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Note

Purification and Characterization of a Novel Prolyl Aminopeptidase from *Maitake* (*Grifola frondosa*)

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We have found a novel prolyl aminopeptidase in *Grifola frondosa*. The enzyme was purified by DEAE-Sepharose CL-6B, Butyl-Toyopearl, Sephacryl S-100, and Mono-Q column chromatographies. The purified enzyme exists as a dimer and gives high activity toward L-proline-*p*-nitroanilide. The enzyme was strongly inhibited by *p*-chloromercuribenzoic acid and iodoacetic acid and markedly inhibited by phenylmethylsulfonyl fluoride and arphamenin A.

Key words: *Grifola frondosa*; prolyl aminopeptidase; purification; characterization

Prolyl aminopeptidase (PAP) [EC 3.4.11.5] specifically releases amino-terminal proline from peptides. The presence of this enzyme has been reported mainly in micooroganisms.[1–3] Hence, we screened more than 30 kinds of fruiting bodies of edible mushrooms and found that *Maitake* (*Grifola frondosa*) had high PAP activity. *G. frondosa* is one of the most popular edible mushrooms in Japan. It has been reported that it contains endo-[4] or amino-peptidase (AP).[5] Moreover, Nishiwaki *et al.*[6] have reported a capability of AP from *G. frondosa* to debitter soy protein and milk casein hydrolysates. But, there is no report about PAP from *G. frondosa*. In the present study, we purified and characterized some properties of a novel PAP from *G. frondosa* (GfPAP).

PAP activity was measured using L-proline-*p*-nitroanilide (L-Pro-pNA, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) as a substrate. An enzyme solution (50 μl) was mixed with 450 μl of 2 mM L-Pro-pNA in 50 mM sodium phosphate buffer, pH 7.0, and incubated at 37 °C for 30 min. After incubation, the reaction was stopped by the addition of 500 μl of 10% acetic acid, and then the absorbance of the mixture at 405 nm was measured. One enzyme unit was defined as the amount of enzyme that produced 1 μmol *p*-nitroaniline per min ($\varepsilon_{405\,nm}$ = 9,620 $M^{-1}cm^{-1}$).[7] Protein concentration was measured by the method of Lowry *et al.*[8] using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli.[9] The molecular weight of the native enzyme was estimated by gel filtration on FPLC equipped with a HiLoad 16/60 Superdex 75 column (1.6 × 60 cm, Amersham Biosciences, Uppsala, Sweden) using 20 mM sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl and 0.02% NaN_3. Aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used as standard proteins. Mass spectrometric analysis of the purified GfPAP was performed by matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using the Voyager-DE STR (Applied Biosystems, Foster City, California, U.S.A.).

The fruiting bodies (3.5 kg) of *G. frondosa* were homogenized in 7 liters of 5 mM sodium phosphate buffer, pH 7.0 (buffer A), and centrifuged at 10,000 × *g* for 30 min. DEAE-Sepharose CL-6B (360 ml, Amersham Biosciences) was added to the supernatant and gently stirred for 16 h. The adsorbed proteins were eluted with 1.8 liters of 20 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The eluate was brought to 80% saturation with ammonium sulfate. After centrifugation at 10,000 × *g* for 30 min, the precipitate was dissolved in a small volume of buffer A, and dialyzed against buffer A. The dialysate was put on a column (4.6 × 10 cm) of DEAE-Sepharose CL-6B that had been equilibrated with buffer A. The enzyme was eluted with 800 ml of a zero to 0.2 M NaCl linear gradient in the same buffer. The fractions showing peptidase activity were pooled. Solid ammonium sulfate

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Abbreviations: PAP, prolyl aminopeptidase; AP, aminopeptidase; GfPAP, *G. frondosa* prolyl aminopeptidase; PAGE, polyacrylamide gel electrophoresis; pNA, *p*-nitroanilide; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PCMB, *p*-chloromercuribenzoic acid; IAA, iodoacetic acid; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; βNA, β-naphthylamide

was added to the pooled fraction at a final concentration of 1.0 M and put on a column (4.6 × 5 cm) of Butyl-Toyopearl 650M (Tosoh, Tokyo, Japan). The column was washed with the same buffer, and the peptidase was eluted with 500 ml of a 1.0 to zero M ammonium sulfate linear reverse gradient in the same buffer. The active fractions were concentrated by ultrafiltration and put on a column (5.0 × 80 cm) of Sephacryl S-100 HR (Amersham Biosciences) which had been equilibrated with 20 mM sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl and 0.02% NaN_3. The fractions showing peptidase activity were pooled, concentrated by ultrafiltration, and dialyzed against 20 mM sodium phosphate buffer, pH 6.0. The sample was applied to FPLC equipped with a Mono-Q HR 5/5 column (0.5 × 5 cm, Amersham Biosciences) equilibrated with the same buffer. The enzyme was eluted with 30 ml of zero to 0.1 M NaCl linear gradient in the same buffer at a flow rate of 1 ml/min. Fractions showing peptidase activity were pooled and used as purified GfPAP.

The purified preparation showed a single protein band on SDS-PAGE. The molecular weight of the purified enzyme was estimated to be 33,000 and 58,000 by SDS-PAGE and native gel filtration, respectively (data not shown). On the other hand, the molecular mass of the enzyme was measured to be 34,090 by MALDI-TOF/MS. These results indicate that the purified GfPAP exists as a dimer. To determine the N-terminal sequence of GfPAP, 1 μmol of the purified preparation was subjected to protein sequencing (PPSQ-10, Shimadzu, Kyoto, Japan). No PTH amino acid, however, was detected. The N-terminal amino acid may be modified by the acyl group or pyroglutamated. We are now trying to determine the internal amino acid sequence(s) of GfPAP using MALDI-TOF/MS for molecular cloning of the gene.

The purified GfPAP showed a bell-shaped pH optimum curve with maximum activity at pH 7.5. For thermal stability of GfPAP, purified enzyme was incubated in 50 mM sodium phosphate buffer, pH 7.0, for 30 min at various temperatures (15–95 °C), and then the remaining PAP activity was measured. The enzyme was very stable. After incubation at 65 °C, more than 60% of the PAP activity remained. The optimum temperature of the enzyme was also determined to be about 60 °C. Thermostable PAP from *Aeromonas sorbia* has been reported.[2] The enzyme was quite stable. Even after incubation for 15 min at pH 8.0 at 57 °C, about 50% PAP activity remained compared to that of the control. Thus, the GfPAP is much more stable than thermostable PAP from *A. sorbia*. Most interestingly, *G. frondosa* contained other thermostable metalloendopeptidase[4] and AP.[5] But the physiological role of these thermostable proteinases is not yet understood.

The effects of several enzyme inhibitors on GfPAP activity are shown in Table 1. PAP activity strongly inhibited by *p*-chloromercuribenzoic acid (PCMB) and iodoacetic acid (IAA), and markedly inhibited by phenylmethylsulfonyl fluoride (PMSF) and arphamenine A. EDTA, 1, 10-phenanthroline, diisopropylfluorophosphate (DFP), pepstatin A and amastatin had no effect on enzyme activity. Hence, the enzyme appeared to be sulfhydryl peptidase. On the other hand, PAPs from *A. sobria* and *Bacillus coagulans* inhibited by PCMB and heavy metal ions. Moreover, DFP and other proteinase inhibitors had no or little effect. It is commonly assumed that these PAPs are sulfhydryl enzymes.[2,3] But the enzymes were identified as a serine peptidase by site-directed mutagenesis.[10] For the above reason, further studies are necessary to identify the active site residue(s) of GfPAP.

Table 1. Effects of Enzyme Inhibitors on GfPAP Activity

Inhibitor	Final concentration	Remaining activity (%)
None		100
EDTA	5.0 mM	103
1, 10-Phenanthroline	5.0 mM	87
PMSF	5.0 mM	42
	1.0 mM	81
	0.1 mM	96
DFP	5.0 mM	94
PCMB	1.0 mM	3
	0.1 mM	52
IAA	5.0 mM	18
	1.0 mM	31
	0.1 mM	90
Pepstatin A	10.0 μg/ml	96
Amastatin	10.0 μg/ml	93
Arphamenine A	10.0 μg/ml	24
	1.0 μg/ml	66
	0.1 μg/ml	85

As shown in Table 2, the enzyme showed high activity toward L-Pro-pNA, moderate activity toward L-Ala-pNA, and weak activity toward Gly-, L-His-, L-Met-, L-Leu-, L-Ile-, L-Glu-, L-Val-, L-Asp-, L-Lys-, and L-Arg-pNAs. The kinetic parameters of GfPAP against relatively well hydrolyzed substrates are also shown in Table 2. The K_m, k_{cat}, and k_{cat}/K_m values of GfPAP

Table 2. Substrate Specificity and Kinetic Parameters of GfPAP

Substrate[a]	Relative activity[b] (%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
L-Pro-pNA	100	0.73	160	220
L-Ala-pNA	31.4	0.34	28	84
Gly-pNA	14.1	0.22	7.8	36
L-His-pNA	11.4	0.24	6.5	28
L-Met-pNA	8.5			
L-Leu-pNA	3.4			
L-Ile-pNA	3.2			
L-Glu-pNA	2.8			
L-Val-pNA	1.8			
L-Asp-pNA	1.5			
L-Lys-pNA	1.5			
L-Arg-pNA	1.4			

[a] Final substrate concentrations were 2 mM.
[b] Relative activity for L-Pro-pNA was taken to be 100%.

Table 3. Characteristics of PAPs and *G. frondosa* AP

Enzyme	GfPAP[a]	*A. sobria* PAP[2)]	*B. coagulans* PAP[3)]	*G. frondosa* AP[5)]
Optimum pH	7.5	8.5	8.0	8.5
pH stability	4.5–9.5	4.5-8.3	5.5–8.2	6.0–10.5
Optimum temperature	60 °C	55 °C	40 °C	65 °C
Thermal stability	65 °C	57 °C	38 °C	55 °C
Molecular weight				
by gel filtration	58,000	205,000	33,000	27,000
by SDS-PAGE	33,000	43,000	33,000	30,000
Inhibitor(s)	PCMB, IAA	PCMB	PCMB, IAA	EDTA
Suitable substrate	L-Pro-pNA	Pro-βNA[b]	Pro-βNA[b]	L-Leu-pNA

[a] This study.
[b] Pro-βNA, proline β-naphthylamide.

were 0.73 mM, $160\,s^{-1}$, and $220\,mM^{-1}s^{-1}$ for L-Pro-pNA, respectively. The K_m value for L-Pro-pNA was about 2 to 3 times higher than that for other pNA substrates. But the k_{cat} values for L-Pro-pNA were 1 or 2 orders of magnitude higher than that for other substrates. Consequently, GfPAP hydrolyzed L-Pro-pNA 3 times or more effectively than other substrates.

Table 3 shows some properties of PAPs[2,3)] and *G. frondosa* AP.[5)] First we compared the properties of GfPAP with relatively well characterized microbial PAPs. The optimum pH, pH dependent stability, and inhibitors of GfPAP were very similar to those of *A. sobria* and *B. coagulans* PAPs, but the molecular weight of GfPAP was very different from that of *A. sobria* and *B. coagulans* PAPs. GfPAP exists as a dimer. On the other hand, *A. sobria* and *B. coagulans* PAPs exists as a tetramer and a monomer, respectively. Then we compared the properties of GfPAP with those of other AP from *G. frondosa*. The optimum temperature and monomeric molecular weight of GfPAP were very similar to those of *G. frondosa* AP. But substrate specificity, native molecular weight, and the inhibitors of GfPAP were completely different from those of *G. frondosa* AP. These results clearly indicate that GfPAP is a novel PAP from *G. frondosa*.

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