Biosci. Biotech. Biochem., **57** (4), 618–622, 1993

Purification and Characterization of Serine Carboxypeptidases from *Absidia zychae*

Byung Rho LEE, Michio TAKEUCHI, and Yasuo KOBAYASHI

Laboratory of Molecular Biology and Microbial Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

Received October 5, 1992

Among 29 strains of zygomycetes screened for serine carboxypeptidases, *Absidia zychae* NRIC 1199 showed the highest enzyme production. Two serine carboxypeptidases, CPZ-1 and CPZ-2, were purified to a homogeneous state from an extract of koji culture of *A. zychae* NRIC 1199. Purified CPZ-1 and CPZ-2 showed similar properties except the isoelectric point (p*I*); The p*I* of CPZ-1 and CPZ-2 were 4.50 and 4.65, respectively. The molecular weights of the CPZ-1 and CPZ-2 were 48,000 by SDS–PAGE and gel filtration. Among the proteinase inhibitors tested, phenylmethylsulfonyl fluoride and monoiodoacetic acid strongly inhibited the enzyme activity. The optimum pHs of CPZ-1 and CPZ-2 were 4.2 towards Z-Glu-Tyr. It is shown that the substrate specificities of CPZ-1 and CPZ-2 were dependent on the presence of bulky amino acid residues in the penultimate position (P_1) for the small Z-peptides. However, in spite of the presence of Gly, Asp, Arg, or Pro in the P_1 position, oligopeptides were hydrolyzed rapidly. CPZ-1 and CPZ-2 had not only carboxypeptidase but also carboxyamidase and amidase activities, and acted preferentially as a carboxyamidase for C-terminal amidated peptides. The hydrophobicity of P_2 and P_3 positions and the bulkiness of P_1 and P_1' positions of the substrate may be important for carboxyamidase and amidase activities.

Serine carboxypeptidases (EC 3.4.16.1) catalyze the sequential hydrolytic liberation of carboxyterminal amino acids of peptides and proteins at acidic pHs. In our previous paper, we reported that the carboxypeptidase from *Aspergillus saitoi* released C-terminal amino acid amides (carboxyamidase activity) and/or ammonia (amidase activity) from C-terminal amidated peptides.[1)]

Serine carboxypeptidases are widely distributed in fungi, higher plants, and animal tissues.[2)] Since fungi secrete products well, they are useful for enzyme production. Fungal serine carboxypeptidases have been isolated and purified from ascomycetes, such as *Asp. saitoi*,[3)] *Asp. oryzae*,[4–6)] and *Penicillium janthinellum*,[7,8)] but limited information is available on the carboxypeptidases of zygomycetes. Tanaka *et al.*[9)] and Ichishima *et al.*[10)] reported that the zygomycete *Rhizopus* produced a small or undetectable amount of its extracellular carboxypeptidase.

We found that the zygomycete *Absidia* produced abundant serine carboxypeptidase in the koji. Since *A. zychae* carboxypeptidases acted more as a carboxyamidase than an amidase, those specificities of the C-terminal amidated peptides differed from *Asp. saitoi* carboxypeptidase. *Absidia* is closely related to *Rhizopus* because of its rhizoids, stolons, apophyses, and indefinite growth of the fertile aerial mycelium and is also related to *Phycomyces* because of the resemblance in sexual reproduction. However, it differs asexually from *Rhizopus* since the sporangiophores always arise along the stolons but never opposite to the rhizoids.[11)] Hesseltine and Ellis described *Absidia* sp. with ovoid spores that are nonpathogenic to animals and man.[12)] *A. zychae* have ovoid, nearly elliptical sporangiospores.[12)]

Wöstemeyer *et al.* are developing a host-vector system in *Absidia*.[13–18)] So the enzymes produced by *Absidia* are very interesting objects of study. This study reports purification and properties of *A. zychae* serine carboxypeptidases with a unique specificity for the C-terminal amidated peptides.

Materials and Methods

Materials. All peptides were purchased from the Peptide Institute, Inc., Osaka, DEAE-Sephadex A-50 and SP-Sephadex C-50 resin were obtained from Pharmacia. Reagents for isoelectric point (p*I*) measurement and polyacrylamide gel electrophoresis were supplied by Bio-Rad Laboratories. Low molecular weight protein standards for SDS–PAGE and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was purchased from Seikagaku Kogyo Co,, and monoiodoacetic acid (IAA) and *p*-chloromercuribenzoic acid (PCMB) were from Wako Pure Chemical Industries. All other chemicals used were of reagent grade.

Organisms. All strains tested were subcultured from the culture collections. Table I is a list of strains tested. Twenty-nine strains of zygomycetes were examined for enzyme production by the koji method.[10)]

Carboxypeptidase assay. Carboxypeptidase activity was measured with a ninhydrin colorimetric assay of Z-Gly-Tyr hydrolysate as described by Ichishima.[3)] One katal of carboxypeptidase was defined as the amount of enzyme required to liberate 1 mol of tyrosine from Z-Glu-Tyr per second at 30°C and pH 4.2.

Enzyme production. Enzyme was produced by the koji method.[10)] Wheat bran (900 g) was thoroughly mixed with 630 ml of tap water, 51 g portions were removed to 1 liter Erlenmeyer flasks, and these was autoclaved at 121°C for 30 min. The sterilized bran was inoculated with spores of *A. zychae* and then incubated at 30°C for 100 h. The flasks were shaken twice a day to redistribute the medium and maintain better temperature control. Carboxypeptidase produced in the solid culture medium was extracted for 2 h at room temperature with 9000 ml of 10 mM acetate buffer at pH 5.0, and 3 ml of toluene. After extraction from the koji, the culture filtrate was pooled and pH was adjusted to 5.0 with 1 N HCl. The filtrate was used for the following purification of the enzyme.

Protein measurement. Protein concentrations were monitored by the measurement of absorbancy at 280 nm during purification. Proteins were also measured by the method of Lowry *et al.*,[19)] using bovine serum albumin as a standard.

Purification of the enzyme. All procedures were done at 4°C. Solid

ammonium sulfate (3120 g) was added to culture filtrate (8000 ml) and the precipitate was discarded. The enzyme was salted out by the addition of 230 g of ammonium sulfate per liter of the supernatant. After centrifugation, the pellet was suspended in 100 ml of 10 mM acetate buffer containing 0.1 M NaCl (pH 5.0: buffer A) and dialyzed against buffer A for 24 h with several changes of the buffer. The precipitate formed during dialysis was discarded by centrifugation, and the supernatant was put on a DEAE-Sephadex A-50 anion-exchange column (2 by 30 cm) which was preequilibrated with buffer A. The column was washed with 100 ml of buffer A and carboxypeptidase was then eluted with a linear gradient of NaCl (0.1 to 0.25 M) in buffer A. Fractions containing the highest carboxypeptidase activity were collected and dialyzed against 10 mM sodium acetate buffer (pH 4.5: buffer B). The dialyzed enzyme solution (200 ml) was put on an SP-Sephadex C-50 cation exchange column (2 by 30 cm) preequilibrated with buffer B. The column was washed with the buffer B, and carboxypeptidase was then eluted with a linear gradient of NaCl (0 to 0.1 M) in buffer B. The carboxypeptidase activities were eluted in two peak fractions (Fig. 1). The first and second peak fractions were termed CPZ-1 and CPZ-2, respectively. To separate CPZ-1 and CPZ-2 perfectly, rechromatography was done with each. To obtain the enzyme of high specific activity, rechromatographies of CPZ-1 and CPZ-2 were done on an SP-Sephadex C-50 column with pH gradient (4.5 to pH 5.0) and NaCl gradient (0 to 0.2 M) in buffer B. The major active fractions of CPZ-1 and CPZ-2 were stored at 4°C until use.

Enzyme characterization. The molecular weights of purified CPZ-1 and CPZ-2 were estimated by gel filtration on a Sephadex G-75 column and SDS–PAGE by the method of Laemmli.[20] Isoelectric focusing was done with 5% ampholine for 4 h at a constant wattage of 12W at 4°C, using a Rotofor preparative isoelectric focusing cell (Bio-Rad Laboratories). The active fraction of carboxypeptidase was diluted to 40 ml with water and then used for second isoelectric focusing. After the second isoelectric focusing, pH of active fraction was measured.

Measurement of kinetic parameters. The K_m and V_{max} for Z-Glu-Tyr, Z-Glu-Phe, Z-Tyr-Leu, Z-Tyr-Glu, Z-Leu-Tyr, and Z-Gly-Pro-Leu-Gly were graphically calculated from Lineweaver–Burk plots. Initial rates toward the substrates were measured at pH 4.5 and 30°C, except that for Z-Glu-Tyr was measured at pH 4.2.

Hydrolysis of oligopeptides and C-terminal amidated peptides. One nkat of CPZ-1 or CPZ-2 was incubated with 100 nmol of substrate at 30°C in 1 ml of 50 mM acetate buffer (pH 4.2). Two hundred μl of the reaction mixture was removed and the enzyme reaction was stopped by the addition of an equal volume of 0.06 N HCl. After centrifugation, the released amino acids and amino acid amides in 50 μl of supernatant were analyzed on an automatic amino acid analyzer (Hitachi Model 835-30) by the previously described method.[1]

Results

Table I shows the production of serine carboxypeptidase by zygomycetes strains. The *Mucor* and *Rhizopus* strains tested produced a low level of serine carboxypeptidase, except *R. oligosporus* NRIC 1502, but the *Absidia* and *Gongronella* strains tested produced a large amount of serine carboxypeptidase. Among them *Absidia zychae* NRIC 1199 showed the highest enzyme activities in the survey using koji.

Using the koji culture method, the maximum enzyme production was obtained after 4 days in the koji. For the purification of the enzyme, *A. zychae* was cultured for 4 days at 30°C. The results of the purification are summarized in Table II. The carboxypeptidase activities were eluted in two fractions by the first SP-Sephadex (Fig. 1). The

Table I. List of Strains of Genera *Absidia*, *Gongronella*, *Mucor*, and *Rhizopus* for Serine Carboxypeptidase Production by the Koji Method

Strain	Activity (*n* kat/ml of filtrate)
Absidia zychae NRIC 1199	6.48
Absidia atrospora NRIC 1163	0.85
Absidia glauca NRIC 1181	3.13
Absidia glauca NRIC 1183	0.42
Absidia heterospora NRIC 1186	1.53
Absidia spinosa NRIC 1197	3.32
Gongronella butleri IFO 8080	4.48
Gongronella butleri IFO 8081	2.12
Mucor hiemalis NRIC 1244	0.08
Mucor pusillus IFO 4579	0.12
Mucor racemosus NRIC 1264	Trace
Rhizopus arrhizus TUA 059M	0.17
Rhizopus batatas TUA 193M	0.09
Rhizopus chinensis TUA 088M	0.03
Rhizopus delemar TUA 056M	0.04
Rhizopus formosensis HUT 1244	0.05
Rhizopus javanicus IFO 5411	Trace
Rhizopus niveus TUA 205M	0.04
Rhizopus oligosporus TUA 085M	0.03
Rhizopus oligosporus IFO 8631	0.05
Rhizopus oligosporus NRIC 1592	2.25
Rhizopus oligosporus NRRL 2710	0.12
Rhizopus oryzae TUA 057M	0.04
Rhizopus oryzae IFO 4785	0.31
Rhizopus stolonifer IFO 6188	Trace
Rhizopus stolonifer IFO 31005	0.05
Rhizopus stolonifer TUA 069M	0.05
Rhizopus tonkinensis TUA 192M	0.14
Rhizopus 14-2	0.10

Table II. Purification of *Absidia zychae* Serine Carboxypeptidases

Step		Total protein (A_{280})	Total activity (*n*kat)	Specific activity (*n*kat/A_{280})	Yield (%)	Purification (-fold)
Filtrate		42,688	46,957	1.1	100	1
60–90% Saturated $(NH_4)_2SO_4$ precipitate		3,974	25,800	6.8	55	6
DEAE-Sephadex A-50 (0.1–0.25 M NaCl)		291	19,857	68.3	42	62
SP-Sephadex C-50 (0–0.1 M NaCl)	CPZ-1	45.5	4,461	98	9.5	89
	CPZ-2	57.8	5,603	97	11.9	88
SP-Sephadex C-50 (0–0.1 M NaCl)	CPZ-1	38.5	4,232	110	9.0	100
	CPZ-2	43.0	4,687	109	10.0	99
SP-Sephadex C-50 (pH 4.5–5.0)	CPZ-1	34.5	4,140	120	8.8	109
	CPZ-2	36.0	4,243	118	9.0	107
SP-Sephadex C-50 (0–0.2 M NaCl)	CPZ-1	28.8	4,048	141	8.6	128
	CPZ-2	26.8	3,880	145	8.3	132

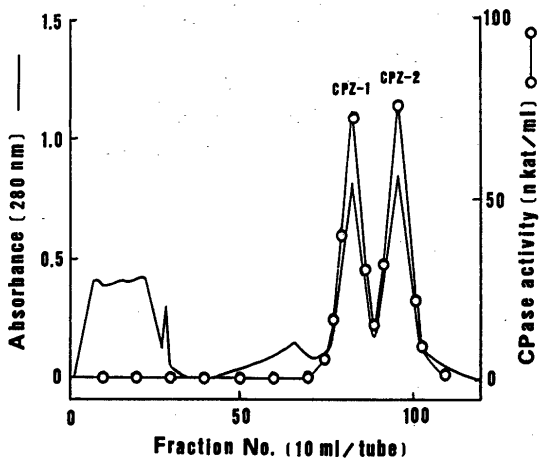

Fig. 1. SP-Sephadex C-50 Chromatography of *Absidia zychae* Serine Carboxypeptidases.

Active fraction (200 ml) of carboxypeptidase from DEAE-Sephadex A-50 chromatography was put on an SP-Sephadex C-50 cation exchange column (2 by 30 cm), and elution was done with 0–0.1 M NaCl gradient in 10 mM acetate buffer (pH 4.5).

Table III. Some Enzymatic and Physicochemical Properties of Serine Carboxypeptidases from *Absidia zychae*

Property	CPZ-1	CPZ-2
Isoelectric point	4.50	4.65
Molecular mass (kDa) estimated by		
Sephadex G-75	48	48
SDS–PAGE	48	48
Optimum pH for hydrolysis of		
Z-Glu-Tyr	4.2	4.2
Z-Tyr-Leu	4.5	4.5
Stable in pH range (30°C, 10 min)	5–8	5–8
Thermal stability (10 min), °C	50	50
$E^{1\%}_{1\,cm,280\,nm}$	17	17
Inhibitor (Inhibition %)		
1 mM PMSF	97	99
1 mM TPCK	57	46
1 mM PCMB	99	96
1 mM IAA	96	99

PMSF, phenylmethylsulfonyl fluoride; TPCK, Tosyl-L-phenylalanine chloromethyl ketone; PCMB, *p*-chloromercuribenzoic acid; IAA, monoiodoacetic acid.

Table IV. The Relative Amino Acid Composition of CPZ-1 and CPZ-2

Amino acid	CPZ-1 Composition ratio (mol%)	CPZ-2 Composition ratio (mol%)
Asp + Asn	12.27	12.40
Thr	4.01	4.02
Ser	8.32	8.22
Glu + Gln	13.50	13.47
Gly	9.48	9.38
Ala	7.59	7.52
Val	6.30	6.37
Met	1.75	1.62
Ile	3.70	3.73
Leu	5.40	5.68
Tyr	5.57	5.64
Phe	5.53	5.46
Lys	6.25	6.28
His	3.04	2.92
Arg	2.45	2.38
Pro	4.94	4.92

The values of analysis of two hydrolysates were obtained after 24 h of hydrolysis in 2 ml of 5.7 N HCl containing 5 μl 2-mercaptoethanol and 2 drops of 5% phenol at 110°C.

Table V. Kinetic Parameters of Carboxypeptidases from *Absidia zychae* toward Z-Peptides.

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
CPZ-1				
	Z-Glu-Tyr	0.19	15.5	81.6
	Z-Glu-Phe	0.27	18.7	69.2
	Z-Tyr-Leu	0.23	20.0	87.0
	Z-Tyr-Glu	0.58	17.0	29.3
	Z-Gly-Pro-Leu-Gly	0.80	19.3	24.1
CPZ-2				
	Z-Glu-Tyr	0.19	16.0	84.2
	Z-Glu-Phe	0.26	16.3	62.7
	Z-Tyr-Leu	0.20	15.7	78.5
	Z-Tyr-Glu	0.62	16.3	26.3
	Z-Gly-Pro-Leu-Gly	0.82	18.8	22.9

carboxypeptidase in the first fraction was termed carboxypeptidase Z-1 (CPZ-1) and that in the second, Z-2 (CPZ-2). CPZ-1 and CPZ-2 were separated perfectly by the second SP-Sephadex. The specific activities of CPZ-1 and CPZ-2 were raised about 30% by the third and forth SP-Sephadex rechromatographies. Purified CPZ-1 and CPZ-2 were homogeneous by SDS–PAGE and native PAGE.

Some enzymatic and physicochemical properties of CPZ-1 and CPZ-2 are summarized in Table III. CPZ-1 and CPZ-2 had similar properties, except the isoelectric point. When the purified CPZ-1 and CPZ-2 were electrophoresed in SDS–PAGE with or without mercaptoethanol, a single band with molecular weight around 48,000 was obtained, indicating that the enzymes were composed of single chain monomer. The amino acid sequences for the N-terminal 11 amino acids of both the enzymes were the same (Tyr-Thr-Ser-Pro-Lys-Leu-Xaa-Asp-Pro-Asp-Val-) and no essential differences were observed between the amino acid compositions of the two enzymes (Table IV). The Ouchterlony double diffusion technique using antibody raised against the CPZ-2 protein demonstrated good cross-reactions between CPZ-1 and CPZ-2. Both the enzymes were inhibited by PMSF, TPCK, PCMB, and IAA. However, the enzymes were not inhibited by EDTA, *o*-phenanthroline, antipain, E-64, and pepstatin.

CPZ-1 and CPZ-2 hydrolyzed Z-Glu-Tyr, Z-Glu-Phe, Z-Tyr-Glu, Z-Tyr-Leu, Z-Phe-Leu, Z-Leu-Tyr, and Z-Gly-Pro-Leu-Gly rapidly. But Z-Val-Glu and Z-Ala-Glu were hydrolyzed at a lower rate, and Z-Gly-Leu, Z-Gly-Phe, and Z-Pro-Pro were not hydrolyzed by the enzymes. CPZ-1 and CPZ-2 preferentially hydrolyzed Z-peptides containing aromatic or aliphatic amino acids in the penultimate position (P_1).

The kinetic parameters for the hydrolysis of some small Z-peptides of the two carboxypeptidases are shown in Table V. Similar values of K_m and k_{cat} were obtained between CPZ-1 and CPZ-2, and the K_m values were variable while k_{cat} values were approximately constant for all of the substrates tested. The K_m for Z-Tyr-Glu was 2.5 to 3.0-fold higher than that for Z-Tyr-Leu.

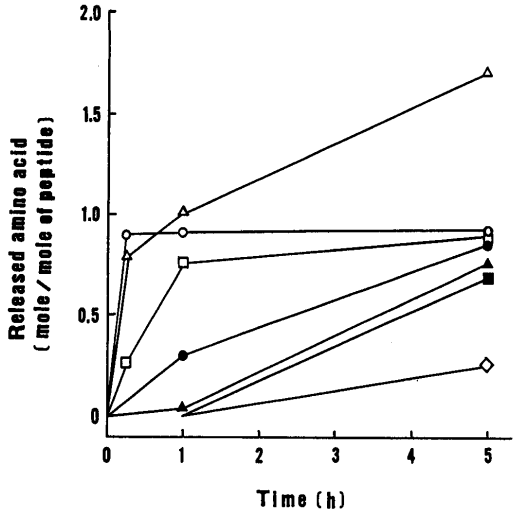

Fig. 2. Hydrolysis of Proangiotensin by CPZ-2.

The action of CPZ-1 was similar to CPZ-2 for the hydrolysis of proangiotensin. The reaction was done at 30°C with a substrate to enzyme ratio of 1200 (mol/mol). See the text for the experimental details. The results indicate the sequence Val-Tyr-Ile-His-Pro-Phe-His-Leu. The amino acid sequence of proangiotensin is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. For the amino acids released from the C-terminus, the following symbols were used: ○, Leu; △, His; □, Phe; ●, Pro; ▲, Ile; ■, Tyr; ◇, Val.

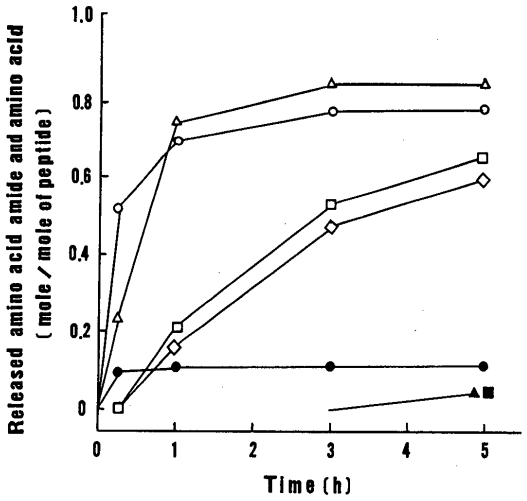

Fig. 3. Hydrolysis of Eledoisin Related Peptide by CPZ-2.

The action of CPZ-1 was similar to CPZ-2 for the hydrolysis of eledoisin-related peptide. The experimental scale is the same to Fig. 3 and the details are described in the text. The amino acid sequence of eledoisin-related peptide is Lys-Phe-Ile-Gly-Leu-Met-NH_2. Symbols: ○, Met-NH_2; ●, Met; △, Leu; □, Gly; ◇, Ile; ▲, Phe; ■, Lys.

Figure 2 indicates that CPZ-2 sequentially released hydrophobic and basic amino acids as well as proline from the C-terminus of proangiotensin. When CPZ-2 hydrolyzed a C-terminal amidated peptide, such as eledoisin related peptide, it released the C-terminal Met-NH_2 and a small amount of Met (Fig. 3). After the C-terminal methionine amide (carboxyamidase activity) and ammonia (amidase activity) were released, carboxyterminal Leu, Gly, Ile, Phe, and Lys were sequentially released (carboxypeptidase activity) by the enzyme. Since CPZ-2 cannot hydrolyze Met-NH_2, Met was not released from liberated Met-NH_2. Furthermore CPZ-2 sequentially released Leu, Ile, Tyr, Pro, Arg, Pro, Cys, Asn, Glu, and Tyr from the C-terminal of neurotensin, and also released Phe-NH_2, Asp, Met, and Trp from the C-terminal of gastrin related peptide. These results indicated that CPZ-2 was able to liberate all amino acids from the C-terminals of peptides. The substrate specificity

Table VI. Comparison of Carboxyamidase and Amidase Activities of Serine Carboxypeptidases toward the Carboxyterminal Amidated Peptides

Substrate	Split site by the enzyme	Relative rate of hydrolysis by		
		A. zychae		*Asp. saitoi**
	S_4 -S_3 -S_2 -S_1 ↓ S'_1			
	P_4 -P_3 -P_2 -P_1 -P'_1	CPZ-1	CPZ-2	CPase
LPLRF-amide	L -P -L -R -FNH_2	1	1	
	L -P -L -R -F -NH_2	0	0	
FMRF-amide	F -M -R -FNH_2	0.9	0.9	0.16
	F -M -R -F -NH_2	0.1	0.1	0.84
Gastrin-	W -M -D -FNH_2	1	1	1
related peptide	W -M -D -F -NH_2	0	0	0
Eledoisin-	K -F -I -G -L -MNH_2	0.8	0.8	0.65
related peptide	-F -I -G -L -M -NH_2	0.2	0.2	0.35
[D-Ala^2, Met^5]-	Y -DA-G -F -MNH_2	0.6	0.6	0
enkephalin amide	Y -DA-G -F -M -NH_2	0.4	0.4	1

Arrow indicates the site split by the enzyme.
* Data from ref. 1.

Table VII. Relative Activities of Carboxypeptidase and Carboxyamidase by Serine Carboxypeptidases from *Absidia zychae* and *Aspergillus saitoi*

Substrate	Relative activity		
	CPZ-1	CPZ-2	*Asp. saitoi* CPase*
Z -E ↓ Y	1000	1000	1000
L -P -L -R -FNH_2	334	324	nd
F -M -R -FNH_2	266	250	27
W -M -D -FNH_2	99	103	82
K -F -I -G -L -MNH_2	164	147	12
Y -DA-G -F -MNH_2	36	34	0

Vertical arrow indicates the site split by the enzyme. The rate of hydrolysis of Z-Glu-Tyr is arbitrarily taken to be 1000.
* Data from ref. 1; nd, not determined; CPase, carboxypeptidase.

of CPZ-1 resembled that of CPZ-2. When CPZ-1 and CPZ-2 acted on amidated peptides, the C-terminal amino acid amide was released faster than ammonia for all the substrates tested (Table VI). This specificity of CPZ-1 and CPZ-2 for C-terminal amidated peptides differed from that of *Asp. saitoi* carboxypeptidase. A comparison of carboxyamidase and carboxypeptidase activities of CPZ-1 and CPZ-2 is shown in Table VII. Carboxyamidase activities of CPZ-1 and CPZ-2 were about one-third of the carboxypeptidase activities for the LPLRF-amide, and were about 10-fold higher than that of *Asp. saitoi* for the FMRF-amide and eledoisin related peptide.

Discussion

Limited information is available on serine carboxypeptidases from zygomycetes. In this report, we found that strains of the zygomycetes *Absidia* and *Gongronella* have high potential for carboxypeptidase production. Among the strains tested, *Absidia zychae* NRIC 1199 was an excellent producer of these enzymes.

The strain produced two forms of serine carboxypeptidase (CPZ-1 and CPZ-2), distinguished in their isoelectric points, but with identical molecular weights, specific activities, optimum pHs, stability, specificity, amino acid composi-

tions, N-terminal amino acid sequences, and immunological properties. These two enzymes may be almost identical proteins. These similarities suggests that CPZ-1 and CPZ-2 may be generated from the same molecule by some different modification, such as deamination.

The molecular weights of CPZ-1 and CPZ-2 were estimated to be about 48,000 by gel filtration and SDS–PAGE, and the enzymes were completely inhibited by PMSF, PCMB, and IAA. These results indicated that the two enzymes were serine carboxypeptidases of the low molecular weight type.[4,6] Hayashi *et al.*[21] reported that carboxypeptidase Y from baker's yeast has one SH group per molecule, Cys-341, as measured by titration with PCMB and assay of S-carboxymethyl cysteine. Since CPZ-1 and CPZ-2 were inhibited by PCMB and IAA, one SH group may be in each molecule of the enzymes like the carboxypeptidase Y.

It is known that serine carboxypeptidase has amidase and/or carboxyamidase activity for carboxyterminal amidated peptides. Previous studies[1] indicated that *Asp. saitoi* serine carboxypeptidase showed carboxyamidase activity only for gastrin-related peptide but the amidase in preference to carboxyamidase activity for [D-Ala^2, Met^5]-enkephalin amide and FMRF-amide. For *Asp. saitoi* serine carboxypeptidase, the P_3 and P_4 positions of the substrate were important for the hydrolysis of carboxyterminal amidated peptides.

When CPZ-1 and CPZ-2 acted on amidated peptides, the C-terminal amino acid amide was released faster than ammonia for all the substrates tested (Table VI). CPZ-1 and CPZ-2 mostly acted as a carboxyamidase for LPLRF-amide, FMRF-amide, and gastrin-related peptide, but had a carboxyamidase activity of about 60% for [D-Ala^2, Met^5]-enkephalin amide. These actions of CPZ-1 and CPZ-2 were considered to be due to the presence of hydrophobic amino acid residues in the P_3 and P_2 positions and bulky amino acid residues in the P_1 and P'_1 positions of the substrate. For LPLRF-amide, the presence of Pro and Leu may be chosen in preference to Leu and Arg in the P_3 and P_2 positions, respectively. For [D-Ala^2, Met^5]-enkephalin amide, the rate of release of C-terminal amino acid amide was low because of the presence of D-Ala and Gly in the P_3 and P_2 positions (Table VI and VII), and amidase activity was increased by 40% due to the presence of Phe in the P_2 position, but in spite of the presence of Phe in the P_2 position, amidase activity was not preferable to carboxyamidase activity (Table VI). CPZ-1 and CPZ-2 acted as a carboxyamidase rather than an amidase and this also suggests that the amino acid residue occupying the P'_1 position was important for the enzyme action. It may be decided whether CPZ-1 and CPZ-2 act as carboxyamidase or if the amidase is dependent on hydrophobicity in P_3 and P_2 positions and bulkiness of amino acid side chains in P_1 and P'_1 positions of the substrate used.

CPZ-1 and CPZ-2 had a unique specificity for carboxyterminal amidated peptides since the enzymes acted preferentially as carboxyamidases. CPZ-1 and CPZ-2 were better enzymes than *Asp. saitoi* serine carboxypeptidase to hydrolyze C-terminal amidated peptides (Table VII). In spite of the presence of Gly, Asp, Pro, or Arg in the P_1 position, CPZ-1 and CPZ-2 rapidly released all carboxyterminal amino acids from oligopeptides. Therefore, the enzyme may be useful for carboxyterminal sequence analysis of not only carboxyterminal-free but also of carboxyterminal-amidated peptides.

Acknowledgment. We thank Drs. M. Kosaki and S. Okada, Tokyo University of Agriculture, for their kindness in supplying zygomycete strains.

References

1) M. Takeuchi and E. Ichishima, *Agric. Biol. Chem.*, **53**, 2301–2306 (1989).
2) K. Breddam, *Carlsberg Res. Commun.*, **51**, 83–128 (1986).
3) E. Ichishima, *Biochim. Biophys. Acta*, **258**, 274–288 (1972).
4) M. Takeuchi and E. Ichishima, *Agric. Biol. Chem.*, **45**, 1033–1035 (1981).
5) M. Takeuchi, T. Ushijima, and E. Ichishima, *Curr. Microbiol.*, **7**, 19–23 (1982).
6) M. Takeuchi and E. Ichishima, *Agric. Biol. Chem.*, **50**, 633–638 (1986).
7) S. Yokoyama, A. Oobayashi, O. Tanabe, and E. Ichishima, *Appl. Microbiol.*, **28**, 742–747 (1974).
8) T. Hofmann, in "Methods in Enzymol.," ed. by L. Lorand, Academic Press, New York, 1976, pp. 587–599.
9) T. Tanaka, N. Okazaki, and M. Kitani, *J. Brew. Soc. Japan*, **77**, 831–835 (1982).
10) E. Ichishima, A. Yamane, T. Nitta, M. Kinoshita, S. Nikkuni, T. Oka, and S. Yokoyama, *Appl. Microbiol.*, **26**, 327–331 (1973).
11) C. W. Hesseltine and J. J. Ellis, *Mycologia*, **56**, 568–601 (1964).
12) C. W. Hesseltine and J. J. Ellis, *Mycologia*, **58**, 761–785 (1966).
13) A. Burmester and J. Wöstemeyer, *Curr. Genet.*, **10**, 435–441 (1986).
14) J. Wöstemeyer and A. Burmester, *Curr. Genet.*, **10**, 903–907 (1987).
15) A. Burmester and J. Wöstemeyer, *Curr. Genet.*, **12**, 599–603 (1986).
16) J. Wöstemeyer, A. Burmester, and C. Weigel, *Curr. Genet.*, **12**, 625–627 (1987).
17) A. Burmester, A. Wöstemeyer, and J. Wöstemeyer, *Curr. Genet.*, **17**, 155–161 (1990).
18) A. Wöstemeyer, H. Teepe, and J. Wöstemeyer, *Curr. Genet.*, **17**, 163–168 (1990).
19) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265–275 (1951).
20) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
21) R. Hayashi, S. Moore, and W. Stein, *J. Biol. Chem.*, **248**, 8366–8369 (1973).