The Initial Attack Sites of a *Streptomyces* Alkalophilic Proteinase on Oxidized Insulin B-Chain

Toshihiro NAKANISHI and Takehiko YAMAMOTO

Faculty of Science, Osaka City University, Sumiyoshi-ku, Osaka

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The sites on oxidized insulin B-chain substrate initially attacked by an alkalophilic proteinase from a *Streptomyces* sp., were investigated under incubation conditions employing one part enzyme to one thousand parts of substrate at 0°C.

Analysis of the peptides produced after 10 to 40 seconds of incubation revealed that the enzyme, which has an optimum pH of around 13, first attacks two peptide linkages “-Leu (15)-Tyr (16)-Leu (17)-” of the oxidized insulin B-chain with equal efficiency.

In our previous papers,^{1,2)} a number of peptides and amino acids were shown to be produced by the alkalophilic proteinase when incubated with oxidized insulin B-chain. However, only two of some 30 synthetic peptides examined, namely Poly-Lys and Cbz-Tyr-Gly·NH₂, were susceptible to this enzyme. Upon analyses of the peptides produced from the oxidized insulin B-chain, it was concluded that the peptide linkages of -Ser(9)·His(10)-, -Leu(11)·Val(12)-, -Phe(25)·Tyr(26)- and -Lys(29)·Ala(30)- of the oxidized insulin B-chain were all susceptible to this enzyme. However, in that investigation, a ratio of one part enzyme to 100 parts substrate and relatively long incubation periods at 37°C were used. Hence, all of the peptide linkages of the substrate susceptible to the enzyme would have been attacked making it impossible to distinguish those peptide linkages which are most sensitive to the enzyme.

The purpose of the present report is to identify those peptide linkages of the oxidized insulin B-chain which are attacked first by the enzyme by employing a higher ratio of substrate to enzyme, shorter incubation times and a lower incubation temperature.

MATERIALS AND METHODS

1. *Enzyme.* The purified *Streptomyces* alkalophilic proteinase used in this study was obtained by

repeated crystallization.¹⁾ The enzyme activity was the same as that described in our previous paper.²⁾

2. *Substrate.* The oxidized insulin B-chain used as the substrate was prepared from crystalline bovine pancreatic insulin (Sigma Chemical Co.) according to the method of Griffin *et al.*,³⁾ and purified by successive chromatography on columns of Dowex 50W-X2 and Sephadex G-25. The homogeneity of the purified B-chain was confirmed by paper chromatography, N-terminal amino acid analysis using the DNP-method and amino acid composition.

3. *Experimental procedure.* The initial attack sites were determined by the following method. To three tubes containing 64 mg (20.6 μmoles) of oxidized insulin B-chain substrate dissolved in 1.5 ml of 1.0% trimethylamine, pH 11.6, 64 μg of the alkalophilic proteinase dissolved in 0.5 ml of 1.0% trimethylamine at 0°C were added. After 10, 20, and 40 seconds of incubation at 0°C, respectively, the enzyme reactions were terminated by the addition of 1.0 ml of acetic acid. For the control, the same amount of B-chain substrate (64 mg) was dissolved directly in 3.0 ml of 10% acetic acid containing 64 μg of the enzyme. The reaction mixtures were then concentrated *in vacuo* to remove as much of the acetic acid as possible and then lyophilized after bringing their total volumes to about 50 ml with water.

4. *Purification of peptides.* Each dried digest was subsequently dissolved in 2.0 ml of 0.2 M pyridine-acetate, pH 3.0, and applied to a Dowex 50W-X2 column previously equilibrated with the same buffer, and eluted by a linear gradient of pyridine-acetate buffer from 0.2 M, pH 3.0 to 2.0 M, pH 5.0. The final elution was carried out using 8.5 M pyridine-acetate buffer, pH 5.5.

5. *Detection of peptides in eluates.* Peptides in the eluates were detected by the alkaline hydrolysis method.⁴⁾ Three tenths ml of each eluate was transferred to a test tube to which 1.0 ml of 2.5 N NaOH was added and the tubes were immersed into a boiling water bath for 2.0 hr. After the resulting hydrolysates were cooled to 4°C, 1.0 ml of 30% acetic acid and 2.0 ml of ninhydrin solution (20 g of ninhydrin dissolved in 750 ml of methyl cellosolve and mixed with 250 ml of 4.0 M acetate buffer, pH 5.5 containing 1 g of SnCl₂) were added. The resulting color densities for the hydrolysates were then measured spectrophotometrically at 570 nm.

6. *Isolation of peptides.* Each eluate containing ninhydrin positive material was subjected to paper chromatography to determine the homogeneity of its contents by the descending method using butanol:pyridine:AcOH:H₂O (15:10:3:12) as the solvent. Preparative paper chromatography was applied to fractions shown to contain more than one peptide using filter paper previously washed with the solvent. The peptides thus isolated on paper were recovered by eluting them with 30% acetic acid.

7. *Peptide and amino acid analysis.* The isolated and purified peptides were then dinitrophenylated and their N-terminal amino acids determined according to the method of Fraenkel-Contrat *et al.*⁵⁾ Amino acid compositions of the peptides were analysed on an auto-amino acid analyzer (034 Hitachi Liquid Chromatograph) after hydrolysis with 6.0 N HCl at 110°C for 24 hr in sealed tubes.

RESULTS

1. Separation of peptides and amino acids

As shown in Fig. 1, the digest obtained by incubating oxidized insulin B-chain with alkalophilic proteinase as described in MATERIALS AND METHODS was separated into seven fractions by chromatography on a column of Dowex 50W-X2. Fraction (F) 6 and F 7, the latter appearing as a shoulder of the former which was oxidized insulin B-chain itself, were further purified by chromatography on a column of Sephadex G-25. As shown in Fig. 2, they were separated clearly into two fractions.

Paper chromatographic analysis (Toyo Roshi No. 50) of each fraction revealed that F 4, F 5, F 6 and F 7 were homogeneous. However, F 1, F 2, and F 3 contained at least two ninhydrin⁶⁾ and Rydon-Smith⁷⁾

FIG. 1. Chromatography of the Proteinase Digest of Oxidized Insulin B-Chain on a Dowex 50W-X2 Column (38°C).

Column, 0.9×98 cm; sample applied, 2.0 ml which contained 21 μ moles each on the basis of B-chain; incubation period: I, no incubation (control); II, 10 sec; III, 20 sec; IV, 40 sec.; buffer used, 0.2 M pyridine-acetate, pH 3.0; *linear gradient from 0.2 M, pH 3.0 to 2.0 M, pH 5.0; **8.5 M, pH 5.5; flow rate, 18.0 ml/hr.

positive spots (Fig. 3). These fractions were then subjected to preparative paper chromatography to isolate each component. F 1 subsequently yielded F 1a and F 1b but the yields of peptides or amino acids contained in F 2 and in F 3 were too small and therefore not subjected to further study.

2. Determination of N-terminal amino acids and amino acid compositions of peptides isolated

Each of the peptides isolated above was dinitrophenylated using one fourth to one half portions of the peptides except for F 6, in which case 0.1 μ mole was employed (amount based on results of amino acid analysis). Phe was determined to be the N-terminus for each peptides in F 5 and F 7. The N-

FIG. 2. Separation of F 6 and F 7 Obtained in Fig. 1 by Gel Filtration on a Column of Sephadex G-25.

Column, 1.6×150 cm; $1/10$ M acetic acid; sample applied, (I)~(IV) 4 ml with 10% acetic acid; flow rate, 30 ml/hr.

terminus for peptides in other fractions were not investigated because of the small quanti-

ties of the peptides available. Instead, those were used to obtain information concerning their amino acid compositions.

The amino acid composition of the peptides was determined on the portions remaining from the above experiment. The analytical data are presented in Table I together with the N-terminal amino acid for each peptide. F 1 and F 3 contained a mixture of two peptides as determined by the molar ratio of their amino acid compositions. F 2, F 4 and F 5 also contained contaminating peptides but their concentrations were too small (3~5% of the major peptide) to be analyzed.

The results presented in Table I show that F 2 and F 7 contained the main peptides produced by the action of *Streptomyces* alkalophilic proteinase on the oxidized insulin B-chain substrate. The peptide linkages therefore involved are “-Leu (15)-Tyr (16)-” and “-Tyr (16)-Leu (17)-” of B-chain, respectively. The implications of these results are discussed in the following section.

DISCUSSION

As shown in Table I, the recovery yield of oxidized insulin B-chain following chromatography on a column of Dowex 50W-X2 under

FIG. 3. Paper Chromatograms of Each Fraction Obtained in Figs. 1 and 2.

Carried out by the descending method using But: Pyr: AcOH: H_2O (15: 10: 3: 12) as the solvent; location, initially sprayed ninhydrin in butanol saturated with H_2O , and then treated according to the method of Rydon and Smith.⁷⁾

TABLE I. RECOVERY YIELDS, N-TERMINAL AMINO ACID AND PROBABLE SEQUENCE OF PEPTIDES PRODUCED FROM OXIDIZED INSULIN B-CHAIN BY THE ENZYME

Incubation, sec.	Fraction	Recovery yield, μ moles	N-terminal amino acid	Site of B-chain			
None (I, control)	1a	15.26	Phe	“ 1-30”			
	1a'						
	1b						
	2						
	3						
	3a'						
	4						
10 (II)	5	11.29	Phe	“ 1-30”			
	6						
	7						
	1a				0.06	—	“19-21”
	1a'				0.03	—	“ 7- 9”
	1b				0.05	—	“16”, (26)
	2				0.62	Phe	“ 1-15”
20 (III)	3	10.97	Phe	“ 1-16”			
	3a'						
	4				0.04	—	“ 1- 9”
	5				0.15	—	“16-30”
	6				0.68	Phe	“ 1-30”
	7				0.08	Leu	“17-30”
	40 (IV)				1a	1.19	Leu
1a'		0.08	—	“19-21”			
1b		0.04	—	“ 7- 9”			
2		0.08	—	“16”, (26)			
3		0.64	Phe	“ 1-15”			
3a'		0.28	Phe	“ 1-16”			
4		0.08	Phe	“ 1- 9”			
	5	10.32	Phe	“ 1-30”			
	6						
	7						
	1a				0.09	—	“19-21”
	1a'				0.04	—	“ 7- 9”
	1b				0.06	—	“16”, (26)
	2				0.82	Phe	“ 1-15”
	3	1.19	Leu	“17-30”			
	3a'				0.02	—	“26-30”
	4				0.07	—	“ 1- 9”
	5				0.26	Tyr	“16-30”
	6				10.32	Phe	“ 1-30”
	7				1.19	Leu	“17-30”

FIG. 4. Initial Cleavage Points of Oxidized Insulin B-Chain by the *Streptomyces* Alkalophilic Proteinase. 1, Hydrolysis at an early stage of enzyme reaction; 2, Hydrolysis, at a later stage of enzyme reaction¹⁾ (The longer arrows represent bonds split more easily.)

paper differs considerably from those in our previous report in which it was reported that the enzyme hydrolyzes most easily the peptide linkages of -Ser (9)·His (10)-, -Leu (11)·Val (12)-, -Phe (25)·Tyr (26)- and -Lys (29)·Ala (30)- of the B-chain. Furthermore, it was stated that the enzyme was capable of splitting additional peptide linkages of the substrate but at reduced rates. However, in those experiments, the ratio of 1/100 of enzyme to substrate and incubation conditions of 37°C for 24 hr were used. Under such conditions, it would be impossible to determine what the initial attack sites of the enzyme were on the B-chain substrate. Results from this and the previous paper do, however, strongly suggest that the peptides produced initially by the enzyme have less sensitive peptide linkages which are subsequently attacked by the enzyme to produce smaller peptides or free amino acids upon longer incubation. If transpeptidation occurred during the incubation periods employed, it is doubtful that the initial attack sites could have been determined by this method. Furthermore, although the purity of the enzyme used in this study has been determined by various standard methods, the possibility of it being contaminated with other proteinases can not be completely excluded.

In the present paper, the reaction products produced by the enzyme were analyzed at a time when major portion of the substrate still remained. Thus, the results obtained, enables us to conclude that the sites of oxidized insulin B-chain initially attacked by the enzyme are as shown in Fig. 4. Furthermore, since smaller peptides were detected under these conditions, it appears highly probable that the enzyme subsequently attacks the peptides initially produced rather than other sites on the original B-chain substrate.

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logical to consider that peptides in F 1a, F 1a', and F 4 are those subsequently produced by the enzyme attacking the peptides resulting from the initial hydrolysis.

The conclusions reached in the present

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