Specificity of Thermophilic *Streptomyces* Alkaline Proteinase

Masashi MATSUE,* Eiji MAJIMA and Eiji ICHISHIMA**

*Laboratory of Enzymology and Microbial Chemistry,
Tokyo Nōkō University, Fuchu, Tokyo 183, Japan*

Received March 3, 1982

The specificity of highly purified alkaline proteinase B (EC 3.4.21.14) from thermophilic *Streptomyces rectus* var. *proteolyticus* was investigated with an oxidized insulin B chain. Hydrolysis of the oxidized insulin B chain in a 4-hr incubation was observed mainly at three peptide bonds (Phe²⁴-Phe²⁵, Leu¹⁵-Tyr¹⁶ and Leu¹¹-Val¹²) and additionally at six others (Leu⁶-CysO₃H⁷, Gln⁴-His⁵, Leu¹⁷-Val¹⁸, His⁵-Leu⁶, Glu¹³-Ala¹⁴, Asn³-Gln⁴).

Hydrolysis of angiotensin (formerly designated angiotensin II) was observed at the Tyr⁴-Ile⁵ bond. Hydrolysis of proangiotensin (formerly designated angiotensin I) was observed at the Tyr⁴-Ile⁵ and Phe⁸-His⁹ bonds.

The extracellular alkaline proteinase B (EC 3.4.21.14), produced by thermophilic *Streptomyces rectus* var. *proteolyticus*, a soil microorganism, shows optimal activity at pH 10.7 for milk casein digestion,¹⁾ and it possesses a single active serine residue²⁾ and a single thiol group.³⁾

In the present study, the specificity of thermophilic *Streptomyces* alkaline proteinase B was investigated towards oxidized insulin B chain, angiotensin and proangiotensin.

MATERIALS AND METHODS

Materials. Crystalline bovine insulin (Lot 24C-3130) was purchased from Sigma Chemical Co. Human angiotensin (Lot 29073) and proangiotensin (Lot 291022) were purchased from the Protein Research Foundation, Osaka. Polyamide layer sheet (Lot 690418) was purchased from the Seikagaku Kogyo Co.

An oxidized insulin B chain was prepared according to the method described previously.⁴⁾

***Streptomyces* alkaline proteinase.** The alkaline proteinase B was assayed according to the method described previously.⁵⁾ One katal of alkaline proteinase B was defined as the amount of enzyme which yields the equivalent of 1 mol of tyrosine per second at 280 nm, using milk casein as substrate at pH 8.0 and 30°C.

Crystalline alkaline proteinase from *Streptomyces rectus*

var. *proteolyticus* was the gift of Dr. K. Mizusawa, Kikkoman Corp.

Determination of time course for the hydrolysis of oxidized insulin B chain. The oxidized insulin B chain (8.75 mg) was dissolved in 12.5 ml of 0.05 M sodium borate buffer, pH 8.0, and incubated for 20 min with 2.2 nkatal (enzyme/substrate (e/s)=1:1600, mol/mol) of *Streptomyces* alkaline proteinase at 30°C. Aliquots (500 μ l) were withdrawn at various time intervals and mixed with 500 μ l 5% acetic acid; a ninhydrin assay⁶⁾ was carried out on these samples. The total numbers of amino groups of the oxidized insulin B chain were determined with a hydrolysate of 6 N HCl at 110°C for 24 hr.

Hydrolysis of oxidized insulin B chain. The oxidized insulin B chain (7 mg) was dissolved in 10 ml 0.18 M NH₄OH, and 2.2 nkatal (e/s; 1:1280, mol/mol) of alkaline proteinase B from *Streptomyces* was added to the solution, and then the pH was adjusted to 8.0 with NH₄OH. The mixture was incubated at 30°C for 4 hr. One drop of concentrated HCl was added to inactivate the enzyme. The samples of hydrolysate were stored at -20°C, and then the frozen digest was lyophilized.

Hydrolysis of angiotensin and proangiotensin. Angiotensin (angiotensin II, 2 mg, 1.98 μ mol) was dissolved in 10 ml of 0.18 M NH₄OH at pH 8.0, and incubated at 30°C with 2.5 nkatal (e/s; 1:1200, mol/mol) of *Streptomyces* alkaline proteinase B for 1 hr.

Proangiotensin (angiotensin I, 2 mg, 1.54 μ mol) was dissolved in 10 ml of 0.18 M NH₄OH at pH 8.0, and incubated at 30°C with 2.5 nkatal (e/s; 1:968, mol/mol) of

* Present address: Miyake High School, Miyake Village, Miyake Island, Tokyo 100-12, Japan.

** To whom inquiries about this paper should be addressed.

Streptomyces alkaline proteinase B for 1 hr.

Separation of peptides from enzymatic digest. The freeze-dried digest was dissolved in 50 μ l 1 M NH_4OH , and was separated on Toyo filter paper, in the first dimension by high-voltage paper electrophoresis, and in the second dimension by paper chromatography by a method previously described.⁴⁾ The letters A, N and B refer to acidic, neutral or basic peptides, respectively.

Further purifications of the B-1 and N-3 peptides shown in Fig. 2 were performed on chromatographic procedures with Bio-Gel P-2 (1 $\times$ 55 cm), respectively. Elution was performed with 0.1 M NH_4OH . The peptide fractions eluted from Bio-Gel P-2 were determined at 230 nm. Further purification of N-4 peptide was performed on DEAE-Sephadex A-25. Gradient elution was performed with NH_4CO_3 from 0.1 M to 1 M.

Amino acid analysis. Freeze-dried peptides were dissolved in 2 ml of 5.7 N HCl and were hydrolyzed at 110°C for 24 hr. Hydrolysates of peptides were examined with a Hitachi amino acid analyzer, Model 835-30.

Determination of N-terminal amino acid of peptides. The N-terminal amino acids of the purified peptides (A-1, A-2, N-1, N-2, N-3a, N-3b, N-4a and B-1a) were determined by the DNP-method of Sanger.⁷⁾

The N-terminal amino acids of peptides obtained from angiotensin and proangiotensin were determined by the DNS-method.⁸⁾ The DNS-amino acids were identified by polyamide layer sheet (7.5 $\times$ 7.5 cm) using the solvent system: 1.5% HCOOH , and benzene-acetic acid (9:1, v/v).

Relative rate of hydrolysis. The relative rate of hydrolysis at the $\text{Phe}^{24}\text{-Phe}^{25}$ was calculated from the peptides recovered.

RESULTS

Hydrolysis of oxidized insulin B chain

The rate of hydrolysis of the oxidized insulin B chain was examined and the results are shown in Fig. 1. The peptides obtained in 4-hr incubation were isolated and purified as described; a total of twelve peptides were obtained. The strong spots shown in the finger print map (Fig. 2) of the digest from the oxidized insulin B chain were the acidic peptide A-2, neutral peptides N-1, N-2, N-3 and the basic peptide B-1. Further purifications of B-1, N-3 and N-4 fractions were performed on chromatographic procedures.

Table I lists the peptides, their amino acid

FIG. 1. Time Course for the Hydrolysis of the Oxidized Insulin B Chain by Thermophilic *Streptomyces* Alkaline Proteinase B.

FIG. 2. Peptide Map of the Digest of the Oxidized Insulin B Chain by Thermophilic *Streptomyces* Alkaline Proteinase B.

compositions and recoveries. All of the peptides were readily identifiable from the known sequence of the oxidized insulin B chain. However, the B-1b fraction contained at least two peptides. In the case of B-1b, it was possible to assume the fraction had two peptides, B-1b-1 and B-1b-2, as indicated in Table I. The recovery of the peptide $\text{His}^5\text{-Leu}^6$ (B-1b-2) was not determined, because the presence of a contaminating B-1a peptide in the B-1b preparation still remained a possibility. A

TABLE I. AMINO ACID COMPOSITION OF PEPTIDES FROM THE 4-hr DIGEST OF OXIDIZED INSULIN B CHAIN BY THERMOPHILIC *Streptomyces* ALKALINE PROTEINASE B^a

Amino acids	Peptides (residues/molecule)												
	A-1	A-2	A-3	N-1	N-2	N-3a	N-3b	N-4a	N-4b	B-1a	B-1b-1	B-1b-2	B-2
CySO ₃ H		0.8 (1)	0.9 (1)			1.0 (1)		0.5 (1)	0.9 (1)				
Asp					0.8 (1)		1.0 (1)						1.0 (1)
Thr										1.0 (1)	1.4 (1)		
Ser			0.9 (1)			0.8 (1)			1.1 (1)				
Glu	1.0 (1)	1.0 (1)					0.9 (1)	2.0 (1)					1.0 (1)
Pro										0.5 (1)	0.9 (1)		
Gly		1.8 (2)	1.1 (1)			1.0 (1)		3.6 (2)	2.2 (1)				
Ala	1.0 (1)			1.0 (1)						1.0 (1)	1.0 (1)		
Val	1.0 (1)	1.0 (1)			1.0 (1)		0.8 (1)	0.5 (1)					1.0 (1)
Leu	1.3 (1)	1.0 (1)	1.0 (1)	1.9 (2)		2.0 (1)			2.0 (2)			0.6 (1)	
Tyr		0.6 (1)		1.0 (1)						0.9 (1)	1.4 (1)		
Phe		0.9 (1)			0.6 (1)		0.9 (1)	1.0 (1)		0.7 (1)	1.1 (1)		0.5 (1)
Lys										1.0 (1)	1.3 (1)		
His			0.7 (1)			1.0 (1)			2.0 (2)			0.4 (1)	0.8 (1)
Arg		0.9 (1)						1.0 (1)					
Recovery (μmol)	0.278	0.862	0.460	0.188	0.142	0.101	0.639	0.091	0.228	0.466	nd	nd	0.106
N-Terminal amino acid	Val	Tyr	nd*	Ala	Phe	Leu	Phe	Val	nd	Phe	Phe	nd	nd
Suggested segment	12~15	16~24	7~11	14~17	1~3	6~11	1~4	18~24	5~11	25~30	25~30	5~6	1~5

* nd, not detected.

^a The values in parentheses denote the theoretical number of residues of a given amino acid in the peptide.

FIG. 3. Summary of the Specificity of Thermophilic *Streptomyces* Alkaline Proteinase B towards the Oxidized Insulin B Chain.

Abbreviations of amino acids are alphabetical. The perpendicular arrows indicate the bond split and relative rate of hydrolysis at the Phe²⁴-Phe²⁵ bond. Thick arrows indicate major sites; thin arrows, minor sites. Horizontal arrows indicate the peptides obtained.

A: Thermophilic *Streptomyces rectus* alkaline proteinase B, 4-hr incubation at pH 8.0 and 30°C (e/s, 1:1260, mol/mol).

B: Subtilisin Carlsberg,¹¹⁾ 2-hr incubation at pH 8.0 and 30°C (e/s, 1:980, w/w).

C: Subtilisin Novo,¹¹⁾ 2-hr incubation at pH 8.0 and 30°C (e/s, 1:1170, w/w).

D: *Streptomyces fradiae* proteinase type III,¹²⁾ at pH 9 and room temperature.

E: *Streptomyces griseus* alkaline proteinase C,¹³⁾ 2-hr incubation at pH 9.2 and 30°C with a carboxymethylated B-chain of insulin.

F: *Streptomyces alkalophilic* keratinase,¹⁴⁾ 24-hr incubation at pH 11.6 and 37°C.

G: Cathepsin G,¹⁰⁾ 4-hr incubation at pH 8.5 and 37°C, (e/s, 1:1000, w/w).

H: *Saccharomyces cerevisiae* proteinase B,¹⁵⁾ 6-hr incubation at pH 7.2 and 25°C at ratio 500:1 (mol insulin B chain/mol proteinase B).

summary of the specificity of *Streptomyces* alkaline proteinase B is shown in Fig. 3.

Hydrolysis of angiotensin and proangiotensin

When angiotensin was digested with *Streptomyces* alkaline proteinase B for 1 hr, the products could be resolved into three spots (a-1, a-2 and a-3) by high-voltage paper electrophoresis and paper chromatography. The digestion products of angiotensin were subjected to amino acid analysis. The results are shown in Table II. The peptide bond that had been hydrolyzed by *Streptomyces* alkaline proteinase B was the Tyr⁴-Ile⁵ bond in angiotensin.

When proangiotensin was digested with *Streptomyces* alkaline proteinase for 1 hr, the products could be resolved into four spots (p-1, p-2, p-3 and p-4) by high-voltage paper electrophoresis and paper chromatography. The peptide bonds that had been hydrolyzed by *Streptomyces* alkaline proteinase B were the Tyr⁴-Ile⁵ and Phe⁸-His⁹ bonds in proangiotensin. The specificities of the alkaline proteinase B towards angiotensin and proangiotensin are shown in Fig. 4.

DISCUSSION

Major cleavage sites at Phe²⁴-Phe²⁵, Leu¹¹-

TABLE II. AMINO ACID COMPOSITION OF PEPTIDES OBTAINED FROM ANGIOTENSIN AND PROANGIOTENSIN BY THERMOPHILIC *Streptomyces* ALKALINE PROTEINASE B^a

Amino acids	Peptides (residues/molecule)						
	Angiotensin			Proangiotensin			
	a-1	a-2	a-3	p-1	p-2	p-3	p-4
Asp	1.0 (1)	1.0 (1)		1.0 (1)	1.0 (1)		
Pro	1.2 (1)		1.4 (1)	1.2 (1)		1.4 (1)	
Val	0.9 (1)	1.0 (1)		1.1 (1)	1.0 (1)		
Ile	1.0 (1)		1.0 (1)	1.0 (1)		1.0 (1)	
Leu							1.0 (1)
Tyr	0.9 (1)	1.0 (1)		0.8 (1)	1.0 (1)		
Phe	0.9 (1)		1.0 (1)	1.0 (1)		1.0 (1)	
His	0.8 (1)		0.9 (1)	0.9 (1)			0.7 (1)
Arg	1.0 (1)	1.1 (1)		1.0 (1)	1.1 (1)	1.0 (1)	
Recovery (μmol)	0.388	0.422	0.499	0.100	0.629	0.559	0.632
N-Terminal amino acid		Asp	Ile	Asp	Asp	Ile	His
Suggested segment	1~8	1~4	5~8	1~8	1~4	5~8	9~10

^a The values in parentheses denote the theoretical number of residues of a given amino acid in the peptide.

FIG. 4. Specificity of Thermophilic *Streptomyces* Alkaline Proteinase B towards Angiotensin (A) and Proangiotensin (B).

The values in parentheses denote the recovery (μmol) of peptides. Arrows indicate the bond split.

Val¹² and Leu¹⁵-Tyr¹⁶ in the oxidized insulin B chain in 4-hr incubation with proteinase were observed. When proangiotensin or angiotensin were digested with the proteinase, the Tyr⁴-Ile⁵ was readily hydrolyzed. Using the numbering system of Schechter and Berger,⁹ the peptide bonds (P₁-P₁'), which have hydrophobic amino acids in the P₁ and P₁' positions, are

preferentially cleaved by the proteinase.

Additional cleavages of the bonds, Gln⁴-His⁵, Leu⁶-CySO₃H⁷, Leu¹⁷-Val¹⁸, His⁵-Leu⁶, Gln¹³-Ala¹⁴ and Asn³-Gln⁴ in the oxidized insulin B chain in 4-hr incubation, were also observed. These results suggest that the proteinase preferentially cleaved the oxidized insulin B chain on the carboxyl side of leucine.

When proangiotensin was digested with the proteinase, the Phe⁸-His⁹ bond also hydrolyzed. The result suggests that the proteinase requires oligopeptide to have the P₂' position.

The cleavage sites at Leu⁶-CySO₃H⁷, Leu¹¹-Val¹², Leu¹⁵-Tyr¹⁶, Leu¹⁷-Val¹⁸ and Phe²⁴-Phe²⁵ in the oxidized insulin B chain by the proteinase were identical with those in the work with cathepsin G.¹⁰ The cleavage sites at Gln⁴-His⁵, Leu¹¹-Val¹², Leu¹⁵-Tyr¹⁶ and Phe²⁴-Phe²⁵ with the proteinase were similar to those reported in the studies of *S. fradiae* proteinase III¹²) and *S. griseus* alkaline proteinase,¹³) respectively. The cleavage sites at Gln⁴-His⁵, Glu¹³-Ala¹⁴, Leu¹⁵-Tyr¹⁶, Leu¹⁷-Val¹⁸ and Phe²⁴-Phe²⁵ by the proteinase were similar to those reported in the studies of *Streptomyces* alkalophilic keratinase.¹⁴)

Major cleavage sites at Leu¹⁵-Tyr¹⁶, Leu¹¹-Val¹², Gln⁴-His⁵ and a minor cleavage site at Leu¹⁷-Val¹⁸ in the oxidized insulin B chain with the proteinase were similar to those reported in the studies of subtilisin Carlsberg (EC 3.4.21.14) at the enzyme substrate ratio (e/s) of 1:980 by Johansen *et al.*¹¹⁾ The proteinase did not split the Tyr¹⁶-Leu¹⁷ and Tyr²⁶-Thr²⁷ bonds in the oxidized insulin B chain, which are known cleavage sites by subtilisin Carlsberg.

Major cleavage sites at Leu¹⁵-Tyr¹⁶ and Phe²⁴-Phe²⁵ and minor sites at Leu¹¹-Val¹² and Leu¹⁷-Val¹⁸ in the oxidized insulin B chain with the proteinase were similar to those reported in the studies of *S. cerevisiae* proteinase B at 25°C at a ratio of 500:1 (mol insulin B chain/mol proteinase B).¹⁵⁾

The single SH residue of the proteinase is essential for catalysis, as shown by inhibition studies with *p*-chloromercuribenzoate.³⁾ Inhibition of the proteinase by DFP indicates that in addition to the sulfhydryl residue³⁾ a serine residue²⁾ is also essential for the catalysis. The reaction of the serine residue with DFP is blocked in the presence of *p*-chloromercuribenzoate, suggesting that the serine and cysteine residues are located close together in the proteinase.^{2,3)} Similar observations are reported with *S. cerevisiae* proteinase B.^{15,16)}

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