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Purification and characterization of a new type of serine carboxypeptidase from *Monascus purpureus*

Abstract Carboxypeptidase produced by *Monascus purpureus* IFO 4478 was purified to homogeneity. The purified enzyme is a heterodimer with a molecular mass of 132 kDa and consists of two subunits of 64 and 67 kDa. It is an acidic glycoprotein with an isoelectric point of 3.67 and 17.0% carbohydrate content. The optimum pH and temperature were 4.0 and 40 °C, respectively. The enzyme was stable between pH 2.0 and 8.0 at 37 °C for 1 h, and up to 50 °C at pH 5.0 for 15 min. The enzyme was strongly inhibited by piperastatin A, diisopropylfluoride phosphate (DFP), phenylmethylsulfonylfluoride (PMSF), and chymostatin, suggesting that it is a chymotrypsin-like serine carboxypeptidase. *Monascus purpureus* carboxypeptidase was also strongly inhibited by *p*-chloromercuribenzoic acid (PCMB) but not by ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline, indicating that it requires cysteine residue but not metal ions for activity. Benzyloxycarbonyl-L-tyrosyl-L-glutamic acid (Z-Tyr-Glu), among the substrates tested, was the best substrate of the enzyme. The K_m, V_{max}, K_{cat}, and K_{cat}/K_m values of the enzyme for Z-Tyr-Glu at pH 4.0 and 37 °C were 0.86 mM, 0.917 mM min^{-1}, 291 s^{-1}, and 339 mM^{-1} s^{-1}, respectively.

Keywords *Monascus purpureus* · Chymotrypsin-like enzyme · Serine carboxypeptidase · Heterodimer · Glycoprotein

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Introduction

Monascus species have been traditionally used in the fermentation industry in East Asia. In China, they are used to produce not only natural colorants but also red wine and red fermented soybean curd. In Okinawa, Japan, they are used for the production of *tofuyo*, which is a vegetable protein food made from soybean curd by the action of microorganisms. *Tofuyo*-making by *Monascus* has been studied in our laboratory [27, 29]. It was found that a large number of free amino acids (e.g. glutamic acid and aspartic acid) were produced during the maturation of *tofuyo*. This was considered to be the result of proteolytic enzymes produced by *Monascus* hydrolyzed soybean protein. However, knowledge of this process is very limited.

In order to elucidate the functions of *Monascus* enzymes in the maturation of *tofuyo*, they must be purified and characterized. In a previous study, the production, purification, and properties of an acid proteinase from *Monascus* were reported [28]. The acid proteinase is an endopeptidase that hydrolyzes proteins from their internal chain to peptides, but only to small amounts of free amino acids [30]. Thus, the action of acid proteinase is not enough to explain the occurrences of a large number of free amino acids during the maturation of *tofuyo*. By contrast, carboxypeptidase is an exopeptidase that releases free amino acids from the carboxyl termini of peptides or proteins. It is considered to serve as a key enzyme in the production of flavorful amino acids during the maturation of *tofuyo*. Although a large number of carboxypeptidases have been isolated from various species of fungi such as *Aspergillus saitoi* [10], *A. oryzae* [17, 19, 20, 21, 22, 23, 24], *A. niger* [4, 14], *Absidia zychae* [16], *Mucor racemsus* [7], *Penicillium janthinellum* [6, 9, 31], and *Pycnoporus sanguineus* [12], the *Monascus* enzyme has not yet been reported. In this study, we describe the purification and characterization of carboxypeptidase produced by *M. purpureus* IFO 4478.

Materials and methods

Organism and cultivation

M. purpureus IFO 4478 was used for the production of carboxypeptidase. The fungus was cultured at 28 °C for 6 days with shaking in a medium containing 2% soybean protein isolate (SPI), 1% glucose, 0.5% $MgSO_4 \cdot 7H_2O$, 0.5% KH_2PO_4 (pH 6.0), with 1% (v/v) mineral solution (per ml: 15 mg $CaCl_2$, 15 mg $FeSO_4 \cdot 7H_2O$, 7.8 µg $CuSO_4 \cdot 5H_2O$, 10 µg H_3BO_3, 10 µg $MnSO_4 \cdot 5H_2O$, 70 µg $ZnSO_4$, and 10 µg MoO_3). The culture broth was filtered through No. 2 filter paper (Advantec, Japan). The filtrate was adjusted to pH 6.0 with 0.1 M citric acid and centrifuged at 10,000 *g* for 30 min. The supernatant was used as a crude enzyme solution for the purification.

Purification of the enzyme

All subsequent manipulations, except HPLC, were carried out at 4 °C.

Step 1. The crude enzyme solution was put on a DEAE-cellulose column (5.0×35 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 6.0). After the column had been washed thoroughly with the same buffer but containing 0.1 M NaCl, the enzyme was eluted with the equilibrating buffer containing 0.2 M NaCl. Active fractions were collected, combined, concentrated by ultrafiltration (UHP-90 K, Advantec), and then dialyzed against 10 mM citrate/20 mM phosphate buffer (pH 6.0).

Step 2. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column (1.5×20 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 6.0). After the column had been washed thoroughly with the same buffer, the enzyme was eluted with a NaCl linear gradient from 0 to 0.4 M in the same buffer. Active fractions were collected, combined, concentrated by ultrafiltration (UHP-90 K, Advantec), and then dialyzed against 10 mM citrate/20 mM phosphate buffer (pH 6.0).

Step 3. The dialyzed enzyme solution was placed on a Q-Sepharose Fast Flow column (1.0×10 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 6.0). After the column had been washed thoroughly with the same buffer, the enzyme was eluted with a NaCl linear gradient from 0 to 0.5 M in the same buffer. Active fractions were collected, combined, concentrated, and the sample buffer exchanged with 10 mM citrate/20 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl by ultrafiltration (Centriprep YM-30, Millipore, USA).

Step 4. The exchanged enzyme solution was injected into a Superdex 200 HR10/30 column equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl, and eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled, concentrated, and the sample buffer exchanged with 25 mM 1-methylpiperazine buffer (pH 5.70) by ultrafiltration (Centriprep YM-30, Millipore, USA).

Step 5. The exchanged enzyme solution was injected into a Mono P HR 5/5 column. Fractions were eluted with a pH gradient from 5.0 to 4.0 generated by 10% Polybuffer 74 (pH 3.97), followed by 20 ml of a NaCl gradient from 0 to 0.4 M in the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled, dialyzed against 10 mM citrate/20 mM phosphate buffer (pH 6.0), and stored at −20°C before use.

Enzyme and protein assay

Carboxypeptidase activity was determined using a modification of the method of Nakadai [18] with Z-Glu-Tyr as the substrate. The reaction mixture, containing 40 µl of enzyme solution and 160 µl of 5.0×10^{-4} M Z-Glu-Tyr in 0.05 M acetate buffer (pH 3.5), was incubated at 37 °C for 30 min. One unit of enzyme activity is defined as the amount of enzyme liberating 1 µmol tyrosine from the substrate per min under the applied conditions.

Protein concentrations were determined by the method of Bradford [2] with bovine serum albumin as the standard. Absorbance at 280 nm was used for monitoring protein concentrations in the fractions during the purification.

Carbohydrate content assay

Carbohydrate content was measured by the phenol sulfuric acid method [8] with D-(+)-mannose as standard.

Electrophoresis

PAGE of the enzyme was carried out by the method of Laemmli [15] using an 8.5% polyacrylamide gel in the presence of SDS (SDS-PAGE), or by the method of Davis [5] using a 10% polyacrylamide gel in the absence of SDS (native PAGE). The protein and carbohydrate bands in the gels were stained with Coomassie Brilliant blue R-250 and periodic acid-Schiff (PAS) reagent according to the method of Zacharius [32], respectively. Isoelectric focusing (IEF)-PAGE was carried out with a pH gradient of 3.5–9.5 generated by ampholine pH 3.5–9.5 (Amersham Pharmacia Biotech, USA). The isoelectric point of the purified enzyme was determined using pI marker proteins from a broad pI calibration kit (Amersham Pharmacia Biotech, USA).

Characterization of the enzyme

The molecular mass of the native and denatured enzyme was estimated by gel filtration on a Superdex 200 HR 10/30 column using the method of Andrews [1] and by SDS-PAGE using the method of Weber [26]. Molecular mass marker proteins (Oriental Yeast, Japan) and polypeptide SDS-PAGE standards (Broad Range, Bio-Rad, Japan), respectively were used for calibration.

To estimate the pH and thermal stabilities, the enzyme was pre-incubated at different pHs at 37 °C for 60 min and at different temperatures at pH 5.0 for 15 min, respectively. Residual activities were determined by the above-described method.

To estimate the effects of various compounds on enzyme activity, the enzyme was pre-incubated with 1 mM of these compounds at 37 °C for 15 min (DFP and PMSF for 2 h), and the residual enzyme activity was measured by the above-described method.

The kinetic parameters of the enzyme for Z-Tyr-Glu at pH 4.0 and 37 °C were calculated from a Lineweaver-Burk plot.

Materials

Monascus purpureus IFO 4478 was obtained from the Institute for Fermentation, OSAKA (Osaka, Japan). Synthetic peptides such as Z-Tyr-Glu, Z-Glu-Tyr and Z-Glu-Phe were purchased from the Peptide Institute, Inc. (Osaka, Japan). DEAE-cellulose and DEAE-Toyopearl 650 M were obtained from Whatman Biosystems (Kent, England) and TOSOH (Tokyo, Japan), respectively. Q-Sepharose Fast Flow, Superdex 200 HR 10/30, and Mono P HR 5/5 were from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals used were of analytical grade.

Results

Production of the enzyme

Preliminarily screening, carried out using 28 strains of *Monascus*, revealed that *M. purpureus* IFO 4478 exhibited the highest ability to produce carboxypeptidase

Table 1 Effects of various nitrogen sources on the production of carboxypeptidase from *Monascus purpureus* IFO 4478. Values are the means of those from three independent assays

Nitrogen source (2% w/v)	Activity (mU/ml filtrate)
Peptone	32.2
Meat extract	19.0
Yeast extract	10.1
Urea	0
Casein	25.2
Polypeptone	32.6
Tryptone	53.3
Soybean protein isolate	64.2
Ammonium sulfate	1.5
Ammonium phosphate monobasic	0.7
Ammonium carbonate	0
Ammonium chloride	0
Ammonium nitrate	0
Sodium nitrate	0
Potassium nitrate	0

Fig. 1A–C Electrophoresis of the purified serine carboxypeptidase from *Monascus purpureus*. **A** SDS-PAGE, **B** Native-PAGE. The gels were stained with CBB. **C** Native-PAGE followed by staining with periodic acid-Schiff. *Lane M* Protein markers, *lane S* purified enzyme

(data not shown); therefore, this strain was chosen for production of the enzyme. The effect of various nitrogen sources in the medium on enzyme production by strain IFO 4478 was examined (Table 1). The enzyme was induced by most organic nitrogen sources (SPI, 64.2 mU/ml; tryptone, 53.3 mU/ml; polypeptone, 32.6 mU/ml; peptone, 32.2 mU/ml), but not by urea or inorganic nitrogen sources. SPI exhibited the highest ability to induce carboxypeptidase, and the addition of SPI at an initial concentration of 2.0% resulted in maximal activity.

Purification of the enzyme

The purification procedures are summarized in Table 2. The enzyme was purified to homogeneity, corresponding to a 213-fold increase compared to the crude enzyme, and a 4% yield. The final enzyme preparation exhibited a single protein band following native-PAGE (Fig. 1B) and IEF-PAGE gel in the absence of urea (native IEF-PAGE, data not shown). However, two protein bands were detected following SDS-PAGE (Fig. 1A) and IEF-PAGE in the presence of urea (denatured IEF-PAGE, data not shown). These results suggested that the enzyme consists of two different polypeptides.

Characterization of the enzyme

The molecular mass of the native enzyme was estimated as 132 kDa by gel filtration. The two protein bands detected on SDS-PAGE gel with (Fig. 1A) or without 2-mercaptoethanol (data not shown) corresponded to molecular mass of 64 and 67 kDa, respectively. These results indicated that the final enzyme preparation is a heterodimer with two subunits linked by a non-disulfide bond.

PAS staining on the native-PAGE gel showed that the purified enzyme is a glycoprotein (Fig. 1C) with 17.0% carbohydrate content. The isoelectric point of the enzyme is estimated to be 3.67 by slab IEF electrophoresis. The optimum pH and temperature of the enzyme for Z-Glu-Tyr were around 4.0 and 40 °C, respectively. The enzyme is stable between pH 2.0 and 8.0 at 37 °C for 1 h, and up to 50 °C at pH 5.0 for 15 min.

The effects of various metal salts and reagents on the enzyme activity were examined at pH 4.0 as shown in Table 3. The enzyme was completely inhibited by Sn^{2+} and SDS, strongly inhibited by Pb^2, Fe^{3+}, DFP, PMSF, piperastatin A, chymostatin, and PCMB, partially inhibited by Hg^{2+} and TPCK, but not inhibited by EDTA and 1,10-phenanthroline. In addition, the enzyme was not activated by metal ions, indicating that it is a chymotrypsin-like serine carboxypeptidase, and that there may be cysteine and histidine residues but no metal ion in or near its active site.

Table 2 Purification of carboxypeptidase from *Monascus purpureus* IFO 4478

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude enzyme	4675	42.4	0.01	1	100
DEAE-cellulose	1046	39.0	0.04	4	92
DEAE-Toyopearl 650 M	69.5	19.1	0.27	27	45
Q-Sepharose Fast Flow	14.7	10.2	0.69	69	24
Superdex 200 HR 10/30	3.7	4.7	1.27	127	11
Mono P HR 5/5	0.8	1.7	2.13	213	4

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Table 3 Effects of various metal salts and reagents on the activity of carboxypeptidase from *Monascus purpureus* IFO 4478. *DTNB* 5,5'-Dithio-bis (2-nitrobenzoic acid), *EPNP* 1,2-epoxy-3-(*p*-nitrophenoxy) propane

Metal salt or reagent (final concentration 1 mM)	Relative activity (%)	Metal salt or reagent (final concentration 1 mM)	Relative activity (%)
None	100	DFP	37
MgCl$_2$	108	PMSF	28
CaCl$_2$	84	Piperastatin A	30
MnCl$_2$	86	TLCK	87
FeCl$_2$	90	TPCK	43
FeCl$_3$	8	Chymostatin	11
CoCl$_2$	93	Elastatinal	113
NiCl$_2$	103	STI	88
CuCl	63	Leupeptin	104
CuCl$_2$	61	PCMB	5
ZnSO$_4$	99	MIA	90
HgCl$_2$	46	NEM	29
PbCl$_2$	5	DTNB	104
CdCl$_2$	103	E-64	109
SnCl$_2$	0	SDS	0
EDTA	98	DTT	101
1,10-Phenanthroline	98	2-Mercaptoethanol	90
r,r'-Dipyridyl	100	Pepstatin A	92
Phosphoramidon	107	EPNP	104

Table 4 Substrate specificity of the enzyme

Substrate (final concentration 0.5 mM)	Relative activity (%)
Z-Glu-Tyr	100
Z-Glu-Phe	119
Z-Tyr-Glu	134
Z-Phe-Ala	77
Z-Phe-Gly	24
Z-Phe-Leu	21
Z-Phe-Tyr	18
Z-Ala-Glu	76
Z-Gly-Pro-Leu-Gly	59
Z-Gly-Leu	8
Z-Gly-Phe	3
Z-Gly-Pro	3
Z-gly-Pro-Leu	0
Bz-Gly-Arg	3
Bz-Gy-Lys	0
Z-Gy-Leu-NH$_2$	4
Z-Gly-Phe-NH$_2$	2
Bz-Tyr-pNA	0
Bz-L-Arg-pNA-HCl	3
Leu-Gly-Gly	2

The substrate specificity of the enzyme is shown in Table 4. The enzyme has a broad substrate specificity catalyzing the hydrolysis of various peptides. Among the substrates tested, Z-Tyr-Glu was the best substrate of the enzyme, followed by Z-Glu-Phe and Z-Glu-Tyr. The enzyme prefers peptides containing an aromatic or glutamic acid in the C-terminal position (P1′) or penultimate position (P1) of the C-terminus, suggesting that it possesses chymotrypsin-like activity. The enzyme did not nearly hydrolyze peptides containing an amide
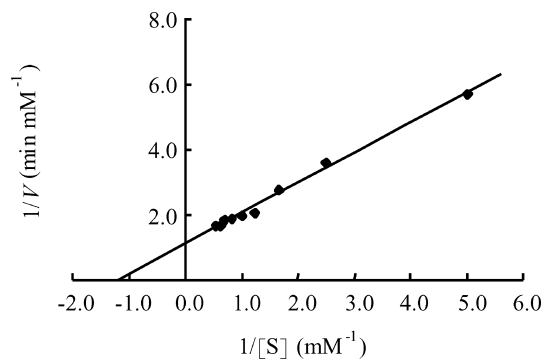
Fig. 2 Lineweaver-Burk plot of *M. purpureus* IFO 4478 carboxypeptidase catalyzing the hydrolysis of Z-Glu-Tyr at pH 4.0, 37 °C

group in the C-terminal position (P1′) or proline or glycine in the penultimate position (P1).

The kinetic parameters of the enzyme for Z-Tyr-Glu at pH 4.0 and 37 °C were calculated from a Lineweaver-Burk plot (Fig. 2). The K_m, V_{max}, k_{cat}, and k_{cat}/K_m of the enzyme were 0.86 mM, 0.917 mM min^{-1}, 291 s^{-1}, and 339 mM^{-1} s^{-1}, respectively.

Discussion

In preliminary experiments, we found that *M. purpureus* IFO 4478 exhibited the highest ability to produce carboxypeptidase among 28 strains of the genus *Monascus*. Further experiments on induction of the enzyme by various nitrogen compounds suggested that SPI was the most potent inducer of carboxypeptidase from *M. purpureus* IFO 4478. It has also been reported that carboxypeptidases from *Aspergillus saitoi* and *Penicillium janthinellum* are induced by defatted soybean [11, 31].

The *M. purpureus* IFO 4478 enzyme was purified to homogeneity and characterized. Inhibitory and specificity studies on the purified enzyme suggested that it is a chymotrypsin-like serine carboxypeptidase. Serine carboxypeptidases are widely distributed in fungi as well as in higher plants and animal tissues, but they are particularly abundant in filamentous fungi [3]. Carboxypeptidase from *M. purpureus* IFO 4478, like most other fungal serine carboxypeptidases, has an acidic optimal pH and requires a serine residue, but not metal ions for its catalytic activity [3]. Serine carboxypeptidase from *M. purpureus* IFO 4478 was strongly inhibited by PCMB, partially by TPCK, indicating that cysteine and histidine residues are located in or near its active site, similar to the serine carboxypeptidase from yeast [13].

SDS-PAGE and molecular mass analysis of the purified enzyme revealed that it is a heterodimer with a native molecular mass of 132 kDa. The enzyme comprises two different subunits, of 64 and 67 kDa, linked by a non-disulfide bond. Although carboxypeptidases from other fungi, such as those from *A. saitoi* [10] and *A. niger* [14], carboxypeptidase Ib, I, II, and O from *A. oryzae* [17, 19, 20, 24], and carboxypeptidase S2 from

P. janthinellum [9] have similar molecular masses, they are not heterodimers, but either homodimers or homotrimers [10] whose subunits are made up of a single polypeptide chain linked by disulfide bridge. The serine carboxypeptidase from *M. racemosus* is also a heterodimer [7], but with a lower native molecular mass of 52 kDa. That enzyme comprises two different subunits, of 31and 18 kDa, linked by a disulfide bridge.

Serine carboxypeptidase from *M. purpureus*, like most other fungal carboxypeptidases, is an acidic glycoprotein with an isoelectric point of 3.67 and 17.0% carbohydrate content. The isoelectric point is similar to that of carboxypeptidase I from *A. niger* [4] and carboxypeptidase S1 from *P. janthinellum* [9]; however, the carbohydrate content is lower than that of carboxypeptidase I from *A. niger* [4] and higher than that of carboxypeptidase S1 from *P. janthinellum* [9].

The relative hydrolysis rates of *N*-acylpeptides indicated that the enzyme, like acid carboxypeptidase from *A. saitoi* [10], prefers substrates with bulky amino acids, such as glutamic acid, tyrosine, and phenylalanine, in the penultimate position (P1) from the C-terminus, but it also prefers substrates with bulky amino acids in the C-terminal position (P1′), in contrast to the enzyme from *A. saitoi* [10], which prefers substrates with neutral and basic amino acids, even proline, in the C-terminal position (P1′). The *M. purpureus* enzyme, like acid carboxypeptidases from *A. oryzae* var. *viride* [17], does not hydrolyze peptides with glycine or proline in the penultimate position (P1). Z-Tyr-Glu was the best substrate of carboxypeptidase from *M. purpureus*, also similar to carboxypeptidase Ib from *A. oryzae* var. *viride* [17]. This is consistent with the findings that glutamic acid is the one of the most abundant free amino acids found in matured *tofuyo* [27, 29]. Z-Glu-Tyr was also reported to be the best substrate of acid carboxypeptidases III, IV, O-1, and O-2 from *A. oryzae* [21, 22, 23], carboxypeptidases Z-1 and Z-2 from *A. zychae* [16], and carboxypeptidase S1 from *P. janthinellum* [9], while Z-Glu-Phe was the best substrate of serine carboxypeptidase from *P. carneus* [25]. In the present study, both Z-Glu-Tyr and Z-Glu-Phe were good substrates of carboxypeptidase from *M. purpureus*. Thus, carboxypeptidase from *M. purpureus* appears to play an important role in removing the bitter compounds from soybean proteins and peptides during the maturation of *tofuyo*.

This is the first report on the purification and characterization of a serine carboxypeptidase from the genus *Monascus*. Although it is similar to other fungal serine carboxypeptidases in its molecular mass, glycoprotein, pI, optimum pH, and substrate specificity, it differs in its protein structure (heterodimer, the two subunits of which are not linked by a disulfide bond). Therefore, it seems to be a new type of fungal serine carboxypeptidase. Cloning and sequencing of the gene encoding the *M. purpureus* serine carboxypeptidase will elucidate the molecular features of the enzyme. In addition, the large-scale synthesis of this enzyme and its application in the food industry will be the subjects of further studies.

References

1. Andrews P (1965) The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem J 96:595–606
2. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
3. Breddam K (1986) Serine carboxypeptidase: a review. Carlsberg Res Commun 51:83–128
4. Dal Degan F, Ribadeau-Dumas B, Breden K (1992) Purification and characterization of two serine carboxypeptidases from *Aspergillus niger* and their use in C-terminal sequencing of proteins and peptide synthesis. Appl Environ Microbiol 58:2144–2152
5. Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427
6. Dey ES, Aasmul-Olsen S (1993) Novel serine penicillocarboxypeptidase CPD-S3 from *Penicillium janthinellum* IBT 3991: purification characterization, and uses in peptide synthesis and modification. Enzyme Microb Technol 15:1042–1050
7. Disanto ME, Li QH, Logan DA (1992) Purification and characterization of a developmentally regulated carboxypeptidase from *Mucor racemosus*. J Bacteriol 174:447–455
8. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugar and related substances. Anal Chem 38:350–356
9. Hofmann T (1976) Penicillocarboxypeptidase S-1 and S-2. Methods Enzymol 45:587–599
10. Ichishima E (1972) Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*. Biochem Biophys Acta 258:274–288
11. Ichishima E, Yamane A, Nitta T, Kinoshita M, Nikkuni S, Oka T, Yokoyama S (1973) Production of a new type of acid carboxypeptidase of molds of the *Aspergillus niger* group. Appl Microbiol 26:327–331
12. Ichishima E, Yoshimura K, Tomoda K (1983) Acid carboxypeptidase from a wood-deteriorating basidiomycete, *Pycnoporus sanguineus*. Phytochemistry 22:825–829
13. Kuhn RW, Walsh KA, Neurath H (1974) Isolation and partial characterization of an acid carboxypeptidase from yeast. Biochemistry 13:3871–3877
14. Kumagai I, Yamasaki M, Ui N (1981) Isolation, purification and some chemical properties of an acid carboxypeptidase from *Aspergillus niger* var. *Macrosporus*. Biochim Biophys Acta 659:334–343
15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
16. Lee BR, Takeuchi M, Kobayashi Y (1993) Purification and characterization of serine carboxypeptidase from *Absidia Zychae*. Biosci Biotechnol Biochem 57:618–622
17. Mikami S, Miura O, Watanabe T, Iwano K, Shinoki S, Shimada T, Nunokawa Y (1987) Purification and some properties of acid carboxypeptidase from Sake koji. Hakkokogaku (in Japanese) 65:37–43
18. Nakadai T, Nasuno S, Iguchi N (1971) Quantitative estimation of activities of peptidases in Koji. Tyoumikagaku (in Japanese) 18:435–441
19. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase I from *Aspergillus oryzae*. Agric Biol Chem 36:1343–1352
20. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase II from *Aspergillus oryzae*. Agric Biol Chem 36:1473–1480
21. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase III from *Aspergillus oryzae*. Agric Biol Chem 36:1481–1488
22. Nakadai T, Nasuno S, Iguchi N (1973) Purification and properties of acid carboxypeptidase IV from *Aspergillus oryzae*. Agric Biol Chem 37:1237–1251

23. Takeuchi M, Ushijima T, Ichishima E (1982) A new acid carboxypeptidase, O-1, from *Aspergillus oryzae*. Curr Microbiol 7:19–23
24. Takeuchi M, Ichishima E (1986) A 155 K acid carboxypeptidase O from *Aspergillus oryzae*. Agric Biol Chem 50:633–638
25. Umetsu H, Hishinuma K, Wake E, Ichishima E (1996) Production, purification, and properties of serine carboxypeptidase from *Paecilomyces carneus*. Curr Microbiol 33:44–48
26. Weber K, Pringle JR, Osborn M (1972) Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol 26:3-27
27. Yasuda M (1990) Studies on manufacturing of tofuyo. Nippon Shokuhin Kogyo Gakkaishi (in Japanese) 37:403–409
28. Yasuda M, Shimabukuro M, Kikuchi S (1991) Production, purification and properties of acid proteinase from genus *Monascus*. Nippon shokuhin kogyo Gakkaishi 38:954–961

29. Yasuda M, Matsumoto T, Sakaguchi M, Kobamoto N (1993) Changes in chemical components of tofuyo prepared by *Monascus* fungus during fermentation. Nippon Shokuhin Kogyo Gakkaishi (in Japanese) 40:331–338
30. Yasuda M, Sakaguchi M (1998) Degradation of soybean protein by an acid proteinase from *Monascus anka*. Food Sci Technol Int Tokyo 4:6–8
31. Yokoyama S, Oobayashi A, Tanabe O, Ichishima E (1974) Submerged production, purification, and crystallization of acid carboxypeptidase from *Penicillium janthinellum* IFO-8070. Appl Microbiol 28:742–747
32. Zacharius RM, Zell TE, Morrison JH, Woodlock JJ (1969) Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem 30:148–152