SUBSTRATE SPECIFICITY OF MELON PROTEINASE (1) DIGESTION OF N-TERMINAL FRAGMENT OF QUAIL EGG-WHITE LYSOZYME BY MELON PROTEINASE

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SUBSTRATE SPECIFICITY OF MELON PROTEINASE

(1) DIGESTION OF *N*-TERMINAL FRAGMENT OF QUAIL EGG-WHITE LYSOZYME BY MELON PROTEINASE

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Abstract

The substrate specificity of the melon proteinase was studied by hydrolyzing the N-terminal dodecapeptide of quail egg-white lysozyme. The peptide was cleaved at the peptide bonds of CM-Cys_6-Glu_7, Glu_7-Leu_8 and Ala_9-Ala_{10} by the enzyme.

Introduction

The purification and some properties of a serine-type proteinase in melon fruit (Cucumis Melo L. var. Prince) were reported in the previous paper from this laboratory (**1**).

The enzyme exhibited wide specificity in the hyrolysis of reduced and carboxymethylated insulin B-chain, however, it appears that the carboxyl side of bonds containing acidic amino acid residues such as carboxymethylated cysteine or glutamic acid is appreciably split by the enzyme.

The present paper describes the hydrolysis of N-terminal dodecapeptide of quail egg-white lysozyme by the melon proteinase.

Materials and Methods

Melon proteinase was purified by batch-wise treatment with CM-cellulose fibers, column chromatography on CM-cellulose and filtration with Sephadex G–75 (1). The enzyme fractions were collected, and dialyzed exhaustively against distilled water, and lyophilized.

Quail egg-white lysozyme was prepared and recrystallized two times from aqueous solution of pH 8.3 containing 2.5% sodium chloride (**2**).

Cyanogen bromide and β-mercaptoethanol were purchased from Wako Pure Chemical Industries. Ltd., Osaka.

Preparation of N-Terminal Fragment of Quail Egg-White Lysozyme — Quail egg-white lysozyme consists of a single polypeptide chain and contains two methionine residues in a molecule (residue No. 12 and 105) (**3**). The N-terminal fragment (residue No. 1–12) of quail lysozyme was prepared by reduction and carboxymethylation and subsequent cleavage with cyanogen bromide. To 100 mg of the lyophilized quail egg-

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white lysozyme, were added 3.6 g of deionized urea, 3 ml of Tris-buffer of pH 8.6 (5.23 *g* of Tris and 9 ml of 1.0 N hydrochloric acid diluted to 30 ml with water), and 0.75 ml of 0.1 M EDTA. The mixture was made up to 7.5 ml with water, and the solution is about 1.3% in lysozyme, 8 M in urea and 0.2% in EDTA. The above mxiture was pre-incubated for 1 hr at 30° under nitrogen stream. Then 0.1 ml of β-mercaptoethanol was added to the reaction mixture. The atomosphere in the flask was replaced with nitrogen and at the top of the vessel was placed a rubber stopper to exclude air. After the mixture was incubated for 4 hr, freshly prepared iodoacetic acid in 1.0 N sodium hydroxide of which pH was adjusted to about 8.5, was added to the reaction mixture and stirred for 20 min at room temperature. The pH of the reaction mixture was maintained at 8.0–8.5 by adding 1.0 N sodium hydroxide. The solution was dialyzed against deionized water in a dark and cold room. The dialysis was continued for two days with two changes of water. The dialyzed solution was lyophilized. One hundred mg of reduced and carboxymethylated lysozyme was obtained.

In order to split the two methionyl bonds in lysozyme, the cyanogen bromide method was used (**4**). The lyophilized reduced and carboxymethylated lysozyme (100 mg) was allowed to react with 100 mg of cyanogen bromide in 10 ml of 70% formic acid at room temperature for 24 hr in the dark room. The reaction mixture was diluted with 200 ml of water and lyophilized. To separate the individual peptide fragments, 20 mg of lyophilized material was dissolved in a small volume of 1.0 M formic acid and applied to a column (2×100 cm) of Bio-Gel P-10 equilibrated with 1.0 M formic acid. Elution was performed with 1.0 M formic acid at room temperature. Three distinct peaks were detected by measurement with the ninhydrin method after alkaline hydrolysis. The amino acid composition of the fragments was analyzed by the method of Moore and Stein (**5**, **6**). Samples were hydrolyzed in constant-boiling hydrochloric acid at 110° for 24 hr. The amino acid composition of the fragment eluted in the second peaks was identical to that of the N-terminal dodecapeptide of whole molecule of lysozyme. Finally, 6 mg of the N-terminal fragment was obtained.

Digestion of N-Terminal Fragment of Lysozyme – The digestion of the N-terminal dodecapeptide of lysozyme with melon proteinase was carried out at 37° for 6 hr in 0.1% ammonium bicarbonate buffer, pH 8.0, using a weight ratio of enzyme to substrate of 1: 50. The digestion was stopped by freezing the solution and the lyophilized material was dissolved in the starting buffer used for chromatography just before it was loaded onto the column.

Chromatography of Peptides – The digest was separated by chromatography on a column (0.9×50 cm) of Dowex 50–X2 equilibrated with pyridine-formic acid buffer, pH 3.1. Elution was carried out at a flow rate of 20 ml/hr with linear gradient composed of 150 ml of 0.1 M pyridine-formic acid buffer, pH 3.1, and 150 ml of 2.0 M pyridine-acetic acid buffer, pH 5.0, at 30°. The column was stripped with 4.0 M pyridine-acetic acid buffer of pH 5.0. Fractions of 3.0 ml were collected.

Results and Discussion

Digestion of N-Terminal Fragment with Melon Proteinase – After digestion of the N-terminal dodecapeptide from quail egg-white lysozyme for 6 hr, the resulting peptides were separated by chromatography of a column of Dowex 50–X2. The elution of the peptides was followed by analyzing 0.2 ml aliquots of each tube after alkaline hydrolysis. Homogeneity of the peptide fractionated by the column chromatography was examined by high-voltage paper electrophoresis. Pyridine-acetic acid-water (1:10: 289 v/v) of pH 3.7 was used as the electrolyte and 1,800 volts was applied for 70 min of a paper (Toyo No. 51) of 60 cm length. The paper was dried at room temperature and the peptide was stained with the ninhydrin reagent. Peptides in all peaks except peak-I in Fig. 1 were nearly homogeneous on paper electrophoresis. Peptides in peak-I were purified further by preparative paper electrophoresis in the above system and named I-1, I-2, and I-3 in order the electrophoretic mobility to cathode. For elution of the peptide from the electrophoretogram, 10% acetic acid was used. The amino acid compositions and yields of the major peptides was given in Table 1.

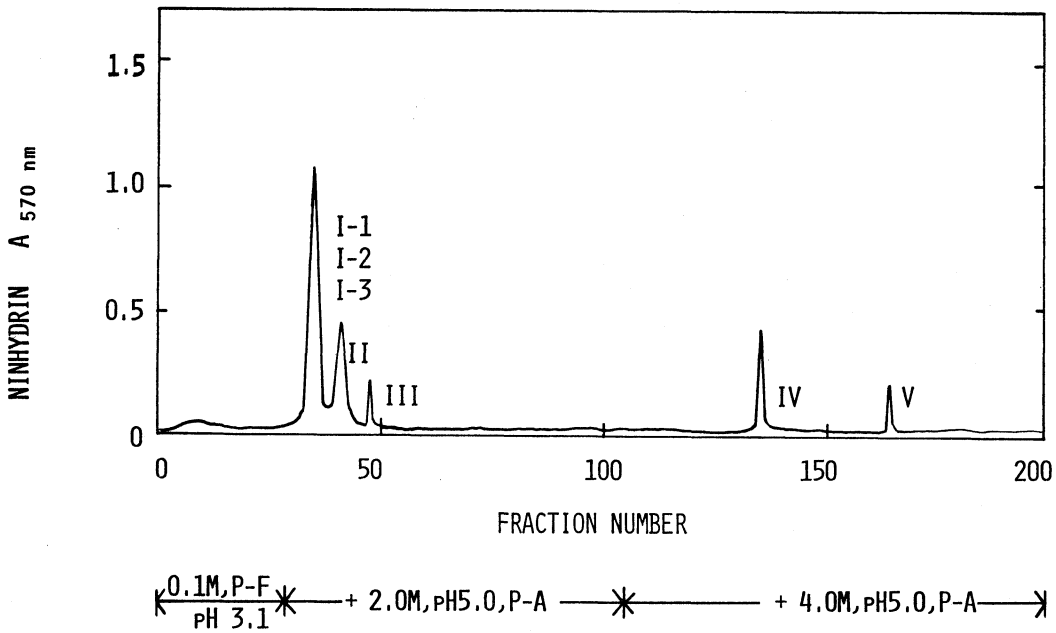

Fig. 1. Chromatography on Dowex 50–X2 of peptides from melon proteinase digest of N-terminal dodecapeptide of quail egg-white lysozyme.

On the basis of the amino acid composition, it was possible to locate each peptide in the primary structure of the N-terminal dodecapeptide of quail egg-white lysozyme (**3**), as shown in Fig. 2. It appears that the carboxyl side of bonds containing acidic amino acid is appreciably split by the enzyme. Similar specificity of the enzyme is observed in the hydrolysis of reduced and carboxymethylated insulin B-chain (**1**).

Table 1. Amino acid compositions of peptides obtained from N-terminal dodecapeptide of quail egg-white lysozyme by melon proteinase

Amino acid	Peptides				
	I–2	I–3	II	III	IV
	residues/molecule				
Lysine					0.8(1)
H·Serine+ H·Serine lactone	1.0(1)	1.0(1)			
Arginine					1.2(1)
CM-Cysteine					1.2(1)
Glutamic acid			0.9(1)		1.0(1)
Glycine					1.1(1)
Alanine	3.0(3)	2.0(2)	1.1(1)	1.0(1)	
Valine					0.9(1)
Leucine	0.9(1)		1.0(1)	1.1(1)	
Tyrosine					1.0(1)
Total	5	3	3	2	7
Yield (%)	5	21	30	15	23

The peptides are numbered by Roman numerals in order of elution. The values in parentheses denote the assumed numbers of residues in the peptides. No corrections have been made for decomposition during hydrolysis.

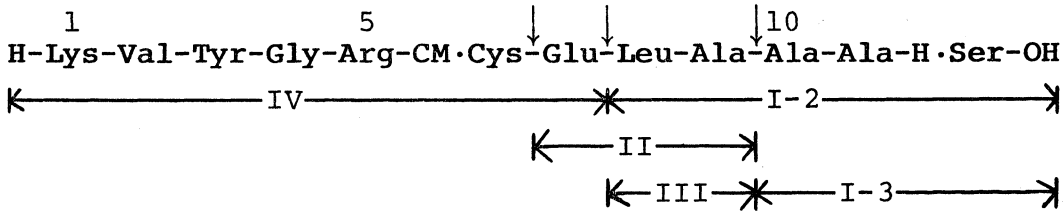

Fig. 2. Summary of the specificity of melon proteinase toward N-terminal dodecapeptide of quail egg-white lysozyme.
Residue number corresponds to that of the whole molecule.
The arrows indicate the sites of splitting by melon proteinase.

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