Note

Substrate Specificity of a Carboxyl Proteinase from *Irpex lacteus*Hideyuki KOBAYASHI, Isao KUSAKABE
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Two carboxyl proteinases with high milk-clotting activity secreted by a basidiomycete, *Irpex lacteus*, have been purified and characterized.¹⁾ The main enzyme, carboxyl proteinase B, has a molecular weight of 36,000, an amino acid composition similar to that of *Endothia parasitica* carboxyl proteinase, and has maximum proteolytic activity on hemoglobin at pH 3.0. The enzyme B exhibits almost the same ratio of milk-clotting activity to proteolytic activity as commercial rennet substitutes from *Mucor pusillus* and *Mucor miehei*. The enzyme was inhibited by carboxyl proteinase inhibitors such as pepstatin, diazoacetyl-D,L-norleucine methyl ester (DAN) and epoxy-p-nitrophenoxypyrone (EPNP). This fact indicates that enzyme B is similar to calf chymosin and microbial

milk-clotting enzymes in their active site structure, having two different carboxyl groups. In this work, the site of cleavage towards the oxidized B-chain of insulin by the carboxyl proteinase B from *Irpex lacteus* was investigated.

Purified carboxyl proteinase B from *Irpex lacteus* was prepared according to the methods described in the previous paper.¹⁾ The oxidized insulin B-chain used as the substrate was prepared from crystalline bovine pancreatic insulin (sigma) according to the method of Sanger²⁾ and the purity of the preparation was ascertained by amino acid analysis and N-terminal amino acid analysis by the method of Hartley.³⁾ The digests of the oxidized B-chain by enzyme B were separated on Dowex 50W-X2 as shown in Fig. 1. The fractions of each peptide shown with brackets in Fig. 1 were concentrated and applied on a Sephadex LH-20 column (0.9 × 90 cm) equilibrated with 0.1 M acetic acid. Each of the fractions was then subjected to thin layer chromatography (silica gel 60, Merck) to determine the homogeneity of its components, using *n*-butanol-pyridine-acetic acid-water = 30:23:6:24 as a solvent. Peptides were detected with fluorescamine. Then the isolated peptides were subjected to analyses of amino acid compositions and N-terminal amino acids. The amino acid compositions and N-terminal amino acids of these peptides are summarized in Table I. In the reaction with fluorescamine, the fluorescent intensity of the peptide having proline as its N-terminal amino acid, such as peptide H, is not increased. Therefore, peptide H appeared as a minor peak, although it is one of the main components, as seen in Table I. The peptide bonds mainly

FIG. 1. Column Chromatography of the Digest of the Oxidized Insulin B-chain on Dowex 50W-X2.

The enzyme B (0.2 mg) was added to a solution of 40 mg of insulin B-chain in 10 ml of 0.1 M acetic acid (pH 3.0, substrate: enzyme = 200:1). After 2 hr incubation at 35°C, the reaction was terminated by lyophilization. The lyophilized powder was dissolved in 1 ml of 50% formic acid and applied on a column of Dowex 50W-X2 (0.9 × 60 cm) equilibrated with 0.2 M pyridine-acetic acid buffer, pH 3.1 at 50°C at a constant rate of 30 ml/hr. After washing with the same buffer, peptides were separated by the linear gradient generated between 0.75 liters of 0.2 M pyridine-acetic acid buffer, pH 3.1 and 0.75 liters of 2 M pyridine-acetic acid buffer, pH 5.0. A 5 ml volume was collected for each tube. Peptides were detected as follows: borate buffer (1.8 ml, 0.5 M, pH 8.5) and 20 µl of each sample were mixed and 0.15 ml of fluorescamine solution (30 mg/100 ml of acetone) was rapidly added with agitation. Fluorescent intensities were measured using a Hitachi fluorescence spectrophotometer model 650-10S and expressed in arbitrary units. The wave lengths of excitation and emission were 390 nm and 480 nm, respectively. ●, relative intensity; —, pyridine concentration.

TABLE I. COMPOSITION OF PEPTIDES FORMED BY THE ACTION OF ENZYME B FROM *Irpex lacteus* ON OXIDIZED INSULIN B-CHAIN

Amino acid compositions were analyzed by a Durrum amino acid analyzer model D-5 after hydrolysis with 6N HCl at 110°C for 24 hr. The dansyl-peptides were hydrolyzed in 6N HCl at 110°C for 20 hr and the dansylamino acids were isolated by the method of Woods and Wang⁴⁾ with polyamide sheets.

Peptide No.	N-Terminal	Amino acid found and its molar ratio	Possible amino acid sequence	Recovery (%) ^a
A	Val	Glu(1.06), Ala(1.00), Val(1.00)	Val(12)-Ala(14)	3
B ^b	—	—	—	—
C	Leu	Cys(0.96), Glu(1.36), Gly(2.14), Val(1.17), Leu(1.85), Tyr(0.61), Arg(0.94)	Leu(15)-Gly(23)	2
D	Leu	Cys(1.03), Glu(1.30), Gly(2.08), Val(1.16), Leu(1.84), Tyr(1.17), Phe(2.05), Arg(1.03)	Leu(15)-Phe(25)	2
E	Leu	Cys(1.05), Glu(0.75), Gly(2.03), Val(1.14), Leu(1.76), Tyr(0.93), Phe(1.08), Arg(1.02)	Leu(15)-Phe(24)	10
Fa	Phe	Cys(1.08), Asp(1.06), Ser(1.03), Glu(1.89), Gly(1.12), Ala(1.06), Val(2.00), Leu(2.20), Phe(0.99), His(2.07)	Phe(1)-Ala(14)	14
Fb	Phe	Thr(0.96), Tyr(0.89), Phe(1.15)	Phe(25)-Thr(27)	5
Ga	Phe	Cys(1.07), Asp(0.80), Ser(0.97), Glu(1.33), Gly(1.04), Ala(0.66), Val(1.11), Leu(2.08), Phe(0.85), His(1.75)	Phe(1)-Leu(11)	7
Gb	Leu	Cys(1.24), Thr(0.76), Glu(1.24), Gly(2.23), Val(1.08), Leu(1.71), Tyr(1.68), Phe(2.16), Arg(1.11)	Leu(15)-Thr(27)	7
H	Pro	Pro(1.00), Ala(1.00), Lys(1.00)	Pro(28)-Ala(30)	11
I	Tyr	Thr(1.01), Pro(1.00), Ala(1.00), Tyr(0.77), Lys(1.02)	Tyr(26)-Ala(30)	2
J	Phe	Thr(1.13), Pro(1.08), Ala(1.11), Tyr(0.74), Phe(0.77), Lys(1.16)	Phe(25)-Ala(30)	5
K	Phe	Thr(1.05), Pro(0.99), Ala(0.97), Tyr(1.00), Phe(1.93), Lys(1.10)	Phe(24)-Ala(30)	3

^a The recovery of peptides was determined by amino acid analysis and expressed as the percentage of total peptide fractions eluted from the Dowex 50W-X2 column.

^b No attempt was made to assign the amino acid composition because of its heterogeneity.

susceptible to the enzyme were the Leu(11)-Val(12), Ala(14)-Leu(15), Phe(24)-Phe(25) and Thr(27)-Pro(28) bonds, and the Ala(14)-Leu(15) bond was hydrolyzed most preferentially. Leu(11)-Val(12), Phe(24)-Phe(25) and Thr(27)-Pro(28) were hydrolyzed to a relatively great extent, whereas the hydrolyses at Gly(23)-Phe(24) and Phe(25)-Tyr(26) were minor. The specificities of several other proteolytic enzymes as determined on the oxidized B-chain of insulin are compared in Fig. 2. The specificity of *Irpex lacteus* enzyme B is distinct from other commercial microbial milk-clotting enzymes. It has a more restricted specificity than chymosin and porcine pepsin.

Kawai⁵⁾ previously reported that *Trametes sanguinea*

(now designated *Pycnoporus coccineus*) produced a proteinase with high milk-clotting activity, to the same degree as *Irpex lacteus*. But the *Irpex lacteus* enzyme was chosen as a rennet substitute because it produced cheddar cheese of good quality.⁵⁾ As shown in Fig. 2, the Ala(14)-Leu(15) and Phe(24)-Phe(25) bonds were commonly cleaved by both enzyme B from *Irpex lacteus* and the carboxyl proteinase from *Pycnoporus coccineus*, but the Leu(11)-Val(12) and Tyr(16)-Leu(17) bonds were only cleaved by the enzyme B and *Pycnoporus coccineus* enzyme, respectively. Moreover, a notable property of enzyme B is that it hydrolyzed the Thr(27)-Pro(28) bond which is hydrolyzed very little by any of the carboxyl proteinases

FIG. 2. Comparison of Cleavage Sites of Oxidized Insulin B-Chain by Some Carboxyl Proteinases.

The arrows indicate the bond split, the degree of hydrolysis being as follows: $\uparrow > \uparrow\uparrow > \uparrow\uparrow\uparrow$

1, *Irpex lacteus* carboxyl proteinase B; 2, calf chymosin⁶; 3, porcine pepsin⁶; 4, *Mucor miehei* carboxyl proteinase⁷; 5, *Endothia parasitica* carboxyl proteinase⁸; 6, *Scytalidium lignicolum* carboxyl proteinase A¹²; 7, *Scytalidium lignicolum* carboxyl proteinase B⁹; 8, *Pycnoporus coccineus* carboxyl proteinase.¹⁰

ever studied.

On the other hand, Ichishima *et al.*¹¹ reported that *Aspergillus sojae* carboxyl proteinase I and *Scytalidium lignicolum* carboxyl proteinase B hydrolyzed the peptide bond of His(6)-Pro(7) of angiotensin I. He classified carboxyl proteinases into four groups¹² (A to D in Fig. 3) according to their specificity toward angiotensin I. The specificity of enzyme B from *Irpex lacteus* on angiotensin I was investigated. As shown in Fig. 3, *Irpex lacteus* enzyme B hydrolyzed the Tyr(4)-Ile(5) bond much more rapidly than the Val(3)-Tyr(4) bond. This specificity resembled that of *Pycnoporus coccineus* carboxyl proteinase.

Using the numbering system of Schechter and Berger,¹³ enzyme B generally required hydrophobic amino acids for exhibiting its activity in the P₁ and P₁' position. Enzyme B did not split the His(6)-Pro(7) bond in angiotensin I in spite of the cleavage of the Thr(27)-Pro(28) bond in oxidized insulin B-chain. This result suggests that the positive charge of histidine in the P₁ position in angiotensin I may inhibit enzyme-substrate complex formation.

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FIG. 3. Comparison of Cleavage Sites of Angiotensin I by Some Carboxyl Proteinases.

Angiotensin I (1 mg, 0.77 nmol) was dissolved in 2 ml of 0.1 M acetic acid at pH 3.0, and incubated at 30°C for 3 hr with enzyme B (substrate:enzyme=2000:1). After that, the reaction was terminated by lyophilization. The N-terminal amino acids were isolated by the method of Hartley.³ The arrows indicate the bond split. Solid arrows, major sites; broken arrows, minor sites. 1, *Aspergillus sojae* carboxyl proteinase I⁶; 1', *Scytalidium lignicolum* carboxyl proteinase B⁶; 2, *Scytalidium lignicolum* carboxyl proteinase A⁶; 3, *Pycnoporus coccineus* carboxyl proteinase⁶; 4, porcine pepsin⁶; 5, *Irpex lacteus* carboxyl proteinase B.

their kind supplying of *Irpex lacteus* rennet powder.

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