000 rpm for 5 min. The absorbance of the supernatant was read at 280 nm against water as blank. One unit of the enzyme activity was defined as an absorbance increase of 0.001 at 280 nm per milliliter of reaction mixture per minute under the experimental conditions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid sequence analysis. The active peak (FP1) was subjected to SDS-PAGE for molecular mass determination in accordance with the procedure of Laemmli (Laemmli, 1970), using a 12% resolving gel and 5% stacking gel. The bands were visualized after staining with Coomassie Brilliant blue. FPLC-gel filtration on a Superdex 75 column, which had been calibrated with molecular mass standards, was conducted to determine the molecular mass of the purified protease.

The band corresponding to the protease in SDS-PAGE gel was destained, digested with trypsin, and then dissolved in 0.1% formic acid and 2% acetonitrile for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using a LTQ–Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany).

Determination of optimal pH and temperature for the isolated protease. In the assay for optimal pH value, a series of assay buffers covering a pH range of 2.5–9.0 (100 mM) were used, including citric acid-sodium citrate buffer (pH 2.5 and pH 3.0), NaOAc-HOAc buffer (pH 4.0 and pH 5.0), Na_2HPO_4-NaH_2PO_4 buffer (pH 6.2) and Tris-HCl buffer (pH 7.0, 8.0 and 9.0). Enzyme activity was determined using the above mentioned method.

To determine the optimal temperature for the protease, the standard protease assay mentioned above was conducted over a temperature range of 10–90°C.

K_m and V_{max} values of the isolated protease. To obtain K_m and V_{max} of the purified protease toward the

Table 1. Yields and protease activities of various chromatographic fractions derived from *Oudemansiella radicata* fruiting body extract.

Chromatographic fraction	Total protein (mg)	Total protease activity (U)	Specific protease activity (U/mg)	Purification fold
Crude extract	36000	1100	0.03	1.00
D1	20000	66	0	0.11
D2	1500	40	0.03	0.87
D3	900	0	0	0
D4	5700	360	0.06	2.07
D5	7200	320	0.04	1.45
Q1	452	88	0.19	6.37
F1	19.50	2.40	0.12	4.03
F2	16.50	7.20	0.44	14.28
F3	12	9.60	0.80	26.18
F4	58.50	1.60	0.03	0.90
F5	40.50	0	0	0
F6	13.50	0	0	0
F7	9	0	0	0
FP1	0.66	1.60	2.42	79.34

casein substrate, a series of casein solutions at different concentrations (10, 5, 2.5, 1.25 and 0.625 mg/mL) were mixed with the protease, followed by an assay of the enzymatic activity at pH 7.0 and 50°C. A double reciprocal (Lineweaver-Burk) plot of the reciprocal of reaction velocity against the reciprocal of substrate concentration was then constructed. The K_m and V_{max} values were calculated from the plot.

Effects of metal ions on the isolated protease. The effects of different metal ions on the protease were investigated by pre-incubating the compounds with the enzyme solution for 60 min at 4°C before the standard protease activity assay was performed. Chlorides of the following metals were used in the experiment at the final concentrations of 10, 5, 2.5 and 1.25 mM: $FeCl_3$, $CuCl_2$, $MnCl_2$, $ZnCl_2$, $HgCl_2$, $MgCl_2$, $PbCl_2$, $AlCl_3$, $CdCl_2$, KCl and $FeCl_2$.

Mechanistic class assay. The purified protease was exposed to the following inhibitors: phenyl methyl sulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), pepstatin A and leupeptin at different concentrations (0.04, 0.2 and 1 mM) to determine the mechanistic class that the protease belongs to. The activity was measured by using the standard assay protocol mentioned above and expressed as percentage of residual activity.

RESULTS

Purification of *Oudemansiella radicata* protease

Protease produced by *Oudemansiella radicata* was purified following a protocol that entailed two consecutive steps of ion exchange chromatography and two steps of gel filtration on a Superdex 75 column, resulting in a specific protease activity of 2.42 U/mg and a purification factor of 79-fold (Table 1). The extract of *Oudemansiella radicata* was applied to a DEAE-cellulose ion exchange column. The enzyme extract was resolved into 5 fractions (D1–D5). Fractions D4 and D5, eluted with 300 mM and 1 M NaCl, respectively, demonstrated the protease activity (Table 1 and Fig. 1a). Since the purification fold of fraction D4 was much higher than that of fraction D5, fraction D4 was further purified on a Q-Sepharose column and eluted with a linear gradient of 0–1 M NaCl. Protease activity was concentrated in fraction Q1 (Table 1 and Fig. 1b). Subsequently, fraction Q1 was resolved into 7 fractions (F1–F7) upon FPLC-gel filtration on Superdex 75. Fraction F3 exhibited a much higher protease activity than the other fractions (Table 1 and Fig. 1c). The active fraction F3 appeared as a single active peak, FP1, upon again FPLC-gel filtration on Superdex 75 (Fig. 1d).

Determination of molecular mass and inner amino acid sequences

The molecular mass of SP1 was 45.7 kDa, as judged from the comparison of its elution volume from the Superdex 75 column with those of molecular mass standards (Fig. 2a). In SDS-PAGE, fraction FP1 with enriched protease activity appeared as a single band with a molecular mass of 39 kDa (Fig. 2b). This result was similar to that obtained from the Superdex 75 column. It indicated that the protease is a monomeric protein with a molecular mass of 39 kDa.

Five inner amino acid sequences of *Oudemansiella radicata* protease were obtained by LC-MS/MS: including AVVLGAAGGIGQPSALLLK, GFHLSSLVALAALLR, DAWIQADVNR, AQLGLGHSYSRAK and SSSIELINPKAESIR. It was found that peptide DAWIQADVNR had a 90% identity with metalloproteases from

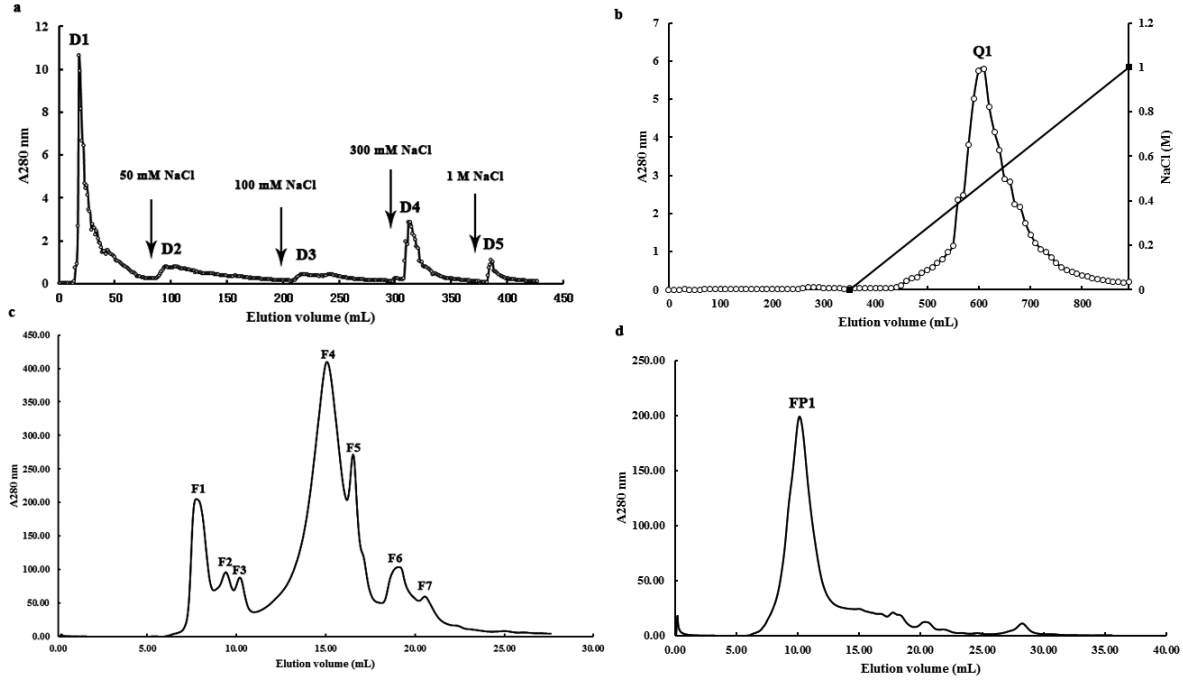

Figure 1. Elution profiles of the *Oudemansiella radicata* protease.
(**a**) Ion exchange chromatography on a DEAE-cellulose column. Fraction D4 was the fraction with the highest protease activity. (**b**) Ion exchange chromatography of fraction D4 on a Q-sepharose column. Fraction Q1 was the fraction with protease activity. (**c**) Gel filtration of fraction Q1 on a Superdex 75 column by FPLC. Fraction F3 was the fraction with protease activity. (**d**) Gel filtration of fraction F3 on a Superdex 75 column by FPLC again. Fraction FP1 represents the purified *Oudemansiella radicata* protease.

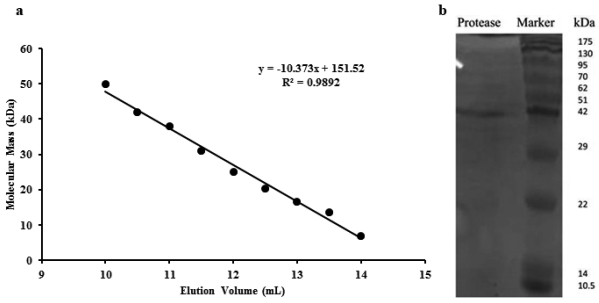

Figure 2. FPLC of molecular mass standards for calibrating Superdex 75 column and SDS-PAGE of fraction FP1 (purified protease) from Superdex 75 column.
(**a**) FPLC of molecular mass standards for calibrating Superdex 75 column. (**b**) SDS-PAGE of fraction FP1 (purified protease) from Superdex 75 column. The molecular mass of FP1 was 39 kDa.

Coprinopsis cinerea (accession number XP_001839300.2, P_001838846.1, XP_001835397.2) by NCBI database searching using BLAST. It also shared 90% identity with a metalloprotease from *Punctularia strigosozonata* HHB-11173 SS5 (accession number XP_007380217.1).

Characteristics of *Oudemansiella radicata* protease

The protease exhibited an optimal pH at pH 7.0 and an optimal temperature at 50°C, respectively (Fig. 3a and Fig. 3b). The pH dependence of the protease activity toward casein is shown in Fig. 3a. When the pH was raised from 2.5 to 7.0, the protease activity increased steadily. However, when the pH was raised beyond 7.0 a rapid decrease was observed. The protease activity vanished when the pH was increased up to 10. It underwent a steady increase when the temperature was increased from 10 to 50°C and a decline in the enzyme activity when the temperature was raised further from 50 to 90°C.

The K_m of purified *O. radicata* protease toward casein was 0.65 mg/mL at pH 7.0 and 50°C. The V_{max} was 125.85 μg/mL/min (Fig. 4). The effects of various metal ions on the purified protease are shown in Table 2. The activity of purified protease was significantly depressed in the presence of cations such as Fe^{3+}, Cu^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+} ions at the concentrations of 1.25–5.0 mM. The inhibitory activity of the protease was dose-dependent. The higher the concentrations of the metal ions: Al^{3+}, Zn^{2+} and Mn^{2+} used, the stronger were the inhibitory effects on the activity of the purified protease. When

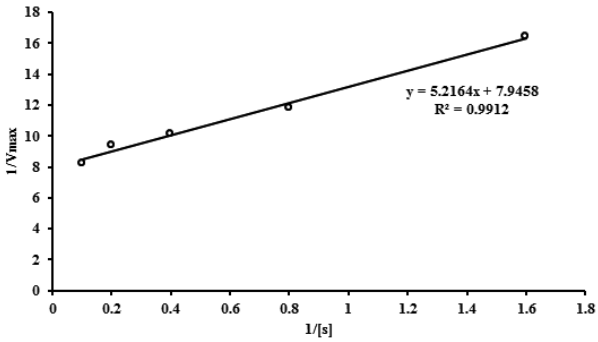

Figure 4. The Lineweaver-Burk plot for *Oudemansiella radicata* protease.

the concentration of the three metal ions was raised to 10 mM, only 37%, 17% and 63%, respectively, of the protease activity were retained, while Mn^{2+} ions elicited slight enhancement of protease activity at the concentrations of 2.5 mM and 1.25 mM. On the contrary, at the high concentration of 10 mM, Fe^{2+} ions demonstrated an enhancing action on the protease activity. The activity of the protease was potentiated by K^+ ions at all of the concentrations examined. Among the protease inhibitors tested, the activity of purified protease was adversely reduced by EDTA, but not affected to any major extent by PMSF, leupeptin and pepstatin A (Table 3), indicating that the enzyme belongs to the metalloprotease family.

DISCUSSION

In the present study, a novel metalloprotease was purified from fruiting bodies of the *O. radicata* mushroom, with two different chromatographic methods- anion exchange chromatography on DEAE-cellulose and anion exchange chromatography on Q-Sepharose. The *O. radicata* protease resembled other mushroom metalloproteases, such as *Armillariella mellea*, *Pleurotus ostreatus* and *Tricholoma saponaceum* metalloproteases, in that it was adsorbed on DEAE-cellulose and could be purified by anion exchange chromatography on DEAE-cellulose (Kim & Kim, 1999, 2001; Shen *et al.*, 2007).

When compared with other mushroom metalloproteases, *O. radicata* metalloprotease displays a number of remarkable differences in characteristics. The molecular mass of *O. radicata* protease (39 kDa) was larger than those of the majority of mushroom metalloproteases (in

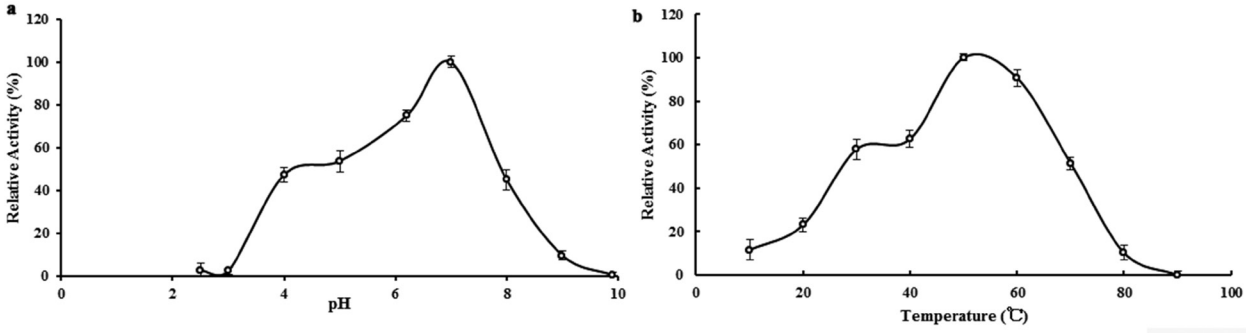

Figure 3. The optimal pH and temperature of *Oudemansiella radicata* protease.
(**a**) The optimal pH of *Oudemansiella radicata* protease was pH 7.0. The maximum value of the protease activity was set as 100%. Results represent mean ±S.D. (n=3). (**b**) The optimal temperature of *Oudemansiella radicata* protease was 50°C. The maximum value of the protease activity was set as 100%. Results represent mean ±S.D. (n=3).

Table 2. Effect of metal ions on the activity of *Oudemansiella radicata* protease.

Metal ion concentration	Relative protease activity (%)			
	10 mmol/L	5 mmol/L	2.5 mmol/L	1.25 mmol/L
Fe^{2+}	138±2.1	82±3.6	93±2.5	97±1.4
K^{+}	147±1.7	125±2.8	111±3.7	103±1.8
Mg^{2+}	95±4.5	98±0.6	125±2.6	116±2.5
Mn^{2+}	63±0.9	70±4.1	115±3.3	113±3.4
Zn^{2+}	17±1.3	34±0.8	65±0.7	102±2.6
Al^{3+}	37±0.8	45±1.3	57±0.9	72±2.8
Pb^{2+}	25±2.9	28±0.9	22±1.3	58±0.5
Hg^{2+}	19±2.4	4±0.2	20±1.4	0
Cd^{2+}	4±0.3	4±0.9	9±1.3	6±0.9
Cu^{2+}	15±3.2	0	11±0.7	5±1.2
Fe^{3+}	0	0	0	52±1.7

Results represent mean ±S.D. (n=3). The protease activity without metal ions added was set as 100%.

the range from 18 to 32 kDa) (Kim & Kim, 1999, 2001; Nonaka *et al*., 1995; Shen *et al*., 2007; Wu *et al*., 2011), and mushroom serine proteases (in the range from 15 to 35 kDa) (Burton *et al*., 1993; Cui *et al*., 2007; Sun *et al*., 2011; Zhang *et al*., 2010). However, it was lower than *Hericium erinaceum* fibirinolytic metalloprotease with a molecular mass of 51 kDa (Choi *et al*., 2013) and much lower than other types of proteases, including a subtilisin-like protease from *Pleurotus ostreatus* (75 kDa) (Palmieri *et al*., 2001), prolyl endopeptidases from *Lyophyllum cinerascens* (76 kDa) (Yoshimoto *et al*., 1988) and *Agaricus bisporus* (78 kDa) (Sattar *et al*., 1990) and an aminopeptidase from *Lyophyllum cinerascens* (150 kDa) (Abdus Sattar *et al*., 1989).

The optimal pH for the activity of the reported mushroom metalloproteases ranged from pH 7.0 to pH 8.0.

Table 3. Effect of inhibitors on the activity of *Oudemansiella radicata* protease.

Inhibitors	Concentration (mmol/L)	Remaining protease activity (%)
leupeptin	0.04	90 ± 3.8
	0.2	87 ± 2.2
	1	86 ± 3.3
PMSF	0.04	95 ± 1.1
	0.2	100 ± 2.1
	1	90 ± 1.5
EDTA	0.04	59 ± 0.8
	0.2	36 ± 2.4
	1	18 ± 3.9
pepstatin A	0.04	88 ± 0.5
	0.2	89 ± 3.1
	1	98 ± 2.9

Results represent mean ±S.D. (n=3). The protease activity without any inhibitors added was set as 100%.

The activity of *Tricholoma saponaceum* metalloprotease reached the maximum at pH 8.0. Both *Lepista nuda* metalloprotease and *Armillariella mellea* metalloprotease were most active at pH 7.0, which was consistent with the metalloprotease from *O. radicata* (Shen *et al*., 2007). The *O. radicata* metalloprotease manifested a temperature optimum at 50°C, which was about quadrupled in comparison to its activity at 20°C. However, its activity at 80°C was only about half of that at 20°C, and its activity vanished at 90°C. Thus, the protease had only moderate thermostability. The optimal temperature of the *O. radicata* metalloprotease was similar to those of its counterparts from *Lepista nuda* (50°C), *Tricholoma saponaceum* (55°C) and *Armillariella mellea* (55°C), which were much higher than that of *Pleurotus ostreatus* metalloprease at 35°C. The effects of metal ions toward protease activity varied considerably among different mushroom metalloproteases. The activity of *Armillariella mellea* metalloprotease was strongly inhibited by Hg^{2+} ions and the activity of *Lepista nuda* metalloprotease was reduced by Cd^{2+}, Hg^{2+} and Fe^{3+} ions. In the presence of Cu^{2+} and Hg^{2+} ions, the activity of *Tricholoma saponaceum* metalloprotease totally disappeared. Many metal ions, including Cd^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+} and Fe^{3+} ions, significantly inhibited the activity of *O. radicata* metalloprotease. It was seen that the activities of mushroom metalloproteases were greatly reduced by Hg^{2+} ions. On the other hand, Mn^{2+}, Zn^{2+} and Ca^{2+} ions enhanced the protease activity of *Grifola frondosa* metalloprotease. Mg^{2+}, Zn^{2+} and Mo^{2+} ions augmented the protease activity of *Armillariella mellea* metalloprotease. Proteases from *Lepista nuda* and *O. radicata* were activated by Fe^{2+} and K^{+} ions, respectively (Kim & Kim, 1999, 2001; Nonaka *et al*., 1995; Shen *et al*., 2007; Wu *et al*., 2011).

In addition, there are reports of several metalloproteases from other sources which had shown some similarity to the purified protease. The purified metalloprotease from *Candida kefyr* 41 PSB demonstrated a molecular mass of 43 kDa (Yavuz *et al*., 2017) which was a little larger than that of *O. radicata* metalloprotease. Its optimal pH was the same as for the *O. radicata* metalloprotease.

Its optimal temperature was 105°C, much higher than the temperature optimum of purified *O. radicata* metalloprotease (50°C). The pH and temperature optimum of a metalloprotease from *Alternaria solani* (Chandrasekaran *et al.*, 2016) exhibited similiarity to the purified *O. radicata* metalloprotease. When compared with other metalloproteases, the *O. radicata* metalloprotease showed several notable differences in characteristics.

In summary, a metalloprotease with some distinctive characteristics was isolated from the wild mushroom *O. radicata*. To the best of our knowledge, this is one of the very few proteins reported on from this mushroom.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was financially supported by Special Fund for Agro-scientific Research in the Public Interest (No. 201303080) and collaborative project of Scientific Research and Graduate Training of Beijing Municipal Education Commission (No. 201502911110426). This work was also financially supported by the 2014 Shanxi Key Scientific and Technology of Coal Basic Research Project (FT2014-03) and The Collaborative Innovation Center Project of Loess Plateau Edible Fungus Quality Improvement in University of Shanxi Province.

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