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Screening, Purification, and Characterization of an Extracellular Prolyl Oligopeptidase from *Coprinopsis clastophylla*

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Culture filtrates of 22 mushrooms were screened for extracellular prolyl oligopeptidase activity. Four strains with relatively high enzyme activity were all from inky cap mushrooms. The production of *Coprinopsis clastophylla* prolyl oligopeptidase was associated with the growth of the fungus and the enzyme was not released by cell lysis. The enzyme was purified 285-fold to a specific activity of 52.05 U/mg. It was purified to a single band on a native polyacrylamide gel. However, the enzyme separated into three bands on a sodium dodecyl sulfate-polyacrylamide gel with mobility corresponding to molecular weights of approximately 84, 60, and 26 kDa. The results of tandem mass spectrometric analysis revealed that the 60 kDa protein was likely a degradation product of the 84 kDa protein. The isoelectric point of the purified enzyme was 5.2. The purified enzyme had an optimal pH and temperature of 8.0 and 37°C, respectively. Diisopropyl fluorophosphate (DFP), *p*-chloromercuribenzoaic acid (PCMB), Hg^{2+}, and Cu^{2+} strongly inhibited *C. clastophylla* prolyl oligopeptidase. This enzyme is a serine peptidase and one or more cysteine residues of the enzyme are close to the active site.

Keywords: *C. clastophylla*, inky cap mushrooms, extracellular prolyl oligopeptidase, serine peptidase, purification

Introduction

Prolyl oligopeptidase (post-proline cleaving endopeptidase; proline-specific endopeptidase; prolyl endopeptidase) [EC 3.4.21.26] preferentially hydrolyzes the peptide bond on the carboxyl side of proline residues only within peptides that are up to 30 amino acids long (Koida and Walter, 1976; Walter and Yoshimoto, 1978). It has been found in animals (Walter *et al.*, 1971; Krishnamurti *et al.*, 1981; Moriyama *et al.* 1988; Rennex *et al.*, 1991), plants (Yoshimoto *et al.*, 1987), and microorganisms (Yoshimoto and Tsuru, 1978; Estera *et al.*, 1992; Szwajcer-Dey *et al.*, 1992; Kanatani *et al.*, 1993; Makinen *et al.*, 1994; Lee *et al.*, 2007). Most prolyl oligopeptidases are intracellular enzymes. A few studies of extracellular prolyl oligopeptidases have been undertaken (Berdal *et al.*, 1983; Sattar *et al.*, 1990). Prolyl oligopeptidases are thought to play a significant role in accelerating the ripening of cheese without causing bitterness (Habibi-Najafi and Lee, 1994). The enzyme also has been used in the preparation of recombinant functional peptides (Xiu *et al.*, 2002), the detoxification of gluten in cases of celiac disease (Gass *et al.*, 2005; Marti *et al.*, 2005; Pyle *et al.*, 2005) and antibody-directed enzyme prodrug therapy (Heinis *et al.*, 2004). New prolyl oligopeptidase-producing microorganisms can be screened for such applications.

Mushrooms belong to the basidomycetes and have the highest protein content of any vegetable. Some mushrooms exhibit a strong autolytic characteristic (Hopple and Vilgalys, 1994; Muraguchi *et al.*, 2008). No research has yet been conducted on the prolyl oligopeptidase from mushrooms with autolytic activity. In this study, an extracellular prolyl oligopeptidase was screened and purified from *Coprinopsis clastophylla*, a member of the fungi that are commonly recognized as the inky cap mushrooms that are known to autolyze soon after complete development of the fruiting body. The biochemical and catalytic properties of the enzyme were elucidated with a view to future applications.

Materials and Methods

Screening of prolyl oligopeptidase-producing strains

All strains were obtained from the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. The strains were cultured on YMA (Difco Lab., USA) plates. The active culture was washed off the plate with 50 ml of a medium that contained 2% glucose, 0.3% yeast extract, 1% polypepton, 0.3% KH_2PO_4, and 0.1% $MgSO_4$ (pH adjusted to 6.0) (Sattar *et al.*, 1990) and blended. Five milliliter of the sheared culture was used to inoculate 50 ml of the same medium in a 250 ml flask. The culture was incubated with agitation at 150 rpm at 25°C for 18 days. Samples were taken at intervals of three days to determine the prolyl oligopeptidase activity of the culture.

Assay of prolyl oligopeptidase activity

The enzyme activity was determined as described by Saito *et al.* (1997) with some modifications. Culture filtrate or enzyme solution (50 µl) was mixed with 50 µl of 10 mM benzyloxycarbonyl-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-*p*-NA) (Fluka, Switzerland) in 40% 1,4 dioxane and 400 µl of 0.1 M citric acid-Na_2HPO_4 buffer (pH 8.0). After incu-

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bation at 37°C for 5–60 min, 500 µl of 1 M HCl were added to the reaction mixture and centrifuged at 13 krpm for 1 min. The absorbance of the resultant supernatant was measured at 410 nm. One unit (U) of prolyl oligopeptidase corresponds to the amount of enzyme that can release 1 µmol of ρ-nitroaniline per min.

Assay of malate dehydrogenase activity

Malate dehydrogenase was used as a cytosolic marker (Cowell *et al.*, 1979) to detect cell lysis. Malate dehydrogenase activity was measured spectrophotometrically from the initial rate of NAD reduction (increase in absorbance at 340 nm) at 25°C. The assay system for malate dehydrogenase contained 63 mM glycine-NaOH buffer, pH 10.0, 100 mM malate, 1.2 mM NAD, and 0.1 ml of enzyme solution in a total volume of 1.0 ml.

Effect of pH and temperature on enzyme activity

Prolyl oligopeptidase activity was analyzed over a pH range from 3.0 to 12.0 at 37°C, as described above. The buffer systems were citric acid-Na_2HPO_4 (pH 3.0–8.0) and glycine-NaOH (pH 9.0–12.0). To determine the optimal temperature for the activity of prolyl oligopeptidase, the activities of the enzyme were measured from 25 to 45°C at pH 8.0. To determine thermal stability, the purified enzyme was incubated at various temperatures for 30 min and its activity was then determined.

Enzyme purification

C. clastophylla was cultivated aerobically in 400 ml of the aforementioned medium in a 3 L flask with shaking at 25°C for 10 days. Culture supernatant (2.4 L) was used for the purification of the prolyl oligopeptidase. The cells were separated from the medium by centrifugation. The supernatant was fractionated using ammonium sulfate from 40 to 60%. The precipitate was dissolved into 0.75 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0). Protease inhibitor (Cocktail set IV; Merck, Germany) was added to the sample. The sample was then applied to a Phenyl Sepharose 6 fast flow column (Amersham Pharmacia Biotech, Sweden) that had been pre-equilibrated with 0.75 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0). The column was eluted with a linear gradient of 0.75 to 0 M ammonium sulfate in the same buffer. The active fractions were combined, concentrated by ultrafiltration on Macrosep centrifugal devices (30 K MWCO, Pall Life Sciences, USA) and then applied to a Sephacryl S-200 column (Amersham Pharmacia Biotech, Sweden) that had been pre-equilibrated with 0.2 M sodium chloride in 50 mM sodium phosphate buffer (pH 7.0). The active fractions were pooled, concentrated, and desalted using Macrosep centrifugal devices. The enzyme solution was applied to a hydroxyapatite column (Sigma-Aldrich, USA) and the adsorbed enzyme was eluted with phosphate buffer using a linear concentration gradient of phosphate from 5 to 400 mM at pH 7.0. Fractions that exhibited activity were combined and concentrated, and the buffers were exchanged by ultrafiltration. The concentrated sample was applied to a Resource Q column (Amersham Pharmacia Biotech, Sweden) for ion-exchange chromatography. The column was then eluted with 20 mM Tris-HCl buffer (pH 8.5) at a linear concentration gradient of sodium chloride from of 75 to 125 mM. Enzyme fractions were combined, concentrated, and stored at -20°C.

Gel electrophoresis

The purity of the enzyme was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and native PAGE. SDS-PAGE was conducted using 10% polyacrylamide gels following the method of Laemmli (1970). The molecular weights of the proteins were estimated using a standard protein marker (Sigma-Aldrich). Part of the native PAGE gel was stained with Coomassie Brilliant Blue G and the other part was sliced. The gel slices were submerged in 1 ml of the enzyme assay reagent that contained Z-Gly-Pro-*p*-NA and then incubated at 37°C overnight.

Mass spectrometry

Protein bands from SDS-PAGE were excised manually. The protein bands were in-gel digested by trypsin and extracted as described by Wilm *et al.* (1996). The mass spectrometry of the peptides was carried out by LC-MS/MS using QSTAR QqTOF that was equipped with a nanoflow liquid chromatograph, an electrospray ionization source and quadruple time-of-flight detection system (*nano*-LC-ESI-Q/TOF) (Applied Biosystem, USA). The mass-to-charge ratio (m/z) and molecular weights of the fragments obtained from the 84 kDa protein were compared with those from the 60 and 26 kDa proteins.

Isoelectric focusing

Isoelectric focusing was performed using an Ampholine PAGplate precasting gel (Amersham Pharmacia Biotech, Sweden) (pH 3.5–9.5), according to the instruction manual that was supplied by Pharmacia Ampholine PAGplate. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, USA) with bovine serum albumin as the standard protein.

Effect of proteinase inhibitors and divalent cations on protease activity

The purified enzyme was preincubated with 0.2 mM protease inhibitors or divalent cations in 0.1 M citric acid-Na_2HPO_4 buffer (pH 8.0) for 10 min at 37°C, and the residual activities were assayed by the standard method using Z-Gly-Pro-*p*-NA as the substrate.

Substrate specificity and kinetic analysis

The hydrolysis of amino acid–*p*-nitroanilide (-*p*-NA) was assayed by the aforementioned method, except that the concentration of the substrate was varied. The degree of hydrolysis of amino acid–nitrophenyl ester (-ONP) was measured by a modified version of a method that was developed by Yoshimoto *et al.* (1980). Fifty microliter of 10 mM amino acid-ONP in dioxane were added to a mixture of 50 µl enzyme solution and 400 µl of 0.1 M citric acid-Na_2HPO_4 buffer (pH 8.0). After incubation at 37°C for 5 to 60 min, 500 µl of 1 M HCl were added to the reaction mixture and centrifuged

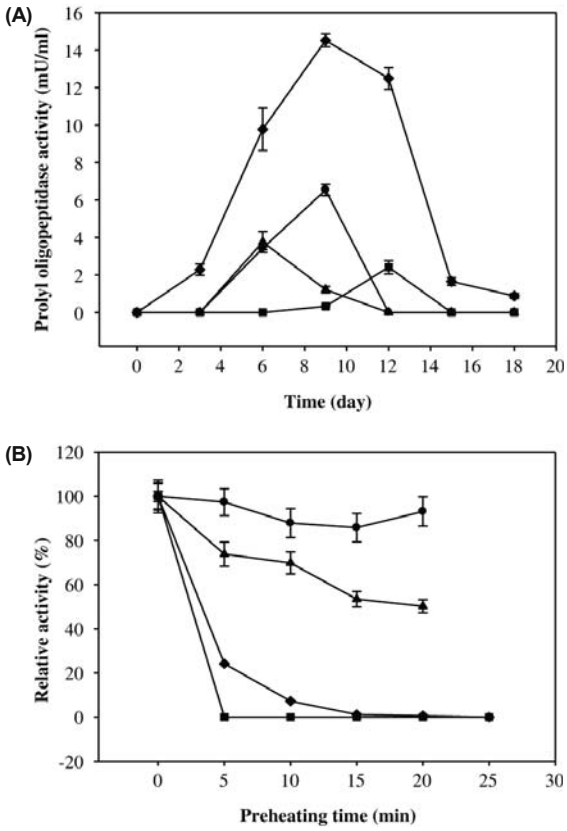

Fig. 1. Extracellular prolyl oligopeptidase activity profiles and thermostabilities of strains with relatively high enzyme activity. (A) Samples were obtained at intervals and their extracellular prolyl oligopeptidase activities were determined. (B) Culture broths of the strains were incubated at 45°C for various times and their activities were then determined. Results are means from duplicate experiments. Symbols: (●) *C. clastophylla*; (■) *C. maysoidisporus*; (▲) *C. radians*; (◆) *C. sclerocystidiosus*.

at 13 krpm for 1 min. The absorbance of the resultant supernatant was measured at 410 nm. The extent of hydrolysis of amino acid-7-amido-4-methylcoumarin (-MCA) was measured using the method of Saito *et al.* (1997) with modification. The enzyme solution (50 µl) was mixed with 50 µl of 10 mM amino acid-MCA and 400 µl of 0.1 M citric acid-Na_2HPO_4 buffer (pH 8.0). After incubation at 37°C for 5 to 60 min, 500 µl of cold methanol were added to the reaction mixture and centrifuged at 13 krpm for 1 min. The release of 4-methylcoumarinylamine was monitored in a Labsystems Fluoroskan II Fluorescent 96 well microplate reader (GMI, USA) using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The kinetic parameters of the prolyl oligopeptidase were determined from Lineweaver-Burk plots.

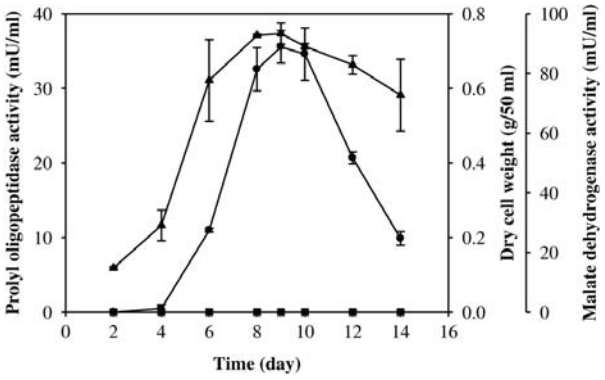

Fig. 2. Cell growth and extracellular prolyl oligopeptidase activity profiles of *C. clastophylla*. Symbols: (▲) dry cell weight; (●) extracellular prolyl oligopeptidase activity; (■) extracellular malate dehydrogenase activity. Results are Means±SD from triplicate experiments.

Results

Screening of prolyl oligopeptidase-producing fungi

Twenty-two strains of *Coprinopsis*, *Coprinellus*, *Coprinus*, *Morchella*, *Volvariella*, *Boletus*, *Agaricus*, and *Dictyophora* species were screened for prolyl oligopeptidase activity using Z-Gly-Pro-*p*-NA as the substrate. Four strains, *Coprinopsis clastophylla* (BCRC 36074), *Coprinus maysoidisporus* (BCRC 36101), *Coprinellus radians* (BCRC 36478), and *Coprinellus sclerocystidiosus* (BCRC 36078), were found to exhibit relatively high prolyl oligopeptidase activity (Fig. 1A). The prolyl oligopeptidase of *C. clastophylla* was selected for further analysis because it was the most thermostable of the enzymes from these strains (Fig. 1B).

Growth and the prolyl oligopeptidase activity profiles of *C. clastophylla*

The growth condition of *C. clastophylla* was optimized based on the medium that was used for screening. A five-fold increase of activity was achieved by growing *C. clastophylla* at 25°C in a medium that contained 2% glucose, 0.3% soybean flour, 1% tryptone, 0.3% KH_2PO_4, and 0.1% $MgSO_4$ (pH 6.0) with an agitation speed of 200 rpm (Fig. 2). Under such growth conditions, the production of prolyl oligopeptidase by the strain was associated with its growth. Little activity was detected in the lag phase. The activity increased during

Table 1. Purification of the prolyl oligopeptidase from *C. clastophylla*

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant (2.4 L)	66.89	366.23	0.18	100	1
$(NH_4)_2SO_4$ precipitation	22.72	56.12	0.40	33.96	2
Phenyl sepharose	48.05	7.54	6.37	71.84	35
Sephacryl S-200	19.57	1.78	11.02	29.26	60
Hydroxyapatite	10.96	0.24	44.85	16.38	246
Resource Q	0.28	0.005	52.05	0.42	285

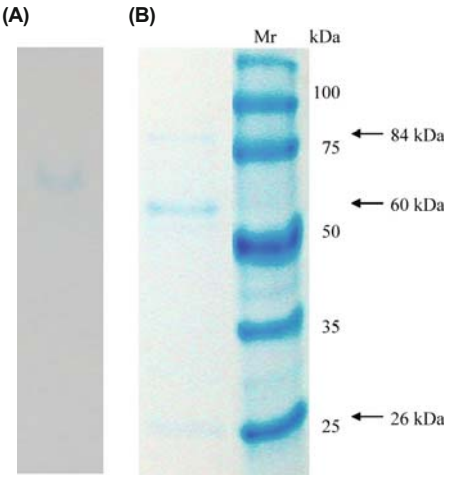

Fig. 3. 10% native (A) and 10% SDS polyacrylamide (B) gel electrophoresis of the purified prolyl oligopeptidase from *C. clastophylla*. Each well was loaded with approximately 0.84 μg of purified enzyme. The 10% SDS polyacrylamide gel was calibrated using standard protein markers.

Table 2. Mass-to-charge ratio (m/z) and molecular weight of 84 and 60 kDa protein fragments

Fragment No.	84 kDa		60 kDa	
	M/Z value	mol wt	M/Z value	mol wt
1	892.59	1783.16	892.59	1783.16
2	799.09	1596.16	-	-
3	744.38	1486.75	-	-
4	717.01	1432.00	717.01	1432.00
5	667.44	1332.87	-	-
6	651.96	1301.93	651.97	1301.91
7	649.46	1296.88	-	-
8	604.93	1207.85	604.94	1207.86
9	595.39	1783.15	595.39	1783.16
10	592.37	1182.73	592.37	1182.73
11	577.72	1730.13	577.71	1730.12
12	574.39	1146.77	574.38	1146.75
13	565.28	1128.55	565.26	1128.51
14	560.43	1118.85	-	-
15	557.28	1112.54	-	-
16	554.72	1661.13	-	-
17	547.91	1093.80	547.91	1093.80
18	547.05	1638.12	-	-
19	541.44	1080.87	541.45	1080.88
20	530.90	1059.78	530.87	1059.72
21	522.39	1564.15	522.39	1564.15
22	516.39	1030.77	-	-
23	515.86	1029.71	-	-
24	497.35	1489.03	-	-
25	478.34	1431.99	478.34	1431.99
26	466.32	930.63	-	-
27	465.32	1392.94	465.32	1392.93
28	458.91	915.81	458.91	915.80
29	446.33	890.65	446.33	890.65
30	438.33	874.65	438.33	874.65
31	431.64	1291.90	431.64	1291.89
32	426.30	1275.89	426.31	1275.89
33	425.64	1273.89	425.64	1273.88
34	420.30	1257.89	420.30	1257.89

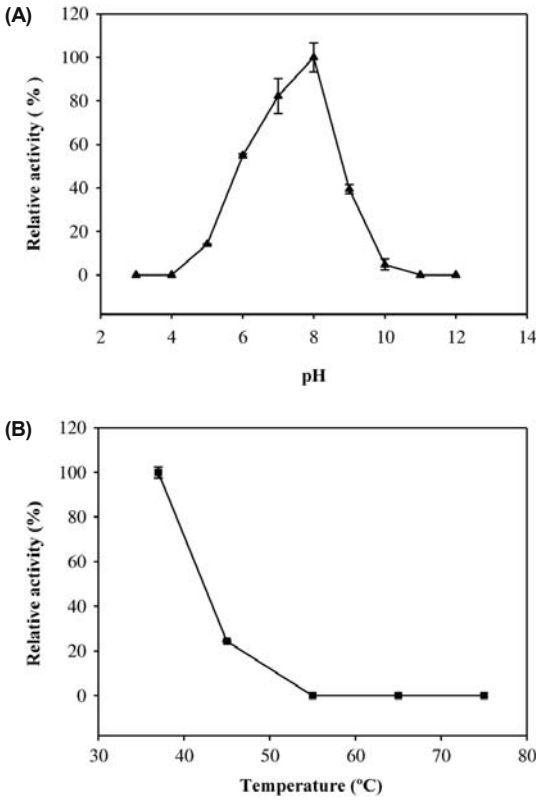

Fig. 4. Effects of pH, temperature (A) and thermostability (B) of purified prolyl oligopeptidase from *C. clastophylla*. Prolyl oligopeptidase activity was analyzed over a pH range from 3.0 to 12.0 at 37°C. To determine effect of temperature, activity was analyzed from 25 to 45°C at pH 8.0. To determine effect of thermostabilty, enzyme was incubated at various temperatures at pH 8.0 for 30 min and activity was then measured. Results are means±SD from triplicate experiments.

the log phase, reaching its maximum detected value in the stationary phase, before decreasing in the death phase. No malate dehydrogenase activity was detected in the growth medium during cultivation, indicating that the prolyl oligopeptidase of *C. clastophylla* was produced extracellularly.

Purification of the extracellular prolyl oligopeptidase from *C. clastophylla*

The extracellular prolyl oligopeptidase in the culture supernatant of *C. clastophylla* was purified by a combination of ammonium sulfate precipitation and serial column chromatography. The final recovery was 0.42% with 285-fold purification and a specific activity of 52.05 U/mg (Table 1). The enzyme was purified to homogeneity as judged by native PAGE (Fig. 3A) and an activity assay of the gel slices, where only the corresponding protein band was active on the substrate. However, the purified enzyme separated into three bands on SDS-PAGE with mobility corresponding to molecular weights of approximately 84, 60, and 26 kDa (Fig. 3B). Tandem mass spectrometric analysis revealed that 14 of the 22 fragments from the 60 kDa protein had identical mass-to-charge ratios (m/z) to those of the cognate fragments from the 84 kDa protein. Furthermore, ten of the 22

Table 3. Effect of inhibitors and divalent cation on prolyl oligopeptidase of *C. clastophylla*

Chemical[a]	Relative activity (%)
None	100
Iodoacetamide	87
N-Ethylmaleimide	50
PCMB	14
3,4-Dichloroisocoumarin	100
TLCK	98
PMSF	93
DFP	13
EDTA	93
o-Phenanthroline	79
$FeCl_2 \cdot 4H_2O$	100
$MnCl_2 \cdot 4H_2O$	93
$SnCl_2 \cdot 2H_2O$	87
$NiCl_2$	86
$ZnSO_4 \cdot 7H_2O$	61
$CoCl_2 \cdot 6H_2O$	48
$CuCl_2 \cdot 2H_2O$	13
$HgCl_2$	0

[a] PCMB, *p*-chloromercuribenzoic acid; TLCK, *p*-toluenesulfonamide; PMSF, phenylmethanesulphonylfluoride; DFP, diisopropyl fluorophosphates

calculated molecular weights of the fragments from the 60 kDa protein were identical to those of the cognate fragments from the 84 kDa protein (Table 2). The results strongly suggest that the 60 kDa protein is derived from the 84 kDa protein.

Effect of pH and temperature

The characterization of the purified prolyl oligopeptidase revealed that the enzyme had an optimal pH and temperature of 8.0 and 37°C, respectively. The activity of the enzyme was reduced by 50% and 60% at pH 6.0 and 9.0, respectively, compared to its maximal activity (Fig. 4A). The effect of temperature on the activity of *C. clastophylla* prolyl oligopeptidase was examined over a temperature range of 25–45°C at pH 8.0. The optimal temperature for the activity of the enzyme was 37°C (Fig. 4A). The enzyme was active over a temperature range of 25–37°C. However, the enzyme was thermolabile, retaining only about 20% of its activity after incubation at 45°C for 30 min (Fig. 4B). The isoelectric point of the prolyl oligopeptidase was 5.2.

Table 4. Substrate specificity of the prolyl oligopeptidase from *C. clastophylla*

Substrate[a]	Specific activity (U/mg)
Substrates with proline	
Pro-*p*-NA	–[b]
Gly-Pro-*p*-NA	–
Z-Pro-ONP	–
Z-Gly-Pro-*p*-NA	52.1
Z-Gly-Pro-MCA	61.3
Substrates without proline	
Z-Gly-Gly-ONP	–
Z-Phe-Arg-MCA	–
Z-Leu-Leu-Glu-MCA	–
Z-Val-Lys-Met-MCA	–
Z-Leu-Leu-Leu-MCA	–
Z-Tyr-Val-Ala-Asp-*p*-NA	–

[a] *p*-NA, *p*-nitroanilide; Z, benzyloxycarbonyl; MCA, 7-amido-4-methylcoumarin; ONP, nitrophenyl ester.
[b] Not hydrolyzed.

Effect of proteinase inhibitors and divalent cation

Table 3 summarizes the effects of proteinase inhibitors and divalent cations on the activity of prolyl oligopeptidase from *C. clastophylla*. Diisopropyl fluorophosphate (DFP), *p*-chloromercuribenzoaic acid (PCMB), Hg^{2+}, and Cu^{2+} strongly inhibited enzyme activity. N-Ethylmaleimide, Co^{2+} and Zn^{2+} partially inhibited the activity of the prolyl oligopeptidase. Phenylmethanesulphonylfluoride (PMSF), iodoacetamide, 3,4-Dichloroisocoumarin, tosyl-L-Lys chloromethyl ketone (TLCK), and metal chealators such as *o*-phenanthroline and EDTA had little effect on the enzyme activity.

Substrate specificity and kinetic analysis

The substrate specificity of prolyl oligopeptidase from *C. clastophylla* was studied using various synthetic substrates (Table 4). The enzyme could not hydrolyze substrates lacking a proline residue. With respect to substrates that contained a proline residue, the enzyme was active on Z-Gly-Pro-*p*-NA and Z-Gly-Pro-MCA, but it was completely inert toward Pro-*p*-NA, Gly-Pro-*p*-NA and Z-Pro-ONP. The Km, Kcat, and Kcat/Km values of the enzyme toward Z-Gly-Pro-*p*-NA were 3.42 mM, 162 S^{-1}, and 47.4 $mM^{-1}S^{-1}$, respectively. The corresponding values for Z-Gly-Pro-MCA were 1.74 mM, 26.6 S^{-1}, and 15.1 $mM^{-1}S^{-1}$, respectively.

Discussion

Most prolyl oligopeptidases are intracellular enzymes. Extracellular prolyl oligopeptidase was previously purified only from a submerged culture of *Agaricus bisporus* (Sattar *et al.*, 1990) and characterized. In this study, culture filtrates of 22 mushrooms were screened for prolyl oligopeptidase activity. Fourteen strains exhibited prolyl oligopeptidase activity, including *A. bisporus*. Although a medium with the same composition as that described by Sattar *et al.* (1990) was used here, *A. bisporus* had a much lower enzyme activity than was determined by Sattar *et al.* (1990), probably because different strains were used in the studies. Interestingly, four of the 14 strains, including *C. clastophylla*, with relatively high prolyl oligopeptidase activity, were strains of inky cap mushrooms that are known to autolyze soon after completing development of the fruiting body. This study demonstrates that the prolyl oligopeptidase activity in the culture filtrate of *C. clastophylla* is associated with growth. Moreover, malate dehydrogenase activity, a cytosolic marker, was not detected during the growth of *C. clastophylla* (Fig. 2). The results indicate that the prolyl oligopeptidase of *C. clastophylla* is produced extracellularly. Recently, Muraguchi *et al.* (2008) demonstrated that the *exp*1 gene is crucial to pileus expansion and autolysis of the inky cap mushroom *Coprinopsis cinerea* (*Coprinopsis cinereus*). The *exp*1 gene,

predicted to encode an HMG 1/2-like protein with two HMG domains, is strongly induced in the pileus 3 h before pileus expansion. *C. cinerea*, which carries recessive mutations in the *exp*1 gene, is defective in both pileus expansion and autolysis. It will be interesting to search for a counterpart of the *exp*1 gene in *C. clastophylla* and study its expression pattern during the submerged culture of *C. clastophylla*.

Prolyl oligopeptidase from *C. clastophylla* was purified using several purification procedures that were developed by Sattar *et al.* (1990) and Yoshimoto *et al.* (1988a) with minor modification. The best purification procedure resulted in 246 fold purification with a specific activity of 44.85 U/mg and a recovery of 16.38% in four steps (Table 1). However, the protein was still separated into six bands on SDS-PAGE (data not shown). Resource Q was then used to purify the protein further. Three more bands were removed on SDS-PAGE, and only one band was present on native PAGE, causing a considerable loss in total activity and reducing the yield to 0.42%. An attempt was made to increase yield by replacing Resource Q with DEAE. Unfortunately, the yield (0.17%) was even less than that of the aforementioned procedure and the protein was still separated into three bands on SDS-PAGE. In the purification steps, the prolyl oligopeptidase activity was inhibited partially by ammonium sulfate, but the enzyme activity was recovered in the next step, which was phenyl sepharose chromatography. Ammonium sulfate at high concentration is known to inhibit transaminase activity, but this activity can be recovered by reducing the ammonium sulfate concentration (Green *et al.*, 1945).

The prolyl oligopeptidase of *C. clastophylla* was purified to a single band on native PAGE. However, the enzyme was separated into three bands with molecular weights of about 84, 60, and 26 kDa on SDS-PAGE. Generally, different proteins have distinct column chromatographic behaviors or electrophoretic mobilities so they can be purified. However, the 60 and 26 kDa proteins herein do not. Although the enzyme was purified through five purification steps in the presence of protease inhibitors, including gel filtration and ion exchange chromatographies, these proteins were still co-purified and co-migrated as a single band on PAGE despite the marked difference between their sizes. Tandem mass spectrometric analysis revealed that the 84 and 60 kDa proteins shared ten common peptides, strongly suggesting that the 60 kDa protein is a degradation product of the 84 kDa protein. Unfortunately, tandem mass data concerning the 26 kDa protein could not be obtained because the purification yield was so low. More studies are required to determine the origin of the 26 kDa protein. The 26 kDa protein might also be a degradation product of the 84 kDa protein, based on the observation of co-purification and the size of the fragments. If the 26 kDa protein were indeed a degradation product of the 84 kDa protein, then some of the 84 kDa molecules might be nicked during the purification process by an unknown, inhibitor-resistant protease(s), generating the 60 and 26 kDa fragments. These two degradation products may somehow retain a conformation that is very similar to that of the 84 kDa protein, resulting in the co-purification. A similar phenomenon is known to occur in plant starch phosphorylases (Rathore *et al.*, 2009), which exist in multiple forms with distinct intracellular localization. Although the L78 insertion regions of the low affinity type starch phosphorylases of sweet potato and potato undergo proteolytic degradation upon maturation, the nicked enzymes retain their native molecular structures and full catalytic activities (Iwata and Fukui, 1973; Brisson *et al.*, 1989; Chen *et al.*, 2002). The proteolytic degradation of L78 may serve as a regulatory switch to initiate the synthesis of amylose or the phosphorolytic degradation of starch (Chen *et al.*, 2002). The mapping of the cleavage site of *C. clastophylla* prolyl oligopeptidase and determination of its effects on the kinetic properties of the enzyme will be of particular interest. Further studies are required to provide more insight into the nature of this observation.

Table 5. Kinetic parameters of prolyl oligopeptidase from various sources with Z-Gly-Pro- p-NA as the substrate

Source	Z-Gly-Pro-p-NA			Reference
	Km (mM)	Kcat (S^{-1})	Kcat/Km ($mM^{-1}S^{-1}$)	
C. clastophylla	3.42	162	47.4	This study
Carrot	0.09	0.83	9.22	Yoshimoto *et al.* (1987)
T. denicola	0.83	113	136	Makinen *et al.* (1994)
L. cinerascens	0.4	21.11	52.8	Yoshimoto *et al.* (1988b)
A. bisporus	0.41	11.8	28.7	Sattar *et al.* (1990)
P. furiosus	0.18	16.9	106	Harris *et al.* (2001)

The prolyl oligopeptidase from *C. clastophylla* was highly susceptible to PCMB and DFP, similar to those from other sources (Yoshimoto *et al.*, 1987, 1988a; Sattar *et al.*, 1990; Ohtsuki *et al.*, 1994; Goossens *et al.*, 1995). DFP has been shown to have high specificity on seryl residues at active sites, whereas PCMB has rather low selectivity toward the sulfhydryl group (Powers and Wilcox, 1970). The result indicates that *C. clastophylla* prolyl oligopeptidase may be a serine protease. One or more cysteine residues of the enzyme are close to the active site. Prolyl oligopeptidase is a member of the serine peptidase group of the prolyl oligopeptidase family. It is a di-domain enzyme, comprising a peptidase domain and a seven-bladed propeller domain. The catalytic triad (Ser554, Asp641, His680) of the enzyme is located in a large cavity at the interface between the two domains, which is covered by the central tunnel of the β-propeller (Fülöp *et al.*, 1998). *C. clastophylla* prolyl oligopeptidase may have similar structures.

The kinetic parameters of *C. clastophylla* prolyl oligopeptidase were compared with those of other enzymes using Z-Gly-Pro-p-NA as the substrate (Table 5). Notably, the Km value of the enzyme from *C. clastophylla* greatly exceeded those of other organisms. The crystalline structures of porcine and two prokaryotic prolyl oligopeptidases have been determined (Fülöp *et al.*, 1998; Shan *et al.*, 2005). Substrate access to the active site requires conformational changes (Polgar, 1991, 1992; Shan *et al.*, 2005). Structural and mechanistic analyses of *M. xanthus* prolyl oligopeptidase confirmed the important role of several domain interfacial residues in the docking of the substrates (Shan *et al.*, 2005). The unusually low substrate affinity may be explained by the fact that *C. clastophylla* prolyl oligopeptidase has a relatively rigid structure that reduces interdomain flexibility. The conformation makes entry by a substrate into the in-

terdomain region of the enzyme difficult. Also notable is the fact that the enzyme has a relatively high Kcat value (Table 5). Given its high Km value, *C. clastophylla* prolyl oligopeptidase had low affinity for substrates, but the substrate was converted rapidly to the corresponding product once it was bound to the enzyme. Apparently, the rate-limiting step of *C. clastophylla* prolyl oligopeptidase is conformational change, rather than acid-base catalysis. Conformational changes are rate-limiting for known prolyl oligopeptidases (Rea and Fülöp, 2006; Gass and Khosla, 2007; Szeltner and Polgár, 2008), except in the case of the enzyme for which general acid-base catalysis is the main rate-limiting factor (Harris *et al.*, 2001). As the native and nicked forms of *C. clastophylla* prolyl oligopeptidase could not be separated from each other, the kinetic parameters cannot represent the kinetics of the native enzyme. Neither whether the enzyme was active nor whether the nick affected the kinetic parameters was known. More studies are required to reveal the kinetic and structural features of *C. clastophylla* prolyl oligopeptidase.

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