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Substrate Specificity of Carboxyl Proteinase from *Pycnoporus coccineus*, a Wood-Deteriorating Fungus

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Abstract. A carboxyl proteinase was purified from submerged-culture filtrate of a wood-deteriorating basidiomycete, *Pycnoporus coccineus*. The purified enzyme was found to be essentially homogeneous in disc gel electrophoresis tests at pH 9.4 and 2.3. The specificity and mode of action of *P. coccineus* carboxyl proteinase I_a were investigated with the oxidized B-chain of insulin. *P. coccineus* carboxyl proteinase I_a hydrolyzed primarily three peptide bonds, Ala^{14}-Leu^{15}, Tyr^{16}-Leu^{17}, and Phe^{24}-Phe^{25} bonds, in the oxidized B-chain of insulin.

The fungi feed entirely by absorption, not by photosynthesis or ingestion. Most fungi secure nitrogen from proteins and their hydrolysates. Fungal growth depends on having cell bound and extracellular proteolytic enzymes to provide the means to satisfy requirements for nitrogen.

The extracellular carboxyl proteinase (EC 3.4.23.6) produced by a wood-deteriorating basidiomycete, *Pycnoporus coccineus* (formerly designated as *Trametes sanguinea*), was initially detected and partially purified from submerged-culture filtrates by measuring its proteolytic activity against milk casein [15,16].The extracellular carboxyl proteinase showed optimal activity at pH 2.8 for milk casein digestion [16]. The partially purified enzyme preparation seemed to be homogeneous by free boundary electrophoresis at pH 6.08 and the sedimentation pattern showed it to be monodisperse [15]. However, the presence of benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z-Glu-Tyr) hydrolase activity in the carboxyl proteinase preparation still remained a possibility, since Z-Glu-Tyr is a sensitive substrate for serine carboxypeptidase (EC 3.4.16.1) of *Aspergillus* [1,5,6] and *Penicillium* [18,19,20].

In the present study, we obtained a further purified preparation of *P. coccineus* proteinase. The specificity of *P. coccineus* carboxyl proteinase was investigated, therefore, using the oxidized B-chain of bovine insulin as the defined polypeptide substrate. We have measured quantitatively the relative rates of hydrolysis by the carboxyl proteinase. The results are discussed in relation to the bond specificities of the other carboxyl proteinases from animal and microbial origins.

Materials and Methods

Materials. Crystalline bovine insulin (lot 57590) was purchased from Fluka AG, Buchs SG, Switzerland. Benzyloxycarbonyl-L-glutamyl-L-tyrosine was purchased from the Protein Foundation, Osaka. After 6 M urea treatment, urea-denatured hemoglobin was dialyzed and lyophilized.

Carboxyl proteinase assay. The carboxyl proteinase activity was assayed by Kunitz's method with a slight modification. To 1 ml of an enzyme solution in a test tube, equilibrated at 30°C, was added 1 ml of 2% urea-denatured hemoglobin in 0.1 M acetate buffer, pH 2.7. After 10 min digestion, 2 ml of 0.4 M trichloroacetic acid solution was added. The precipitated protein was removed by filtration with Toyo filter paper no. 2, and absorbance at 280 nm of the filtrate was determined. Carboxyl proteinase activity is expressed as the katal (abbreviation: kat) according to the IUPAC and IUB recommendations. One katal of the carboxyl proteinase is defined as the amount of enzyme which yields the equivalent of 1 mol of tyrosine per second at 280 nm, using urea-denatured hemoglobin as substrate at pH 2.7 and 30°C.

Purification of the carboxyl proteinase. The carboxyl proteinase was isolated and crystallized from the culture filtrate of *P. coccineus* (=*Trametes sanguinea*) by a previously published method [16]. Further purification of the crystalline carboxyl proteinase was performed on chromatographic procedures with DEAE-Sephadex A-50 and SP-Sephadex C-50, and by isoelectric focusing [17]. The column of DEAE-Sephadex A-50 (2 × 55 cm) was equilibrated with 0.01 M acetate buffer (pH 5.0). Elution was performed with 0.01 acetate buffer (pH 2.7) containing 0.1 M NaCl. The active fraction eluted from DEAE-Sephadex A-50 was run through a

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column of SP-Sephadex C-50 (2 × 55 cm; Fig. 1). The column of SP-Sephadex was equilibrated with 0.01 M acetate buffer (pH 3.5). Elution was performed with 0.01 M acetate buffer (pH 5.0) containing 0.1 M NaCl (Fig. 2).

Isoelectric focusing was used as the final purification step. Ampholines, pH 4.0–6.0, were incorporated into a sucrose gradient to 1%. The LKB electric focusing column (110 ml volume) was used. The focusing time was generally 60 h with the column at 4°C and 500 V. Fractions of 1.6 ml were collected. Absorbance at 280 nm in each fraction was estimated using microcuvettes. The carboxyl proteinase activity was eluted into two fractions, I_a and I_b. For peak I_a 26.2 μkat (122 mg) and for peak I_b 30.7 μkat (169 mg) of activities were recovered. The carboxyl proteinase I_a having a higher specific activity was used in this work. Immediately after the pH had been determined using a Radiometer titrater TTT type pH meter, the purified enzyme was dialyzed against 0.05 M acetate buffer (pH 3.2) to remove the ampholines and then stored at 4°C.

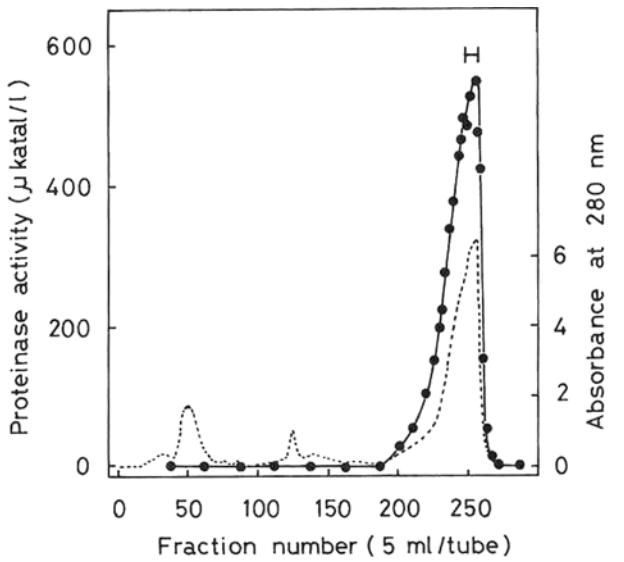

Fig. 1. DEAE-Sephadex A-50 chromatography of *Pycnoporus* carboxyl proteinase from crude crystalline preparation. (●) Carboxyl proteinase activity; (-----) absorbance at 280 nm.

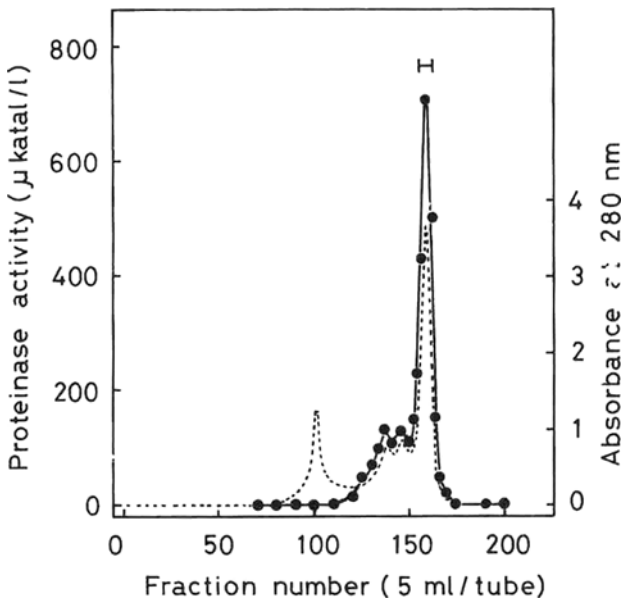

Fig. 2. SP-Sephadex C-50 chromatography of *Pycnoporus* carboxyl proteinase from DEAE-Sephadex A-50 run. (●) Carboxyl proteinase activity; (-----) absorbance at 280 nm.

Portions of purified fractions eluted from the column were incubated for 48 and 72 h with the sensitive carboxypeptidase substrate, Z-Glu-Tyr, at pH 3.1 and 30°C to detect serine carboxypeptidase activity [5]. Hydrolysis of Z-Glu-Tyr was detected by paper chromatography, using a solvent system of *n*-butanol/acetic acid/water, 4:1:2. The purified fraction did not contain any carboxypeptidase activity. The purified fractions were incubated for 3 h with angiotensin II at pH 2.7 and 30°C to detect aminopeptidase activity. The purified fractions did not contain any aminopeptidase activity.

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed at 3°C with the standard pore formulation of Williams and Reisfeld at pH 9.5 and pH 2.3, and 3 mA constant for all gels [5]. Gels were stained with 0.25% Coomassie brilliant blue dissolved in methanol–glacial acetic acid–H_2O (5:1:5, vol/vol) and destained in methanol–acetic acid–H_2O (5:1:4 and 1:1:8, vol/vol) and 7% acetic acid.

Protein concentration. Protein concentrations were usually estimated with the absorbance at 280 nm ($A^{1\%}_{1cm}$ = 15.0) using a Hitachi model 101 spectrophotometer.

Oxidation of insulin. Oxidation of insulin was performed as described by Craig, Konigsberg, and King [2]. Oxidized B-chain of insulin was prepared as described by Griffin, Wagner, and Prescott [4]. Oxidized B-chain preparation was pure as judged by the N-terminal analysis with 1-fluoro-2,4-dinitrobenzene [3] and amino acid analysis.

Separation and identification of peptides from the digest of oxidized B-chain. Oxidized B-chain of insulin (2 μmol, 7 mg) was dissolved in 10 ml dilute HCl, 1.6 nkat of carboxyl proteinase I_a from *P. coccineus* was added to the solution, and then the pH was adjusted to 2.7 with dilute HCl. The mixture was incubated at 30°C for 20 min. One drop of concentrated NH_4OH was added to inactivate the enzyme. The samples of hydrolysates were stored at −20°C, and then the frozen digest was lyophilized.

The freeze-dried digest was then dissolved in 50 μl of 1 M NH_4OH and was separated on Toyo filter paper (60 × 60 cm) in the first dimension by high voltage paper electrophoresis in pyridine/acetic acid/water, 10 : 0.4 : 90, at pH 6.5 for 180 min at 36 V/cm. After drying, at room temperature ascending chromatography was carried out in the solvent system *n*-butanol/acetic acid/water, 4 : 1 : 2 for 60 h. The peptide maps were stained with 0.2% ninhydrin-acetone reagent. Ninhydrin-positive zones were cut out and peptides on paper were washed with acetone and eluted with water. The eluates were freeze-dried in vacuo.

Amino acid analysis. Freeze-dried peptides were dissolved in 2 ml 6 M HCl and were hydrolyzed at 110°C for 24 h. Hydrolysates of peptides were examined with a Hitachi amino acid analyzer, model KLA-3B or 835-30.

Determination of N-terminal amino acid of peptides. The N-terminal amino acids of the purified peptides (B-1, N-1, N-2, N-3, A-1, and A-3) (Fig. 4) were determined by the DNP method of Sanger

[3]. The DNP-amino acids were identified by paper chromatography with the solvent system 1 M NaH_2PO_4/0.5 M Na_2HPO_4 buffer (pH 6.0).

Estimation of the extent of hydrolysis of the various peptide bonds. The hydrolysis rates of the individual peptide bonds which were split were then calculated by summation of the yields of the peptides located on either side of the bond being opened.

Results

The specific activities of *Pycnoporus coccineus* carboxyl proteinase I_a (isoelectric point pI, 4.72) and I_b (pI, 4.58) were 0.215 kat/kg of enzyme and 0.182 kat/kg of enzyme, respectively. The final preparation of the carboxyl proteinase I_a did not have any exopeptidase activities. The highly purified preparation of *P. coccineus* carboxyl proteinase I_a migrates as a single band on disc gel electrophoresis at pH 9.4 and 2.3 (Fig. 3). *P. coccineus* carboxyl proteinase I_a has the $A^{1\%}_{1cm}$ value of 15.0 at 280 nm.

Fig. 3. Disc gel electrophoresis of *Pycnoporus coccineus* carboxyl proteinase I_a (11 μg). (A) Anodic run at pH 9.4 gel; (B) cathodic run at pH 2.3 gel.

Time course for the hydrolysis of oxidized B-chain of insulin by *P. coccineus* carboxyl proteinase I_a was examined. Seven peptides were obtained with both the 20-min and 40-min incubation (Fig. 4). The yield of all peptide segments was 60–65%. The strong spots were B-1, N-2, N-3, A-1, and A-3. The N-terminals of B-1, N-1, N-2, N-3, A-1, and A-3 were identified. The results of the 20-min incubation are shown in Table 1. During the rapid hydrolysis at 20-min incubation, three peptide bonds in the oxidized B-chain of insulin molecule might be hydrolyzed. The results in Fig. 5 show that *P. coccineus* carboxyl proteinase I_a hydrolyzed primarily three peptide bonds in the oxidized B-chain of insulin, the Phe^{24}-Phe^{25}, Tyr^{16}-Leu^{17}, and Ala^{14}-Leu^{15} bonds. With the 40-min incubation, the bond Phe^{24}-Phe^{25} in the oxidized B-chain of insulin appears to be a relatively faster cleaving site than the Tyr^{16}-Leu^{17} and Ala^{14}-Leu^{15} bonds of hydrolysis by *P. coccineus* carboxyl proteinase I_a.

The peptide map obtained from *P. coccineus* carboxyl proteinase I_b was identical with that of the carboxyl proteinase I_a. The specificity of the proteinase I_b seemed to be similar to the proteinase I_a.

Discussion

Splitting sites at 24-25, 16-17, and 14-15 in oxidized B-chain of insulin with *Pycnoporus coccineus* car-

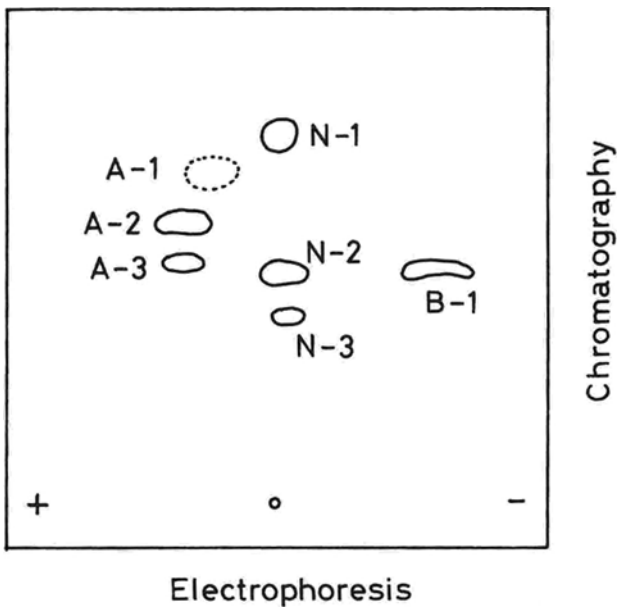

Fig. 4. Peptide map for the digest of oxidized B-chain of insulin by *Pycnoporus coccineus* carboxyl proteinase I_a.

Table 1. Amino acid compositions of peptides obtained from the 20-min digest of oxidized insulin B-chain by *Pycnoporus coccineus* carboxyl proteinase. The values in parentheses denote the theoretical number of residues of a given amino acid in the peptide.

	Peptides (residues/molecule)						
Amino acid	B-1	N-1	N-2	N-3	A-1	A-2	A-3
Lys	0.83(1)						
His			1.59(2)	1.63(2)			
Arg					0.85(1)	1.37(1)	0.74(1)
CySO			1.03(1)	0.92(1)	0.91(1)	1.30(1)	0.82(1)
Asp			1.00(1)	1.00(1)			
Thr	0.99(1)						
Ser			0.76(1)	0.79(1)			
Glu			1.96(2)	1.90(2)	1.00(1)	0.31(1)	1.00(1)
Pro	1.03(1)						
Gly			1.25(1)	1.00(1)	1.93(2)	1.48(2)	1.68(2)
Ala	1.00(1)		1.05(1)	0.89(1)			
Val			1.90(2)	1.80(2)	1.06(1)	1.00(1)	1.00(1)
Leu		1.00(1)	2.70(3)	1.85(2)	1.63(2)	0.76(1)	0.90(1)
Tyr	0.44(1)	0.76(1)	0.20(1)		0.57(1)		
Phe	0.60(1)		0.94(1)	0.74(1)	0.89(1)	0.58(1)	0.80(1)
Recovery (μmol; uncorrected)	0.109	0.067	0.202	0.211	0.207	0.043	0.237
N-terminal amino acid	Phe	Leu	Phe	Phe	Leu		Leu
Suggested segment	25–30	15–16	1–16	1–14	15–24	17–24	17–24

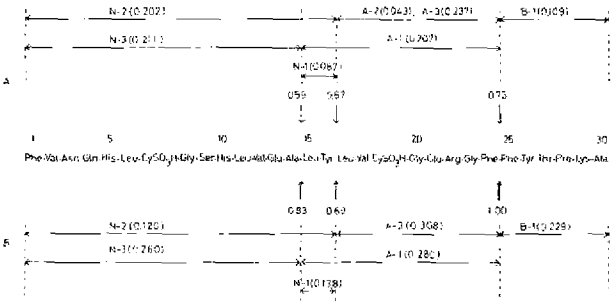

Fig. 5. Summary of the specificity of *Pycnoporus coccineus* carboxyl proteinase I_a towards oxidized B-chain of insulin. Two micromoles of oxidized B-chain of insulin was digested with 1.6 nkat of the enzyme (e/s; 1:289). The values in parentheses denote the uncorrected recovery (μmol) of a peptide. The arrows indicate the bonds split, and the relative rate of hydrolysis at Phe^{24}-Phe^{25} bond and 40-min incubation is arbitrarily taken to be 1.00. (A) 20-min incubation at pH 2.7; (B) 40-min incubation at pH 2.7.

boxyl proteinase I_a were similar to those reported in the work on porcine pepsin C (EC 3.4.23.3) by Ryle, Leclerc, and Falla [12]. However, *P. coccineus* carboxyl proteinase could not split the 10-11 and 13-14 bonds, which are known splitting sites with porcine pepsin C [12]. In this experiment, we found the characteristic small dipeptide N-1 in the hydrolysate of oxidized B-chain of insulin with *P. coccineus* carboxyl proteinase. The dipeptide seemed to be a secondary product of enzymatic reaction.

Primary splitting sites at Phe^{24}-Phe^{25} and Leu^{15}-Tyr^{16} and a secondary splitting site at Tyr^{16}-Leu^{17} in the oxidized B-chain of insulin with *Aspergillus sojae* carboxyl proteinase were reported in a previous paper [7]. *P. coccineus* carboxyl proteinase has a slightly different substrate-specificity towards the middle part of the oxidized B-chain of insulin.

Like *P. coccineus* carboxyl proteinase I_a, *Aspergillus sojae* carboxyl proteinase [7], *Aspergillus saitoi* carboxyl proteinase [14], human erythrocyte cathepsin D [9], porcine pepsin C [12], have high affinity for the Phe^{24}-Phe^{25} bond in the C-terminal area of oxidized B-chain of insulin. On the contrary, human pepsin 3 [11] and human non-pepsin proteinase 2 [10] have high affinity for the Phe^{25}-Tyr^{26} bond.

In the present study, residues in peptides and the corresponding subsites in the enzyme are numbered according to Schechter and Berger [13]. From the results of peptide bond specificity of *P. coccineus* carboxyl proteinase I_a, the peptide bonds which have hydrophobic amino acids such as phenylalanine and leucine in the P_1' position are preferentially cleaved by *P. coccineus* carboxyl proteinase I_a. *P. coccineus* carboxyl proteinase could not hydrolyze the Leu^{14}-Tyr^{15} and Phe^{25}-Tyr^{26} bonds. The results suggest that the hydroxyl group of tyrosine in the P_1' site may inhibit the enzyme action of *P. coccineus* carboxyl proteinase. These results in this experiment show that the S_1'-P_1' interactions might be important in increas-

ing enzyme-substrate affinity and turnover rate. The present study also shows that the *P. coccineus* carboxyl proteinase I_a exhibited a preference for a hydrophobic amino acid such as tyrosine and valine in the P_2' position. Furthermore, the present results show that *P. coccineus* carboxyl proteinase I_a exhibited a lower preference for a hydrophobic amino acid in the P_1 position, but that pepsin exhibited a strong preference for a hydrophobic amino acid in the P_1 position. The S_1 subsite of pepsin is obviously the primary determinant of specificity [8].

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