J Ind Microbiol Biotechnol (2004) 31: 572–580
DOI 10.1007/s10295-004-0190-1

ORIGINAL PAPER

Fang Liu · Shinjiro Tachibana · Toki Taira
Masanobu Ishihara · Fumio Kato · Masaaki Yasuda

Purification and characterization of a high molecular mass serine carboxypeptidase from *Monascus pilosus*

Received: 21 May 2004 / Accepted: 20 October 2004 / Published online: 9 December 2004

Abstract Two serine carboxypeptidases, MpiCP-1 and MpiCP-2, were purified to homogeneity from *Monascus pilosus* IFO 4480. MpiCP-1 is a homodimer with a native molecular mass of 125 kDa composed of two identical subunits of 61 kDa, while MpiCP-2 is a high mass homooligomer with a native molecular mass of 2,263 kDa composed of about 38 identical subunits of 59 kDa. This is unique among carboxypeptidases and distinguishes MpiCP-2 as the largest known carboxypeptidase. The two purified enzymes were both acidic glycoproteins. MpiCP-1 has an isoelectric point of 3.7 and a carbohydrate content of 11%, while for MpiCP-2 these values were 4.0 and 33%, respectively. The optimum pH and temperature were around 4.0 and 50°C for MpiCP-1, and 3.5 and 50°C for MpiCP-2. MpiCP-1 was stable over a broad range of pH between 2.0 and 8.0 at 37°C for 1 h, and up to 55°C for 15 min at pH 6.0, but MpiCP-2 was stable in a narrow range of pH between 5.5 and 6.5, and up to 50°C for 15 min at pH 6.0. Phenylmethylsulfonylfluoride strongly inhibited MpiCP-1 and completely inhibited MpiCP-2, suggesting that they are both serine carboxypeptidases. Of the substrates tested, benzyloxycarbonyl-L-tyrosyl-L-glutamic acid (Z-Tyr-Glu) was the best for both enzymes. The K_m, V_{max}, K_{cat} and K_{cat}/K_m values of MpiCP-1 for Z-Tyr-Glu at pH 4.0 and 37°C were 1.33 mM, 1.49 mM min^{-1}, 723 s^{-1} and 545 mM^{-1} s^{-1}, and those of MpiCP-2 at pH 3.5 and 37°C were 1.55 mM, 1.54 mM min^{-1}, 2,039 s^{-1} and 1,318 mM^{-1} s^{-1}, respectively.

F. Liu · S. Tachibana · T. Taira · M. Ishihara · M. Yasuda (✉)
Department of Bioscience and Biotechnology,
Faculty of Agriculture, University of the Ryukyus,
1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan
E-mail: yasuda@agr.u-ryukyu.ac.jp
Tel.: +81-98-8958807
Fax: +81-98-8958734

F. Kato
Department of Applied Biological Sciences,
Faculty of Agriculture, Saga University,
1 Honjo-machi, Saga 880-8502, Japan

Keywords *Monascus pilosus* · Red koji · Serine carboxypeptidase · Homodimer · Homooligomer

Introduction

Species in the genus *Monascus* have traditionally been 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 using *Monascus* has been studied in our laboratory [40, 43]. It was found that a large number of free amino acids (e.g., glutamic acid and alanine) are produced during the maturation of tofuyo. This is thought to be the result of the hydrolysis of soybean protein by the various proteolytic enzymes produced by *Monascus*.

In order to elucidate the functions of these enzymes in the maturation of tofuyo, it is necessary to purify and characterize these enzymes. In a previous study, the production, purification and properties of an acid proteinase from the genus *Monascus* were reported [42]. Acid proteinase is one of the endopeptidases that hydrolyze proteins at internal sites to peptides, but to only small amounts of free amino acids [41]. Therefore, the action of the acid proteinase was not sufficient to explain the occurrence of the large quantity of free amino acids during the maturation of tofuyo. On the other hand, carboxypeptidase is an exopeptidase that releases free amino acids from the carboxyl termini of peptides or proteins. It is thought to serve as a key enzyme in the production of the taste-conferring amino acids during the maturation of tofuyo [40, 43]. Although a large number of carboxypeptidases have been isolated from various species of fungi, such as *Aspergillus saitoi* [13, 35], *Aspergillus oryzae* [28–32, 34, 36], *Aspergillus niger* [5, 22, 23], *Absidia zychae* [25], *Mucor racemosus* [8], *Penicillium janthinellum* [7, 12, 44, 45], and *Pycnop-*

orus sanguineus [15], and carboxypeptidase Y from yeast [11, 17, 20], reports on *Monascus* enzymes are very few.

In a recent report, we described the purification and characterization of a new type of serine carboxypeptidase (MpuCP) from *Monascus purpureus* [26]. MpuCP is a serine carboxypeptidase purified from a submerged culture broth of this strain. We now report the purification and characterization of the enzyme from solid station fermentation of *Monascus pilosus*.

Materials and methods

Materials

M. pilosus IFO 4480 was obtained from the Institute for Fermentation Osaka (IFO) (Osaka, Japan). Synthetic peptides such as benzyloxycarbonyl-L-tyrosyl-L-glutamic acid (Z-Tyr-Glu), Z-Glu-Tyr and Z-Glu-Phe were purchased from the Peptide Institute, (Osaka, Japan). DEAE-cellulose was purchased from Whatman Biosystem (Kent, UK). Phenyl-Toyopearl 650 M and SP-Toyopearl 650 M were obtained from Tosoh (Tokyo, Japan). Superdex 200 HR 10/30 and Superose 6 10/300 GL were from Amersham Pharmacia (Uppsala, Sweden), and all other chemicals used were of analytical grade.

Organism and cultivation

Screening tests were performed with 28 strains of genus *Monascus*. *M. pilosus* IFO 4480 was selected for the production of carboxypeptidase. *M. pilosus* IFO 4480 was cultivated on steamed rice at 32°C for 8 days, obtaining red molded rice (red koji). The enzyme was extracted from red koji with three volumes of 10 mM citrate/20 mM phosphate buffer (pH 5.0) at 4°C for 24 h with stirring. The mixture was filtered through nylon cloth and then centrifuged at 10,000 *g* for 30 min. The supernatant was used as a crude enzyme solution for purification.

Enzyme purification

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

Step 1

The crude enzyme solution was loaded onto a DEAE-cellulose column (2.5×30 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 5.0). After washing the column thoroughly with the same buffer containing 0.1 M NaCl, the enzyme was eluted with the same buffer containing 0.3 M NaCl. Active fractions were collected and adjusted to 4 M NaCl.

Step 2

The adjusted enzyme solution was applied to a Phenyl-Toyopearl 650 M column (2.5×20 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 5.0) containing 4 M NaCl. After washing the column thoroughly with the same buffer, the enzyme was eluted with a decreasing linear gradient of NaCl from 4 to 0 M in 10 mM citrate/20 mM phosphate buffer (pH 5.0). Active fractions were collected and concentrated by ultrafiltration (UHP-90 K; Advantec, Japan). The concentrate was dialyzed against 10 mM citrate/20 mM phosphate buffer (pH 3.5).

Step 3

The dialyzed enzyme solution was applied to a SP-Toyopearl 650 M column (2.5×15 cm) equilibrated with 10 mM citrate/20 mM phosphate buffer (pH 3.5). After washing the column thoroughly with the same buffer, the enzyme was eluted with an increasing linear gradient of NaCl from 0 to 0.1 M in the same buffer. Active fractions were collected and concentrated, followed by exchange with 10 mM citrate/20 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl by ultrafiltration (Centriprep YM-30; Millipore, Bedford, Mass.).

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 0.4 ml min^{-1}. Active fractions were pooled and concentrated, followed by exchange with 10 mM citrate/20 mM phosphate buffer (pH 6.0) by ultrafiltration (Centriprep YM-30) and stored at −20°C until used.

Enzyme and protein assay

Carboxypeptidase activity was determined by the method of Nakadai [29] using Z-Glu-Tyr as the substrate, with a slight modification. The reaction mixture, containing 40 µl enzyme solution and 160 µl 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 was defined as the amount of enzyme that liberates 1 µmol tyrosine from the substrate per minute under the conditions described above. When the other substrates were used, the appropriate amino acids were measured, for example, glutamic acid for Z-Tyr-Glu.

Protein concentration was determined with a DC protein assay kit (Bio-Rad, Japan) 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 [9] with D(+)-mannose as standard.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the enzymes was performed by the method of Laemmli [24] with 8.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (SDS-PAGE), or by the method of Davis [6] with 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 (CBB) and periodic acid-Schiff (PAS) reagent, respectively, according to the method of Kapitany [18]. Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) was carried out with a gradient of pH 3.5–9.5 generated by ampholine pH 3.5–9.5 (Amersham Pharmacia). The isoelectric points of the purified enzymes were determined with a broad p*I* calibration kit (Amersham Pharmacia) used as p*I* marker proteins.

Enzyme characterization

The molecular masses of the native enzymes were estimated by gel filtration on a Superose 6 10/300 GL column using the methods of Whitaker [39] and San-Marina and Nicholls [33]. The column was calibrated with high and low molecular weight gel filtration calibration kits as described in the supplier's instructions (Amersham Biosciences), and the apparent molecular masses were calculated from their elution positions relative to standard proteins. The molecular masses of the denatured enzymes were estimated by SDS-PAGE using the method of Weber et al. [38] with polypeptide SDS-PAGE standards (Broad Range; Bio-Rad, Japan) for calibration.

To estimate the pH and thermal stabilities, MpiCP-1 and MpiCP-2 were pre-incubated at different pH values at 37°C for 60 min and at different temperatures at pH 6.0 for 15 min, respectively. The residual activities were determined by the method described above.

To estimate the effects of various metal salts and compounds on enzyme activity, MpiCP-1 and MpiCP-2 were pre-incubated with 1 mM of various compounds at 37°C and pH 6.0 for 15 min. The residual activities were measured by the method described above.

The kinetic parameters of MpiCP-1 and MpiCP-2 for Z-Tyr-Glu at pH 4.0 and pH 3.5, respectively, at 37°C were calculated from Lineweaver-Burk plots.

Results

Enzyme production

A screening test of 28 strains of *Monascus* in solid culture revealed that *Monascus* sp. G-1 exhibited the highest ability to produce carboxypeptidase but a much lower ability to produce proteinase. *M. pilosus* IFO 4480 showed the second highest ability to produce carboxypeptidase and the highest ability to produce proteinase (Table 1). Because it was considered that it is the combined actions of proteinase and carboxypeptidase in the solid culture product, red koji, that play an important role in the formation of taste-conferring free amino acids during the maturation of tofuyo, rather than their individual actions, we selected *M. pilosus* IFO 4480 for production of the enzymes.

Enzyme purification

The enzyme purification procedures are summarized in Table 2. The two carboxypeptidases, MpiCP-1 and MpiCP-2, were purified 22-fold with yields of 5% and 1%, respectively. Both MpiCP-1 and MpiCP-2 exhibited a single protein band on SDS-PAGE gel (Fig. 1), native-PAGE (data not shown) and IEF-PAGE gel (data not

Table 1 Production of carboxypeptidase by various strains of genus *Monascus* in solid culture

Strains	Proteinase activity (U mg^{-1} protein)	Carboxypeptidase activity (U mg^{-1} protein)
M. pilosus IFO 4480	0.484	0.447
M. pilosus IFO 4521	0.114	0.008
M. pilosus IFO 5965	0.028	0.102
M. pilosus IFO 8201	0.064	0
M. purpureus IFO 4478	0.052	0.062
M. purpureus IFO 4482	0.103	0.030
M. purpureus IFO 4485	0.100	0.265
M. purpureus IFO 6540	0.044	0.042
M. ruber IFO 4483	0.234	0.118
M. ruber IFO 4492	0.113	0.182
M. ruber IFO 4532	0.348	0.338
M. ruber IFO 7537	0.145	0.119
Monascus sp. 4303	0.043	0.059
Monascus sp. 4820	0.365	0.238
Monascus sp. C-1	0.028	0.148
Monascus sp. C-1-1	0.084	0.022
Monascus sp. C-3	0.055	0.036
Monascus sp. C-4	0.084	0.055
Monascus sp. C-6	0.047	0.051
Monascus sp. C-11	0.068	0.038
Monascus sp. G-1	0.078	0.474
Monascus sp. H-1	0.178	0.078
Monascus sp. H-2	0.021	0.012
Monascus sp. H-3	0.145	0.043
Monascus sp. HK	0.033	0.002
Monascus sp. N	0.107	0.052
Monascus sp. T-1	0.057	0.268
Monascus sp. TSG	0.057	0.198

Table 2 Purification of carboxypeptidases from *Monascus pilosus* IFO 4480

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	1,017	181	0.18	1	100
DEAE-cellulose	350	166	0.47	3	92
Phenyl-Toyopearl 650 M	55	83	1.51	8	46
SP-Toyopearl 650 M					
Peak 1	13.5	47	3.48	19	26
Peak 2	3.2	5.5	1.72	10	3
Superdex 200 HR 10/30					
MpiCP-1	2.3	9.0	3.91	22	5
MpiCP-2	0.5	2.0	4.00	22	1

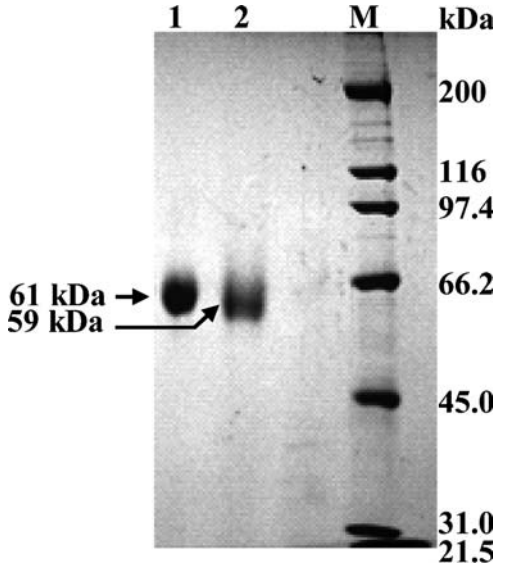

Fig. 1 SDS-PAGE, stained with coomassie brilliant blue (CBB), of carboxypeptidases purified from *Monascus pilosus* IFO 4480. Lanes: *1* Purified MpiCP-1, *2* purified MpiCP-2, *M* protein markers

shown), suggesting that both were purified to homogeneity and consisted of only a single polypeptide.

Enzyme characterization

Some properties of MpiCP-1 and MpiCP-2 are summarized in Table 3. Although MpiCP-1 and MpiCP-2 had the same optimum temperature of 50°C, they had many different properties from each other. The molecular masses of native MpiCP-1 and MpiCP-2 were estimated as 125 kDa (Fig. 2a) and 2,263 kDa (Fig. 2b) by gel filtration, while the protein bands detected on SDS-PAGE gels with 2-mercaptoethanol corresponded to molecular masses of 61 and 59 kDa, respectively (Fig. 1), indicating that MpiCP-1 and MpiCP-2 are a homodimer and a homooligomer, respectively. Purified MpiCP-1 and MpiCP-2 are glycoproteins, as demonstrated by the fact that the carbohydrate bonds stained by PAS had the same mobilities as those of the protein bands stained by CBB on SDS-PAGE gels (data not shown). The carbohydrate contents of MpiCP-1 and MpiCP-2 were estimated at 11 and 33%, respectively.

Table 3 Properties of *Monascus* carboxypeptidases

Property	MpuCP[a]	MpiCP-1	MpiCP-2
Molecular mass (kDa)			
Gel filtration	132	125	2,263
SDS-PAGE	64; 67	61	59
Optimal pH	4.0	4.0	3.5
pH stability (37°C, for 1 h)	2.0-8.0	2.0-8.0	5.5-6.5
Optimal temperature (°C)	40	50	50
Thermal stability (°C, for 15 min)	50	55	50
Isoelectric point	3.7	3.7	4.0
Carbohydrate content (%)	17	11	33
Preferred substrate	Z-Tyr-Glu	Z-Tyr-Glu	Z-Tyr-Glu

[a]From reference [26]

The isoelectric points were estimated at 3.7 and 4.0, respectively, by IEF-PAGE (data not shown). The optimum pH values for activity of MpiCP-1 and MpiCP-2 against Z-Glu-Tyr were around 4.0 and 3.5, respectively. MpiCP-1 was stable between pH 2.0 and 8.0 at 37°C for 1 h and up to 55°C at pH 6.0 for 15 min, but MpiCP-2 was stable between pH 5.5 and 6.5 at 37°C for 1 h and up to 50°C at pH 6.0 for 15 min.

The effects of various metal salts on the activities of MpiCP-1 and MpiCP-2 are summarized in Table 4. No metal salt activated both MpiCP-1 and MpiCP-2; MpiCP-2 was completely inhibited by Hg^{2+} and strongly inhibited by Fe^{3+}, Cu^{+}, Fe^{2+} and Cu^{2+} ions, while no metal salt significantly affected MpiCP-1.

The effects of various reagents on the activities of MpiCP-1 and MpiCP-2 are summarized in Table 5. MpiCP-1 was strongly, and MpiCP-2 completely, inhibited by phenylmethylsulfonylfluoride (PMSF); moreover, diisopropylfluorophosphate (DFP) also inactivated both of them to some extent, suggesting that they are both serine carboxypeptidases. MpiCP-2 was completely inhibited by chymostatin and strongly by *p*-chloromercuribenzoate (PCMB), MpiCP-1 was also slightly affected by these reagents, suggesting that they are both chymotrypsin-like serine carboxypeptidases and may have a cysteine residue in or near their active sites. On the other hand, neither MpiCP-1 nor MpiCP-2 were inhibited by 1,10-phenanthroline, suggesting that no metal ion is in or near their active sites. In addition, MpiCP-1 was strongly inhibited by piperastatin A and

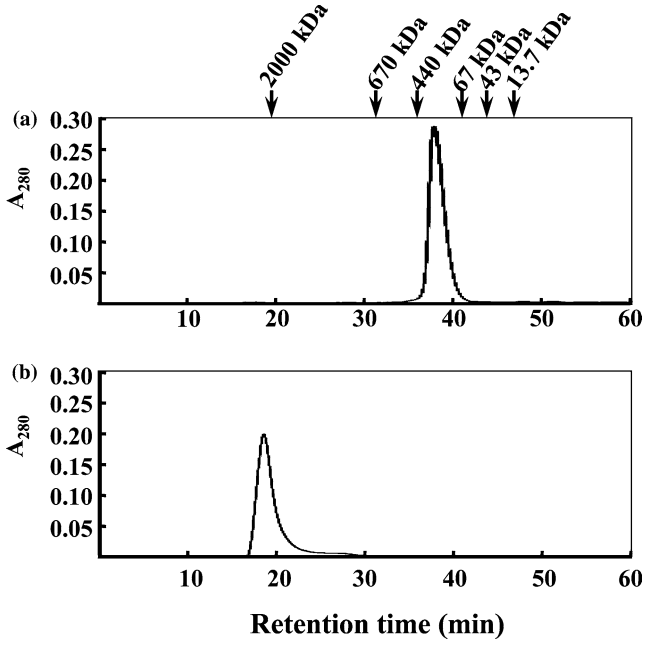

Fig. 2a,b Chromatograms of carboxypeptidases purified from *M. pilosus* IFO 4480 on a Superose 6 gel filtration column. The relative elution positions of blue dextran (2,000 kDa) and various standard proteins: thyroglobulin (670 kDa), ferritin (440 kDa), albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease (13.7 kDa) are indicated. **a** MpiCP-1, **b** MpiCP-2

Table 4 Effects of various metal salts on the activities of carboxypeptidases from *M. pilosus* IFO 4480

Metal salt (1 mM)	Relative activity (%)	
	MpiCP-1	MpiCP-2
None	100	100
$MgCl_2$	85	105
$CaCl_2$	82	107
$MnCl_2$	94	92
$FeCl_2$	65	23
$FeCl_3$	63	14
$CoCl_2$	77	93
$NiCl_2$	104	88
CuCl	67	16
$CuCl_2$	64	27
$ZnSO_4$	102	53
$HgCl_2$	86	0
$PbCl_2$	96	53
$CdCl_2$	104	82
$SnCl_2$	102	53

SDS, but MpiCP-2 was not significantly affected by these two reagents.

The substrate specificities of MpiCP-1 and MpiCP-2 are shown in Table 6. Both had broad substrate specificities, catalyzing the hydrolysis of various peptides. They had some similar specificities: of the substrates tested, Z-Tyr-Glu was the best, and Z-Phe-Ala and Z-Glu-Tyr were also good substrates for both enzymes. Moreover, both preferred peptides containing an aromatic or glutamic acid in the C-terminal (P1′) or penultimate (P1) position of the C-termini, but scarcely hydrolyze peptides containing an amide group or proline in the P1′ or P1 position. On the other hand, they also had some very different specificities from each other. MpiCP-1 exhibited a good reaction with Z-Glu-Phe, but

Table 5 Effects of various reagents on the activities of carboxypeptidases from *Monascus pilosus* IFO 4480. *DFP* Diisopropylfluorophosphate; *PMSF* phenylmethylsulfonylfluoride; *TLCK* –N^{α}-tosyl-L-lysine-chloromethyl ketone; *PCMB* *p*-chloromercuribenzoate; *MIA* monoiodacetic acid; *NEM* N-ethylmaleimide; *DTNB* 5,5′-dithio-bis (2-nitrobenzoic acid); *EPNP* 1,2-epoxy-3-(*p*-nitrophenoxy) propane

Reagent (1 mM)	Relative activity (%)	
	MpiCP-1	MpiCP-2
None	100	100
EDTA	62	97
1,10-Phenanthroline	94	101
r, r′-Dipyridyl	79	78
Phosphoramidon	83	59
DFP	61	33
PMSF	39	0
Piperastatin A	13	83
TLCK	94	87
TPCK	65	88
Chymostatin	64	0
Elastatinal	94	54
STI	92	102
Leupeptin	76	40
PCMB	72	3
MIA	86	75
NEM	99	95
DTNB	92	115
E-64	78	90
SDS	50	99
DTT	100	95
2-Mercaptoethanol	96	87
Pepstatin A	81	89
EPNP	99	86

Table 6 Substrate specificities of carboxypeptidases from *M. pilosus* IFO 4480

Substrate (0.5 mM)	Relative activity (%)	
	MpiCP-1	MpiCP-2
Z-Ala-Glu	39	182
Z-Glu-Tyr	100	100
Z-Glu-Phe	53	0
Z-Gly-Pro-Leu-Gly	12	228
Z-Gly-Leu	0	0
Z-Gly-Phe	0	0
Z-Gly-Pro	0	0
Z-Gly-Pro-Leu	0	0
Z-Gly-Leu-NH_2	0	0
Z-Gly-Phe-NH_2	0	0
Z-Phe-Ala	101	216
Z-Phe-Gly	8	448
Z-Phe-Leu	31	92
Z-Phe-Tyr	6	5
Z-Tyr-Glu	108	792
Bz-Gly-Arg	2	160
Bz-Gly-Lys	0	214
Leu-Gly-Gly	0	0

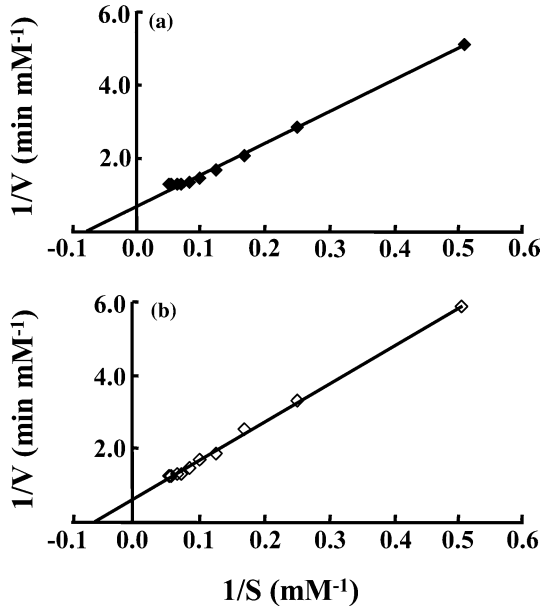

Fig. 3a,b Lineweaver-Burk plots of carboxypeptidases from *M. pilosus* IFO 4480 catalyzing the hydrolysis of Z-Tyr-Glu at 37°C. **a** MpiCP-1 at pH 4.0, **b** MpiCP-2 at pH 3.5

MpiCP-2 did not hydrolyze this peptide at all. In addition, MpiCP-2 reacted highly to peptides containing basic amino acids (Arg and Lys) in the P1′ position, even glycine in the P1 position, and to peptides containing glycine in the P1′ position when a hydrophobic amino acid was in the P1 position, but MpiCP-1 scarcely hydrolyzed these peptides. Interestingly, like MpiCP-1, except when basic amino acid (Arg and Lys) residues were found in the P1′ position, MpiCP-2 did not hydrolyze peptides containing a glycine residue in the P1 position at all.

The kinetic parameters of MpiCP-1 and MpiCP-2 for Z-Tyr-Glu at pH 4.0 and 3.5, respectively, at 37°C were calculated from Lineweaver-Burk plots (Fig. 3). The K_m, V_{max}, K_{cat} and K_{cat}/K_m of MpiCP-1 were 1.33 mM, 1.49 mM min^{-1}, 723 s^{-1} and 545 mM^{-1} s^{-1}, respectively; and the K_m, V_{max}, K_{cat} and K_{cat}/K_m of MpiCP-2 were 1.55 mM, 1.54 mM min^{-1}, 2,039 s^{-1} and 1,318 mM^{-1} s^{-1}, respectively.

Discussion

We recently reported that, among 28 strains of genus *Monascus*, *M. purpureus* IFO 4478 was the best producer of carboxypeptidase in submerged culture [26]. As part of a systematic study of the proteolytic enzymes produced by *Monascus*, we examined the carboxypeptidase produced by *Monascus* in solid culture. The screening test carried out using 28 strains of the genus *Monascus* for proteolytic enzymes activities in solid culture revealed that *Monascus* sp. G-1 exhibited the highest ability to produce carboxypeptidase, followed by *M. pilosus* IFO 4480, but that of *M. purpureus* IFO 4478 was much lower. However, *M. pilosus* IFO 4480 exhibited the highest ability to produce proteinase, but *M. purpureus* IFO 4478 and *Monascus* sp. G-1 were much lower. Furthermore, in a preliminary experiment, we also found that *M. pilosus* IFO 4480 was one of the six best strains for carboxypeptidase production in submerged culture among the 28 *Monascus* strains tested. The above results revealed that the genus *Monascus* has different abilities to produce proteolytic enzymes in submerged and solid cultures. We postulated that the combined actions of proteinase and carboxypeptidase in a solid culture product, red koji, not their individual actions, played an important role in the formation of tasty free amino acids during the maturation of tofuyo. Therefore, we selected *M. pilosus* IFO 4480, which had good ability to produce not only proteinase but also carboxypeptidase in solid culture and could produce carboxypeptidase well in submerged culture, as our study strain.

Two enzymes, MpiCP-1 and MpiCP-2, were purified to homogeneity from extracts of red koji produced by *M. pilosus* IFO 4480, and characterized. Inhibitor studies on the purified enzymes suggested that they are both serine carboxypeptidases. Serine carboxypeptidases are widely distributed in fungi as well as in higher plants and animal tissues, but they are particularly abundant in filamentous fungi [4]. MpiCP-1 and MpiCP-2, like most other fungal serine carboxypeptidases, had an acidic optimal pH and required serine residues, but did not require metal ions for its catalytic activity [4]. MpiCP-2 was completely inhibited by Hg^{2+} and strongly by PCMB. These results suggest the existence in or near the active sites of a sulfhydryl group, which is important for the tertiary structures of proteins like serine carboxypeptidases from *M. purpureus* (MpuCP) [26], carboxypeptidase Y from yeast [11] and carboxypeptidase S3 from *P. janthinellum* [7], carboxypeptidase from *Paecilomyces carneus* [37] and CPZ-1 and CPZ-2 from *Absidia zychae* [25]. However, since MpiCP-1 was only slightly inhibited by Hg^{2+} and PCMB, indicating a higher resistance to the actions of Hg^{2+} and PCMB actions than MpiCP-2 and MpuCP [26], their active centers may be slightly different structurally.

MpiCP-1 and MpiCP-2 were distinguishable by their molecular mass. MpiCP-1 had a molecular mass of 125 kDa. This value is the same as that of carboxypeptidase from *A. saitoi* [35] and similar to those of other most reported fungal carboxypeptidases, such as MpuCP [26] and carboxypeptidases from *A. niger* [14, 19, 23], carboxypeptidase Ib, I, II, and O from *A. oryzae* [28–30, 34], and carboxypeptidase S2 from *P. janthinellum* [12]. Although MpuCP was reported to be a heterodimer with a native molecular mass of 132 kDa composed of two different subunits of 64 and 67 kDa [26], most of the carboxypeptidases mentioned above [12, 23, 34, 35] are homodimers. In the present study, MpiCP-1 also seemed to be a homodimer composed of two identical subunits of 61 kDa. This distinguishes MpiCP-1 from MpuCP. Larger molecular mass forms of

fungal carboxypeptidase, such as carboxypeptidase Ia from *A. oryzae* (230 kDa) [28] and carboxypeptidase S3 from *P. janthinellum* (360 kDa) [7], have also been reported. The latter is a homohexamer composed of six identical subunits of 62 kDa. MpiCP-2, like most other fungal carboxypeptidases, also seems to have a homomeric structure composed of a single polypeptide chain of about 59 kDa. However, it is a high molecular mass homooligomer with a native molecular mass of 2,263 kDa, which is remarkably higher than those of other carboxypeptidases, even that of the larger protease (900 kDa) [16], although lower than that of the largest protease (5,500 kDa) [2], and comprises about 38 identical subunits. Thus, MpiCP-2 may be the largest serine carboxypeptidase reported.

MpiCP-1 and MpiCP-2, like MpuCP and most other fungal carboxypeptidases, are acidic glycoproteins and exhibit maximal activity in the acidic pH range [4]. MpiCP-1 has an isoelectric point of 3.7 and a carbohydrate content of 11%, whereas MpiCP-2 has an isoelectric point of 4.0 and a carbohydrate content of 33%. The isoelectric point of MpiCP-1 is same as that of MpuCP [26], and similar to those of carboxypeptidase I from *A. niger* [5], carboxypeptidase Y from yeast [17] and carboxypeptidase S1 from *P. janthinellum* [12]. However, its carbohydrate content is higher than that of carboxypeptidase S1 from *P. janthinellum* [12], but lower than that of MpuCP [26], carboxypeptidase I from *A. niger* [5] and carboxypeptidase Y from yeast [17]. The isoelectric point of MpiCP-2 is similar to those of carboxypeptidase S2 from *P. janthinellum* [12] and the carboxypeptidase from *A. niger* var. *macrosporus* [23]; however, its carbohydrate content is higher than either of the latter. In addition, although the carbohydrate content of MpiCP-2 is similar to that of carboxypeptidase S3 from *P. janthinellum* [7], their isoelectric points are different.

The results of the relative hydrolysis rates of the two enzymes to *N*-acylpeptides revealed that both MpiCP-1 and MpiCP-2 have broad substrate specificities. Like MpuCP [26] and carboxypeptidase from *A. saitoi* [13], they prefer substrates with bulky amino acids, such as glutamic acid, tyrosine and phenylalanine, in the penultimate position (P1). They also prefer substrates with bulky amino acids in the C-terminal position (P1′), like MpuCP [26], but unlike carboxypeptidase from *A. saitoi* [13], which prefers substrates with neutral and basic amino acids, even proline, in the P1′ position. Moreover, both MpiCP-1 and MpiCP-2 only poorly hydrolyze peptides containing an amide group or proline in the P1′, proline or glycine in the P1 or P1′ position, like MpuCP [26] and carboxypeptidases from *A. oryzae* var. *viride* [28], but unlike carboxypeptidase from *A. saitoi* [13], carboxypeptidase O-1 from *A. oryzae* [36] and carboxypeptidase Y from yeast [11]. Both MpiCP-1 and MpiCP-2 exhibited the highest reactivity to Z-Tyr-Glu, MpiCP-1 also exhibited a good reaction with Z-Glu-Phe, like MpuCP [26]; MpiCP-2 did not hydrolyze the latter substrate at all. Otherwise, MpiCP-2 also exhibited high activity towards peptides containing basic amino acids (Arg and Lys) in the P1′ position, even glycine in the P1 position, or a glycine residue in the P1′ position when there was a hydrophobic amino acid in the P1 position, like the carboxypeptidase from *A. saitoi* [13], but MpiCP-1 scarcely hydrolyzed these peptides. Although Z-Tyr-Glu was found to be the best substrate for both MpiCP-1 and MpiCP-2, like MpuCP [26] and carboxypeptidase Ib from *A. oryzae* var. *viride* [28], and both MpiCP-1 and MpiCP-2 had similar K_m and V_{max} values toward Z-Tyr-Glu, MpiCP-2 had higher K_{cat}, and K_{cat}/K_m values than MpiCP-1. This may be because MpiCP-2 has more catalytic sites in its macromolecule than MpiCP-1. The result that both MpiCP-1 and MpiCP-2 exhibited higher activity towards Z-Tyr-Glu, Z-Ala-Glu and Z-Phe-Ala is consistent with findings that glutamic acid and alanine were the two most abundant free amino acids in the matured tofuyo [40, 43]. Z-Glu-Tyr was reported to be the best substrate for acid carboxypeptidase III, IV, O-1 and O-2 from *A. oryzae* [31, 32, 36], carboxypeptidases Z-1 and Z-2 from *A. zychae* [25], and carboxypeptidase S1 from *P. janthinellum* [12], and Z-Glu-Phe was the best substrate for serine carboxypeptidase from *P. carneus* [37], while Z-Phe-Leu was the best for the carboxypeptidase from *Mucor racemsus* [8] and carboxypeptidase Y from yeast [11, 17]. In the present study, Z-Glu-Tyr and Z-Phe-Leu were also found to be good substrates for MpiCP-1 and MpiCP-2, and Z-Glu-Phe also for MpiCP-1, like the substrates of MpuCP [26]. From this point of view, it may be considered that MpiCP-1 and MpiCP-2, like MpuCP [26] and *Aspergillus* acid carboxypeptidase [1], play an important role in removing the bitter compounds, which are predominantly composed of hydrophobic amino acids and peptides with tyrosine or related amino acids at their C-termini [27], from soybean protein and peptides during maturation of tofuyo. Furthermore, the broad specificity of MpiCP-1 and MpiCP-2 mentioned above maybe make them applicable to sequence determination of the C-termini of peptides and proteins like carboxypeptidase Y from yeast [11] and carboxypeptidase from *A. niger* [5].

This is the first report on the purification and characterization of a serine carboxypeptidase from a member of the genus *Monascus*. Two forms of serine carboxypeptidases, MpiCP-1 and MpiCP-2, were purified form *Monascus pilosus* and characterized. MpiCP-2 is a macromolecule with a native molecular mass of 2,263 kDa composed of about 38 identical subunits of 59 kDa. It is the largest form of serine carboxypeptidase reported to date. Although some high molecular mass multimers of the proteasome [10], tripeptidyl peptidase [27], tricon protease [3] and metalloproteinase [2, 16], have been reported, this is the first report of a high molecular mass multimer of carboxypeptidase. In addition to having the highest molecular mass, it is also different from other forms of serine carboxypeptidase from *Monascus* in optimum pH, pH and thermal stability, p *I* and substrate specificity, and especially

carbohydrate content. It remains unclear whether the differences in physicochemical and enzymatic properties of the enzymes are related to their differences in molecular mass, carbohydrate content or amino acid sequence. Therefore, cloning and sequencing of the genes encoding these enzymes will be the subject of further studies to clearly elucidate their molecular features and functions. Large-scale process studies with these enzymes and their application in the food industry are also necessary directions for further studies.

References

1. Arai S, Noguchi M, Shukuko K, Kurosawa S, Kato H, Fujimaki M (1970) Applying proeteolytic enzymes on soybean. 6. Deodorization effect of Aspergillopeptidase A and debittering effect of *Aspergillus* acid carboxypeptidase. J Food Sci 35:392–395
2. Bertenshaw GP, Norcum MT, Bond JS (2003) Structure of homo- and hetero-oligomeric Meprin metalloproteases. J Biol Chem 278:2522–2532
3. Brandstetter H, Kim JS, Groll M, Huber R (2001) Crystal structure of the tricorn protease reveals a protein disassembly line. Nature 414: 466–470
4. Breddam K (1986) Serine carboxypeptidase: a review. Carlsberg Res Commun 51:83–128
5. Dal Degan F, Ribadeau-Dumas B, Breddam 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
6. Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427
7. 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
8. Disanto ME, Li QH, Logan DA (1992) Purification and characterization of a developmentally regulated carboxypeptidase from *Mucor racemosus*. J Bacteriol 174:447–455
9. 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
10. Groll M, Ditzel L, Lowe J, Stock D, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386:463–471
11. Hayashi R (1976) Carboxypeptidase Y. In: Colowick SP, Kaplan NO, Lorand L (eds) Methods in enzymology, vol XLV. Proteolytic enzymes, part B. Academic, New York, pp 568–587
12. Hofmann T (1976) Penicillocarboxypeptidase S-1 and S-2. In: Colowick SP, Kaplan NO, Lorand L (eds) Methods in enzymology, vol XLV. Proteolytic enzymes, part B. Academic, New York, pp 587–599
13. Ichishima E (1972) Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*. Biochim Biophys Acta 258:274–288
14. 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
15. Ichishima E, Yoshimura K, Tomoda K (1983) Acid carboxypeptidase from a wood-deteriorating basidiomycete, *Pycnoporus sanguineus*. Phytochemistry 22:825–829
16. Ishmael F, Norcum MT, Benkovic SJ, Bond JS (2001) Multimeric structure of the secreted Meprin A metalloproteinase and characterization of the functional protomer. J Biol Chem 276: 23207–23211
17. Johansen JT, Breddam K, Ottesen M (1976) Isolation of carboxypeptidase Y by affinity chromatography. Carlsberg Res Commun 41:1–13
18. Kapitany RA, Zebrowski EJ (1973) A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal Biochem 56:361–369
19. Krishnan S, Vijayalakshmi MA (1986) Purification and some properties of three serine carboxypeptidases from *Aspergillus niger*. J Chromatogr 370:315–326
20. Kuhn RW, Walsh KA, Neurath H (1974) Isolation and partial characterization of an acid carboxypeptidase from yeast. Biochemistry 13:3871–3877
21. Kukman LI, Zelenik-Blatnik M, Abram V (1995) Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein hydrolysates by reversed-phase high-performance liquid chromatography. J Chromatogr A 704:113–120
22. Kumagai I, Yamasaki M (1981) Enzymatic properties of an acid carboxypeptidase from *Aspergillus niger* var . *Macrosporus*. Biochim Biophys Acta 659:344–350
23. 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
24. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
25. Lee BR, Takeuchi M, Kobayashi Y (1993) Purification and characterization of serine carboxypeptidase from *Absidia zychae*. Biosci Biotechnol Biochem 57:618–622
26. Liu F, Tachibana S, Taira T, Ishihara M, Yasuda M (2004) Purification and characterization of a new type of serine carboxypeptidase from *Monascus purpureus*. J Ind Microbiol Biotechnol 31:23–28
27. Macpherson E, Tomkinson B, Balow RM, Hoglund S, Zetterqvist O (1987) Supramolecular structure of tripeptidyl peptidase II from human erythrocytes as studied by electron microscopy, and its correlation to enzyme activity. Biochem J 248:259–263
28. 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 (in Japanese). Hakkokogaku 65:37–43
29. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase I from *Aspergillus oryzae*. Agric Biol Chem 36:1343–1352
30. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase II from *Aspergillus oryzae*. Agric Biol Chem 36:1473–1480
31. Nakadai T, Nasuno S, Iguchi N (1972) Purification and properties of acid carboxypeptidase III from *Aspergillus oryzae*. Agric Biol Chem 36:1481–1488
32. Nakadai T, Nasuno S, Iguchi N (1973) Purification and properties of acid carboxypeptidase IV from *Aspergillus oryzae*. Agric Biol Chem 37:1237–1251
33. San-Marina S, Nicholls DM (1996) Metal binding and ferritin immunoreactivity in a high molecular weight fraction from rat brain. Biochim Biophys Acta 1310:277–283
34. Takeuchi M, Ichishima E (1986) A 155 K acid carboxypeptidase O from *Aspergillus oryzae*. Agric Biol Chem 50:633–638
35. Takeuchi M, Ichishima E (1986) Improved purification and further characterization of acid carboxypeptidase from *Aspergillus saitoi*. Agric Biol Chem 50:1403–1407
36. Takeuchi M, Ushijima T, Ichishima E (1982) A new acid carboxypeptidase, O-1, from *Aspergillus oryzae*. Curr Microbiol 17:19–23
37. Umetsu H, Hishinuma K, Wake E, Ichishima E (1996) Production, purification, and properties of serine carboxypeptidase from *Paecilomyces carneus*. Curr Microbiol 33:44–48
38. Weber K, Pringle JR, Osborn M (1972) Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. In: Colowick SP, Kaplan NO, Hirs CHW, Timasheff SN (eds) Methods in enzymology, vol XXVI. Enzyme structure, part C. Academic, New York, pp 3–27

39. Whitaker JR (1963) Determination of molecular weights of proteins by gel filtration on Sephadex. Anal Chem 35:1950–1956
40. Yasuda M (1990) Studies on manufacturing of tofuyo (in Japanese). Nippon Shokuhin Kogyo Gakkaishi 37:403–409
41. Yasuda M, Sakaguchi M (1998) Degradation of soybean protein by an acid proteinase from *Monascus anka*. Food Sci Technol Int Tokyo 4:6–8
42. Yasuda M, Shimabukuro M, Kikuchi S (1991) Production, purification and properties of acid proteinase from genus *Monascus*. Nippon Shokuhin Kogyo Gakkaishi 38:954–961
43. Yasuda M, Matsumoto T, Sakaguchi M, Kobamoto N (1993) Changes in chemical components of tofuyo prepared by *Monascus* fungus during fermentation (in Japanese). Nippon Shokuhin Kogyo Gakkaishi 40:331–338
44. Yokoyama S, Oobayashi A, Tanabe O, Sugawara S, Araki E, Ichishima E (1974) Production and some properties of a new type of acid carboxypeptidase of *Penicillium* molds. Appl Microbiol 27:953–960
45. 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