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ACID CARBOXYPEPTIDASE FROM A WOOD-DETERIORATING BASIDIOMYCETE, *PYCNOPORUS SANGUINEUS*

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Abstract—An acid carboxypeptidase (EC 3.4.16.1) has been isolated from the culture filtrate of a wood-degrading Basidiomycete, *Pycnoporus sanguineus* and the molecular and enzymatic properties of the enzyme were determined. The extracellular acid carboxypeptidase was homogeneous on polyacrylamide gel electrophoresis at pH 9.4 and SDS-disc gel electrophoresis. The MWs as determined by gel filtration and SDS-gel electrophoresis were 50 000 and 54 000, respectively. The isoelectric point was pH 4.78 using electrofocusing. The purified enzyme had a pH optimum of 3.4, a K_m of 0.74 mM and a k_{cat} of 16/sec with benzyloxycarbonyl-L-glutamyl-L-tyrosine. The K_m and k_{cat} values for bradykinin at pH 3.4 and 30° were 2.0 mM and 25/sec. Values for angiotensin at pH 3.4 and 30° were 0.76 mM and 2.4/sec, respectively.

INTRODUCTION

Growth of wood-deteriorating Basidiomycetes depends on having cell bound and extracellular proteolytic enzymes to provide the means to satisfy requirements for nitrogen; this is true because the fungi feed entirely by absorption, not by photosynthesis or ingestion. In previous papers [1–3] from this laboratory, the specificity and mode of action of *Pycnoporus sanguineus* aspartic proteinase I_a (formerly designated *Trametes sanguinea* acid proteinase [4] were investigated with native insulin [3], the oxidized B-chain of insulin [1], oxidized insulin peptide B1-B16 and B15-B23, angiotensin and proangiotensin [2].

The present report deals with isolation of an acid carboxypeptidase from *Pycnoporus sanguineus* and the mode of action on peptides which release neutral and basic amino acids as well as proline at pH 3.4.

RESULTS

Purification of acid carboxypeptidase

A crude enzyme, prepared from the submerged culture filtrate of *P. sanguineus* ATCC 14622 (= *Trametes sanguinea* IFO 7045) by a previously published method [4], using 10 mM sodium acetate buffer pH 5, was precipitated with cold methanol. The 60–75% methanol precipitate was collected at 4°. The enzyme precipitate was dissolved in 10 mM sodium acetate buffer pH 5. Ion-exchange filtration was conducted on a DEAE-Sephadex A-50 column (2 × 50 cm) equilibrated with the enzyme extraction buffer (pH 5) containing 0.1 M sodium chloride and resulted in a 12.5-fold purification. Further CC (2 × 70 cm) of this enzyme preparation using Bio-Gel P-100 yielded an additional two-fold purification. The enzyme was eluted with 0.1 M sodium acetate buffer pH 5 containing 0.2 M sodium chloride. Re-chromatography on DEAE-Sephadex A-50 (2 × 50 cm) gave active fractions; the enrichment of the enzyme was 1.1-fold in this step. Finally, SP-Sephadex chromatography (1 × 40 cm) brought about an additional 1.3-fold purification. The enzyme was eluted with 10 mM sodium acetate buffer pH 5 which contained 0.1 M sodium chloride. The final preparation of the acid carboxypeptidase had no endopeptidase activities such as aspartic proteinase I_a using milk casein, trypsinogen and the oxidized insulin B-chain [1–3].

Results of the purification are summarized in Table 1. Active fractions from the final step purification were dialysed against 10 mM sodium acetate buffer pH 5, and freeze-dried *in vacuo*.

The protein (5.3 μg) produced from SP-Sephadex C-50 migrates as a single band on polyacrylamide gel electrophoresis (PAGE) at pH 9.4. Further confirmation of the enzyme purity was performed by SDS-disc gel electrophoresis. The protein (5 μg) also migrates as a single band on SDS-disc gel electrophoresis.

General properties

The substrate used for determining general properties was benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z–Glu–Tyr). The purified enzyme was active between pH 1.5 and 5.5, showing a well-defined optimum pH of 3.4.

At 30° for 1 hr the enzyme was stable at between pH 2.0 and 7.0. The enzyme activity in 10 mM sodium acetate buffer, pH 5, was stable at 50° for 10 min. Activity decreased by *ca* 40% at 60° after 10 min; 90% or more of the activity was lost at 70° for 10 min.

The enzyme was completely inhibited by 1 mM phenylmethane sulfonyl fluoride (PMSF). Phenylpyruvic acid at 1 mM was a powerful inhibitor. The nature of the inhibitory effect by this acid was competitive (K_i = 74 μM). At 1 mM, monoiodoacetic acid and TPCK

Table 1. Purification of *Pycnoporus* acid carboxypeptidase

Step	Enzyme activity (mkat)	Total protein (mg)	Specific activity (mkat/mg)	Purification (fold)	Yield (%)
1. Crude enzyme extract	75.4	138 000	0.546	1.0	100
2. MeOH precipitate	39.0	15 700	2.48	4.5	52
3. DEAE-Sephadex A-50	20.2	642	31.5	54.7	27
4. Bio-Gel P-100	12.5	199	62.8	115	17
5. DEAE-Sephadex A-50	6.4	92	70.0	128	8.5
6. SP-Sephadex C-50	1.8	22	81.5	149	2.3
7a. Freezing and thawing*	—	—	81.5	149	2.3
7b. Lyophilization†	—	—	75.0	137	2.1

*In 0.01 M sodium acetate buffer, pH 5.

†From 0.01 M sodium acetate buffer, pH 5, 0.01 M sodium citrate buffer, pH 5, or distilled water.

inhibited the enzyme by 40 and 14 %, respectively. PCMB, EDTA, TLCK and *p*-hydroxycinnamic acid had no apparent effect on its activity at 1 mM. Thus, the inhibition experiment suggests involvement of an active serine group in the enzyme activity.

Molecular properties

The isoelectric point of the enzyme was pH 4.78 using isoelectric focusing. Specific enzymatic activity by the isoelectric focusing procedure was 83.8 mkat/kg of enzyme. The enzyme has the $A^{1\%}_{1\,cm}$ value of 19 at 280 nm. According to gel filtration the MW of the enzyme was 50 000. The MW was also determined by SDS-disc gel electrophoresis and the apparent value was 54 000. Marker proteins were bovine serum albumin (68 000), egg albumin (45 000), bovine trypsinogen (23 000), myoglobin (17 800); the values in parentheses are the respective MWs.

The purified enzyme has 14 % neutral sugars. The enzyme has the following amino acid composition and a total of 362 residues: $CySO_3H_3$, Asp_{46}, Thr_{28}, Ser_{27}, Glu_{30}, Pro_{18}, Gly_{29}, Ala_{27}, Val_{18}, Met_5, Ile_{15}, Leu_{21}, Tyr_{17}, Phe_{16}, Lys_9, His_5, Arg_9, Trp_{39}, $GlcNH_{24}$ and neutral $sugars_{37}$.

Specificity and mode of action

Substrates of the type of Z–Gly–*X* (*X* = L-amino acid) were incubated at pH 3.4 with the enzyme and initial rates of release of *C*-terminal residues determined. These results and the rate of release of proline from Z–Gly–Pro and Z–Gly–Pro–Leu–Gly–Pro are shown in Table 2. Glycine is the penultimate amino acid from the *C*-terminus and the enzyme shows a preference for leucine, phenylalanine and lysine in the *C*-terminal position, lysine being favored.

Initial rates of release of leucine by the enzyme from substrates of the type Z–*X*–L-Leu (*X* = L-amino acid)

Table 2. Comparative rate of hydrolysis for *Pycnoporus* acid carboxypeptidase on a range of peptides

Peptides	Relative activity	Peptides	Relative activity
1. *X* = leucine		5. *X* = phenylalanine	
Z–Gly–Pro–Leu–Gly	136	Z–Phe–Leu	19
2. *X* = glutamic acid		Ac–Phe–Tyr(I_2)	0.7
Z–Glu–Tyr	100	6. *X* = tyrosine	
Z–Glu–Phe*	71	Z–Tyr–Leu	2
3. *X* = valine		Z–Tyr–D–Leu*	0
Z–Val–Glu	65	7. *X* = proline	
Z–Val–Gly	10	Z–Gly–Pro–Leu	0.6
4. *X* = glycine		8. *X* = histidine	
Z–Gly–Leu	30	Bz–Gly–His–Leu	0.01
Z–Gly–Phe	25		
Bz–Gly–Lys	41		
Z–Gly–Arg	15		
Z–Gly–His	9		
Z–Gly–Pro†	1		
Z–Gly–Pro–Leu–Gly–Pro†	26		

The rate of hydrolysis of Z–Glu–Tyr is arbitrarily taken to be 100. *C*-Terminal amino acid residues of the substrate are expressed as *R*–*X*–*Y*.

*Partially insoluble at pH 3.4.

†Proline released was estimated from *A*, at 440 nm.

Table 3. Release of *C*-terminal amino acid residues from 146 nmol of proangiotensin* by *Pycnoporus* acid carboxypeptidase at pH 3.4 and 30°

Enzyme	Incubation time (hr)	Amino acid released (nmol)						
		Val	Tyr	Ile	Pro	Phe	His	Leu
1.56	6	0	0	0	0	5	7	6
1.56	12	0	0	0	0	10	12	11
1.91	48	0	0	0	0	21	25	20
9.55	24	0	1.9	2.1	tr†	52	58	54
9.55	48	0	4.9	5.2	tr†	65	75	66

*Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu.
†tr, Trace.

were determined (Table 2). The influence of the residue in the penultimate position is striking, there being some 3000-fold or more difference between initial rates of release of leucine from Z–Gly–Leu and Z–Gly–His–Leu. The slow liberation of leucine from Bz–Gly–His–Leu indicated that the enzyme exhibited a non-preference for the imidazole group in the penultimate position to the *C*-terminus.

The results presented in Table 2 indicate that the enzyme has a preference for neutral aliphatic residues and acidic glutamic acid in the penultimate position.

Sequential release of C-terminal amino acid residues

The determination and confirmation of the release of *C*-terminal amino acid residues from proangiotensin after prolonged incubation at pH 3.4 and 30° by the enzyme are shown in Table 3. *C*-Terminal leucine, histidine and phenylalanine of proangiotensin were sequentially released by the enzyme but only a trace amount of proline, which is in the fourth position from the *C*-terminus of proangiotensin. No indication of endopeptidase activity could be detected, nor was any release of free amino acids by autodigestion of the enzyme detected after a prolonged period of incubation.

Kinetic studies

The initial rates of release of *C*-terminal amino acid from Z–Glu–Tyr, bradykinin and angiotensin by the purified enzyme are shown in Table 4. The kinetic data in this experiment indicate that the K_m values do not differ greatly and that the differences in the rates of hydrolysis were largely due to widely varying values of k_{cat}. Thus, whereas the K_m values vary over 0.67 and 2.0 mM, the k_{cat} values vary over a 10-fold range.

DISCUSSION

Although serine carboxypeptidases (EC 3.4.16.1) are well-known enzymes in many higher plants (citrus fruit [5–7], barley [8], wheat bran [9], wheat germ [10], rice bran [11], French bean [12], cotton seed [13], pineapple [14], watermelon [15], tomato [16]) and fungi (*Saccharomyces cerevisiae* [17], *Aspergillus saitoi* [18–22], *Aspergillus oryzae* [23, 24] and *Penicillium janthinellum* [25–27]), the enzyme from a wood-degrading Basidiomycete was only recently found in this laboratory.

The work reported here shows that at least two different types of enzyme with proteolytic activity in the acidic pH range are found in the culture filtrate of the wood rotting Basidiomycete, *Pycnoporus sanguineus* (*Trametes sanguinea*). One of these enzymes readily hydrolyses carboxypeptidase substrates in the acidic pH range and differs markedly from the other in its hemoglobinolytic activity. The other, aspartic proteinase I (MW 34 000, EC 3.4.23.6), has hemoglobinolytic activity at pH 2.5 and does not hydrolyse either of the small synthetic α amino substituted peptides [1, 2]. The aspartic proteinase I_a from *P. sanguineus* hydrolysed primarily three peptide bonds, Ala^{14}–Leu^{15}, Tyr^{16}–Leu^{17} and Phe^{24}–Phe^{25}, in the oxidized B-chain of insulin [1] and the B-chain of native insulin [3].

A. saitoi acid carboxypeptidase [18] with a MW

Table 4. Kinetic parameters of *Pycnoporus* acid carboxypeptidase towards angiotensin, bradykinin and Z–Glu–Tyr at pH 3.4 and 30°

Substrate	Concentration (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Angiotensin –His^6–Pro^7↓Phe^8	0.10–1.0	2.4	0.67	3.6
Bradykinin –Pro^7–Phe^8↓Arg^9	0.19–1.0	25	2.0	13
Synthetic peptide Z–Glu↓Tyr	0.2–2.0	16	0.74	22

The arrow indicates the split bond.

139 000 was purified from koji culture and *A. oryzae* acid carboxypeptidases, 0–1 and 0–2, with MWs 60 000, were also purified from this culture [24]. We previously reported that, in koji culture, *P. janthinellum* produced a major component of acid carboxypeptidase with a MW of 51 000 and a minor component of acid carboxypeptidase of MW 160 000 [25]. *P. janthinellum* acid carboxypeptidase with a MW 51 000 was purified and crystallized from submerged culture. In the present study, we obtained *Pycnoporus* acid carboxypeptidase with a MW of 50 000 from a submerged culture.

The optimum pH of the enzyme resembles that of some acid carboxypeptidases from *A. saitoi* [18], *A. oryzae* [23, 24] and *P. janthinellum* [25]. The optimum pH of the enzyme indicates that this wood-degrading fungal enzyme is different from those from higher plants such as citrus [5–7], French bean [12], barley [8], wheat germ [10] and cotton seed [13], but nevertheless shows important similarities in enzyme inhibition studies. All of these enzymes from micro-organisms and the higher plants mentioned above were inhibited by DFP and PMSF.

It was shown that the enzyme exhibited a preference for leucine, valine and the carboxyl group in the penultimate position to the *C*-terminus of the substrate. However, the specificity and mode of action of the enzyme differed from those of *A. saitoi* acid carboxypeptidase [18]. Previous studies had indicated that the *A. saitoi* acid carboxypeptidase exhibited a preference for aromatic and carboxyl groups in the penultimate position of the *C*-terminus [18]. The two *A. oryzae* enzymes, 0–1 and 0–2, appeared to be identical in their specificities for *N*-acylpeptides, optimum pH, MW, pH- and thermal stabilities, and sensitivity to various inhibitors [24]. But their modes of action for substrates having a –Pro–*X* bond differed.

The K_m value of 0.74 mM of the enzyme for Z–Glu–Tyr is similar when compared with those for other carboxypeptidases possessing similar specificity.

It is noteworthy that the enzyme could release the *C*-terminal proline. The serine carboxypeptidases have similar properties. Characterization of molecular and enzymatic properties of the enzyme suggested that it is a member of the serine carboxypeptidases (EC 3.4.16.1).

EXPERIMENTAL

Materials. Human proangiotensin (Lot 291022), bradykinin (Lot 310311) and benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z–Glu–Tyr) (Lot 290519) were purchased from the Protein Research Foundation, Osaka.

The crude enzyme preparation was prepared from the culture filtrate of *Pycnoporus sanguineus* (*Trametes sanguinea*) by the method previously described [4].

Assay of acid carboxypeptidase. The incubation mixture consisted of 0.5 ml 50 mM NaOAc buffer, pH 3.4, and 0.5 ml 1 mM Z–Glu–Tyr in 50 mM NaOAc buffer pH 3.4. Reaction time was 20 min (unless otherwise stated) at 30°. The reactions were stopped by adding 0.2 ml 2.5% HOAc. The amount of amino acid released was measured at 570 nm according to the previous paper [18].

Sequential determination of released C-terminal amino acid residues from proangiotensin. The incubation mixture consisted of 146 nmol proangiotensin in 1.5 ml aq. HCl adjusted to pH 3.4 with 0.1 M HCl and 0.5 ml *Pycnoporus* acid carboxypeptidase (including 1.56, 1.91 and 9.55 nkat) in aq. HCl adjusted to pH 3.4 with 0.1 M HCl. The mixture was incubated at 30° for the indicated time. An equal vol. of 10% CCl_3COOH was added to inactivate the enzyme. After extraction with Et_2O to remove CCl_3COOH, the aq. layer was evaporated to dryness *in vacuo*. Samples of the hydrolysate were investigated on the column of a Hitachi Model KLB 835-30 automatic amino acid analyser as described earlier [28].

Determination of kinetic parameters. To determine the initial rates of the enzyme activity for Z–Glu–Tyr, bradykinin and angiotensin in 0.5 ml 50 mM NaOAc buffer, pH 3.4, a procedure enabling the respective estimation of 3.21, 0.39 and 1.64 μg of the enzyme in 0.5 ml 50 mM NaOAc buffer, pH 3.4, was developed. 0.25 ml 0.06 M HCl was added to inactivate the enzyme. Amino acids liberated were determined on the column of the Hitachi amino acid analyser. By this means, 6–8 points could be determined during the first 2.5–10 min of the incubation at 30°, and the initial rate could be determined with considerable precision. In all cases, satisfactory Michaelis–Menten kinetics were observed and a plot of $1/V$ vs $1/[S]$ permitted the fitting of unambiguous straight lines.

Analytical determinations. Protein concns were usually estimated from the A at 280 nm ($A^{1\%}_{1\,cm} = 19.0$) using a Hitachi Model 101 spectrophotometer.

PAGE was performed at 4° with the standard pore formulation of Davis at pH 9.4 and 3 mA constant for gel [26]. Gels were stained with 0.4% Coomassie brilliant blue dissolved in 50% MeOH–9.5% HOAc and destained in 5% MeOH–9.5% HOAc and 7% HOAc [29].

SDS-disc gel electrophoresis was performed at 4° with 10% acrylamide gel as described in ref. [29].

For the determination of neutral sugars, 0.5 ml enzyme (0.263 mg/ml) dialysed against H_2O was added to 0.5 ml 5% PhOH. After addition of 2.5 ml 18 M H_2SO_4, neutral sugars were measured at 490 nm and calibrated against D-mannose (Komuro Chem. Industries).

For the determination of amino acid composition, 1 ml enzyme (102 μg/ml) dialysed against H_2O was added to 1 ml 11 M HCl (containing 5 μl mercaptoethanol and 10 μl 5% PhOH). The mixtures were hydrolysed at 110° for 24, 48 and 72 hr. Hydrolysates of the enzyme were examined with the Hitachi amino acid analyser as described earlier [30]. Tryptophan was determined spectrophotometrically according to the method described in ref. [31].

For the determination of amino sugars, hydrolysates of the enzyme with 3 M HCl at 100° for 16 hr were examined with the Hitachi amino acid analyser.

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