Purification and characterization of a novel prolyl endopeptidase from *Aspergillus niger*

By Keiko KUBOTA,^{*)} Masaru TANOKURA,^{**)} and Kenji TAKAHASHI^{*)},^{†)}

(Communicated by Masanori OTSUKA, M. J. A.)

Abstract: A novel prolyl endopeptidase has been purified to homogeneity and characterized from the crude extract powder of the culture filtrate of *Aspergillus niger* var. *macrosporus*. It was shown to be a monomeric protein with a molecular weight of approximately 54,500, cleaving Pro-X bonds not only in oligopeptides but also in proteins. It has a pH optimum at around 3.7 and is strongly inhibited by diisopropylfluorophosphate, thus being an acid serine endopeptidase. Comparison of the amino acid sequences of related peptidases in the database indicates that it belongs, not to the serine peptidase family S9 which includes prolyl oligopeptidase, but to the serine peptidase family S28 which includes Pro-specific exopeptidases, such as lysosomal Pro-X carboxypeptidase and dipeptidyl peptidase II.

Key words: *Aspergillus niger*; characterization; prolyl endopeptidase; purification.

Introduction. The endopeptidases so far known that are capable of hydrolyzing Pro-X bonds include only prolyl oligopeptidase¹⁾ and IgA1-specific endopeptidase.^{2),3)} The former is a serine peptidase acting only on low molecular mass peptides of less than about 30 residues, and the latter is a sequence-specific serine or metallo-peptidase acting only on a restricted Pro-X bond(s) in the hinge region of IgA1. Therefore, there is no prolyl endopeptidase known which is capable of hydrolyzing Pro-X bonds in both proteins and peptides specifically and extensively. Such a prolyl endopeptidase would be useful for specific hydrolysis of proteins and peptides as well as for extensive degradation of proteins and peptides in combination with peptidases of different specificities. In the present study, we have found and purified a novel prolyl endopeptidase hydrolyzing Pro-X bonds in both proteins and peptides specifically and extensively from the crude enzyme mixture of *Aspergillus niger* var. *macrosporus*. The enzyme has been shown to be an acid serine endopeptidase belonging, not to the serine peptidase family S9 which

includes prolyl oligopeptidase,¹⁾ but to the serine peptidase family S28 which includes Pro-specific exopeptidases, such as lysosomal Pro-X carboxypeptidase⁴⁾ and dipeptidyl peptidase II.⁵⁾

Materials and methods. The enzyme was purified from the crude extract powder (Proctase, Meiji Seika Co. (Tokyo)) obtained from the culture filtrate of *Aspergillus niger* var. *macrosporus*. All purification procedures were performed at 4 °C. The crude extract powder (2.5 g) dissolved in 25 ml of 50 mM sodium acetate buffer (SAB), pH 4.0, was centrifuged at 10,000 rpm for 10 min and the supernatant fraction was applied to a DEAE-Toyopearl 650S (Tosoh) column (0.7 × 13 cm) equilibrated with the same buffer. The enzyme was eluted with 50 mM SAB, pH 2.5, after washing with the pH 4.0 buffer. The active fractions were pooled and concentrated by centrifugal dialysis to change the buffer to 50 mM SAB, pH 4.0, containing 100 mM NaCl. The sample was applied to a Sephacryl S-100 (Amersham Biosciences) column (2.6 × 60 cm) equilibrated with 50 mM SAB, pH 4.0, containing 100 mM NaCl. The active fractions were pooled, concentrated and rechromatographed on the Sephacryl S-100 column under the same conditions. The enzyme fraction was finally applied to a pepstatin-agarose (Sigma) column (1.4 × 1.8 cm) equilibrated with 50 mM SAB, pH 4.0, to remove any contaminating aspergillopepsin I⁶⁾ which is the major pepstatin-sensitive aspartic peptidase abundant in the crude extract powder. The enzyme was eluted

^{*)} Laboratory of Molecular Biochemistry, School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

^{**)} Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.

^{†)} Correspondence to: K. Takahashi.

E-mail: kenjitak@ls.toyaku.ac.jp

Fig. 1. Purification of the peptidase. (A) DEAE-Toyopearl 650S chromatography. 25 ml of the crude extract (0.1 g/ml) of the culture filtrate powder of *Aspergillus niger* var. *macrosporus* was applied to a DEAE-Toyopearl 650S column equilibrated with 50 mM SAB, pH 4.0. The enzyme was eluted with the 50 mM SAB, pH 2.5. Fraction size, 1.5 ml. (B) First Sephacryl S-100 chromatography. The pooled active fraction was concentrated and applied to a Sephacryl S-100 column equilibrated with 50 mM SAB, pH 4.0, containing 100 mM NaCl. Fraction size, 4.0 ml. (C) Second Sephacryl S-100 chromatography. The conditions were the same as in (B). (D) Pepstatin-agarose chromatography. The pooled active fraction was applied to a pepstatin-agarose column equilibrated with 50 mM SAB, pH 4.0, and eluted with the same buffer containing 1 M NaCl and then (after 40 ml) with 100 mM sodium borate buffer, pH 8.0, containing 1 M NaCl. Fraction size, 1.0 ml. In each chromatography, the fractions under the bar were pooled.

ed with the same buffer containing 1 M NaCl and then with 100 mM sodium borate buffer (SBB), pH 8.0, containing 1 M NaCl.

SDS-PAGE was used for purity check and molecular weight determination of the purified enzyme. The final acrylamide concentration in the separation gel was 10%, and the protein was stained with Coomassie Brilliant Blue.

The enzyme activity was assayed in a mixture containing 100 μ l of the enzyme solution and 505 μ l of Boc-Gly-Pro-Leu-Gly-Pro-MCA (Sigma) (99 μ M) in 100 mM SAB, pH 4.0, as a substrate. The reaction was per-

formed at 37 $^{\circ}$ C for 30 min and then stopped by the addition of 500 μ l of 100 mM SBB, pH 10.0. The reaction mixture was analyzed in a HITACHI F-2000 spectrofluorimeter with $\lambda_{\text{ex}} = 380$ nm/ $\lambda_{\text{em}} = 460$ nm. Protein concentration was determined from the absorbance at 280 nm assuming A_{280} (1 mg/ml) = 1.0.

The pH-dependence of the enzyme activity was determined under the same assay conditions with 0.86 μ M enzyme at pH 1.6–8.8. The following 100 mM buffers, containing 200 mM NaCl, were used: KCl/HCl, pH 1.6–2.0, glycine/HCl buffers, pH 2.5–3.0, potassium acetate/HCl, pH 2.9–5.8, and $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 5.7–7.6.

Table I. Purification of the peptidase from the crude enzyme mixture of *Aspergillus niger*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude extract	2,140	69,300	32.5	100	1
DEAE-Toyopearl 650S	59.4	54,230	913	78.3	28.1
1st Sephacryl S-100	13.4	34,900	2,580	50.0	79.5
2nd Sephacryl S-100	10.2	29,300	2,870	42.2	88.5
Pepstatin-Agarose	6.5	15,200	2,340	22.0	72.1

Fig. 2. SDS-PAGE of the purified peptidase. Electrophoresis was performed under reducing conditions.

Fig. 3. Determination of the molecular weight of the enzyme by gel filtration. (A) The enzyme was applied to a Sephacryl S-100 column equilibrated with 50 mM SAB, pH 4.0, containing 100 mM NaCl with the standards: a, blue dextran (MW 2,000,000); b, apoferritin (MW 44,300); c, ribonuclease A (MW 13,700). (B) The molecular weight of the peptidase was estimated from the effluent volume. The peptidase is shown as an open circle, and the closed circles represent apoferritin, bovine serum albumin (MW 67,000) and ribonuclease A. * The void volume is not included.

Fig. 4. Effects of pH and temperature on the activity. (A) The pH dependence of the activity toward Suc-GPLGP-MCA as a substrate at 37 °C at pH 1.6–8.8. The assay conditions are described in “Materials and methods”. The buffers used were: ▲, KCl/HCl buffers; △, glycine/HCl buffers; ●, potassium acetate/HCl buffers; □, $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffers. (B) The temperature dependence of the enzyme activity toward Suc-GPLGP-MCA as a substrate at pH 4.0 and 10–80 °C.

The temperature dependence of activity was also determined under the same assay conditions, except at different temperatures (10–80 °C).

The B chain of oxidized insulin (Sigma) (0.1 mM) was digested with the purified enzyme (1.4 μM) in 30 μl of 100 mM SAB, pH 4.0 at 30 °C for 10 min to 5 h. The reaction was stopped by the addition of 500 μl of 100 mM SBB, pH 10.0, and the resulting peptides were analyzed by LC-MS (LCQTMDUO mass spectrometer, ThermoQuest Co.). Each peptidyl-MCA (0.1 mM) and the enzyme (4.2 μM) were also incubated at 37 °C for 22 h in 505 μl of 20 mM SAB, pH 4.0. The reaction was stopped and the reaction mixture analyzed in the same manner as above. Casein α chain (Sigma) (21 μM) was digested with the enzyme (1.9 μM) in 100 mM SAB, pH 4.0, at 37 °C for 0–20 h. The reaction was stopped by the addition of 30 μl of 100 mM SBB, pH 10.0, and the mixture was analyzed by SDS-PAGE.

In order to estimate the molecular weight of the enzyme, it was applied to a Sephacryl S-100 (Amersham Biosciences) column (2.6 $\times$ 60 cm) equilibrated with 50 mM SAB, pH 4.0, containing 100 mM NaCl

with blue dextran, apoferritin, bovine serum albumin, and ribonuclease A as marker proteins.

Results and discussion. An enzyme hydrolyzing Boc-Gly-Pro-Leu-Gly-Pro-MCA was found in the crude extract powder from *Aspergillus niger* and purified as shown in Fig. 1 and Table I. The purified enzyme obtained after the second Sephacryl S-100 chromatography was essentially homogeneous and about 10 mg of the purified enzyme was obtained from 2.5 g of the crude enzyme mixture in a yield of 42% with an 88-fold purification. This sample was further treated with pepstatin-agarose to completely remove any contaminating aspergillopepsin I although the specific activity and yield were lowered somewhat by this treatment. The purified enzyme was shown to be homogeneous and had a molecular weight of approximately 55,000 as examined by SDS-PAGE (Fig. 2). The results of gel filtration of the enzyme are given in Fig. 3, which show that the enzyme is a monomeric 54,500 dalton protein. The enzyme had an optimal pH at around 3.7 (Fig. 4a) and the maximum activity at about 60 °C (Fig. 4b). The enzyme was most strongly inhibited by diisopropylfluorophosphate, indi-

Table II. Effects of various proteinase inhibitors on the activity

Inhibitor	Conc. (mM)	Activity (%)	Inhibitor	Conc. (mM)	Activity (%)
Aprotinin	0.002	69	1,10-phenanthroline	10	55
DFP	10	4	E64	0.01	100
PMSF	1	87	Pepstatin A	0.1	83
AEBSF	1	100	Bestatin	10	100

DFP, diisopropylfluorophosphate; PMSF, phenylmethanesulfonyl fluoride; AEBSF, 4-amidinoethylbenzenesulfonyl fluoride; E-64, 1-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

Table III. Activity toward various MCA substrates by the enzyme

Peptidyl-MCA substrate	Activity (%)
Boc-Ala-Gly-Pro-Arg-MCA	0
Leu-MCA	0
Z-Val-Lys-Met-MCA	0
Boc-Ala-Ala-Pro-Phe-MCA	0
Glt-Ala-Ala-Phe-MCA	0.8
Phe-MCA	0.2
Boc-Gly-Pro-Leu-Gly-Pro-MCA	92
Boc-Gly-Pro-MCA	30
Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA	0
Z-Leu-Arg-Gly-Gly-MCA	12
Suc-Ala-Glu-MCA	0.1

Boc, *t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Glt, glutaryl; suc, succinyl; MCA, 4-methylcoumaryl-7-amide.

Fig. 5. Cleavage sites in oxidized insulin B chain by the enzyme. Solid arrowhead, major cleavage site; open arrowhead, minor cleavage site.

Fig. 6. Time course of digestion of casein α chain by the enzyme as analyzed by SDS-PAGE.

cating that it is a serine peptidase (Table II). Table III shows the activity of the enzyme toward various peptidyl-MCA substrates. The results show that the enzyme has a highly restricted specificity toward Pro-X bonds although some cleavage was observed at a Gly-X bond. When the B chain of oxidized insulin was digested with the enzyme, the Pro-Lys bond was cleaved very rapidly, followed by much slower cleavages at a few other sites (Fig. 5). In 1 h of digestion, about 90% of the Pro-

Lys bond was cleaved while the extents of cleavage of other peptide bonds were less than 10%. The enzyme hydrolyzed casein α chain extensively as can be seen from Fig. 6. It was also shown to hydrolyze myoglobin, bovine serum albumin, gelatin and collagen (data not shown). Taken together, these results indicate that the enzyme is a prolyl endopeptidase capable of hydrolyzing not only oligopeptides but also proteins. So far, prolyl oligopeptidase is the only ordinary enzyme known to

Fig. 7. Comparison of the amino acid sequences of prolyl endopeptidase and homologous enzymes. DPP, human dipeptidyl peptidase II (accession No. Q9UHL4); PCP, human lysosomal proline carboxypeptidase (accession No. NP_005031); TSSP, human thymus-specific serine peptidase protein (accession No. Q9NQE7); PEP, *Aspergillus niger* prolyl endopeptidase (accession No. AX534874). The amino acid sequences were aligned using the program Clustal W.

hydrolyze Pro-X bonds endoproteolytically, which can hydrolyze only low molecular mass peptides, and no peptidase extensively hydrolyzing prolyl bonds in high molecular mass peptides and proteins is known. Therefore, this is the first time that such an enzyme was found, purified and characterized. The N-terminal sequencing of the purified enzyme gave an 18-residue sequence: Thr-Thr-Gly-Glu-Ala-Tyr-Phe-Glu-Gln-Leu-Leu-Asp-His-His-Asn-Pro-Glu-Lys. From the protein database search, this sequence was found to be identical with the N-terminal region sequence of a protein predicted from a cDNA clone obtained from *Aspergillus niger*, which is thus presumed to be identical with the present enzyme. Therefore, the result also showed that the enzyme is syn-

thesized as a precursor protein and that the activation cleavage site is the Ala-Thr (residue 42-43) bond in the precursor protein. Database searches for the homologous proteins revealed that this enzyme is not homologous with prolyl oligopeptidase belonging to the family S9, but homologous with some of the enzymes belonging to the family S28, such as dipeptidyl peptidase II, lysosomal Pro-X carboxypeptidase and thymus-specific serine peptidase protein as shown in Fig. 7. It is interesting to note that the present enzyme is a monomeric protein whereas dipeptidyl peptidase II and lysosomal Pro-X carboxypeptidase were reported to be dimeric proteins.

During the preparation of the present paper, Edens *et al.*⁷⁾ have reported partial purification of a pro-

lyl endoprotease from *Aspergillus niger*, its overexpression from the cDNA and partial characterization of the overexpressed enzyme with major interest in its use for debittering protein hydrolysates. This enzyme is thought to be the same one as the present enzyme.

Acknowledgments. We thank Drs. Toshiaki Kono and Yuichi Sato (Bioscience Laboratories, Meiji Seika Co.) for the generous supply of crude extract powder of the culture filtrate of *Aspergillus niger* var. *macrosporus*. This study was supported in part by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Japan Society for the Promotion of Science.

References

- 1) Ito, K., Kitazono, A. A., and Yoshimoto, T. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 2, Elsevier Academic Press, London, pp. 1897-1900.
- 2) Poulsen, K., and Kilian, M. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 2, Elsevier Academic Press, London, pp. 1758-1761.
- 3) Plaut, A. G. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 1, Elsevier Academic Press, London, pp. 772-776.
- 4) Tan, F., and Erdős, E. G. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 2, Elsevier Academic Press, London, pp. 1936-1938.
- 5) McDonald, J. K., and Ohkubo, I. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 2, Elsevier Academic Press, London, pp. 1938-1943.
- 6) Ichishima, E. (2004) *In Handbook of Proteolytic Enzymes*, 2nd ed. (eds. Barrett, A. J., Rawlings, N. D., and Woessner, J. F.). Vol. 1, Elsevier Academic Press, London, pp. 92-99.
- 7) Edens, L., Dekker, P., van der Hoeven, R., Deen, F., de Roos, A., and Floris, R. (2005) *J. Agric. Food Chem.* **53**, 7950-7957.

(Received Nov. 22, 2005; accepted Dec. 12, 2005)