SUBSTRATE SPECIFICITY OF ACTINIDIN, A THIOL PROTEASE FROM ACTINIDIA CHINENSIS

Rep. Fac. Sci., Kagoshima Univ., (Math., Phys. & Chem.),
No. 19. p. 29–34, 1986.

SUBSTRATE SPECIFICITY OF ACTINIDIN, A THIOL PROTEASE FROM *ACTINIDIA CHINENSIS*

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(Received Sep. 10, 1986)

Abstract

Substrate specificity of actinidin, a thiol protease from kiwi fruit, was tested by using reduced and carboxymethylated insulin B-chain and N- and C-terminal fragment of hen egg-white lysozyme. Actinidin exhibited wide specificity for these fragments. However, free amino acids were not liberated from the fragments by the enzyme, except one leucine from the carboxyl terminal of the C-terminal fragment of lysozyme.

Introduction

Actinidin [EC 3.4.22.14] isolated from the sarcocarp of chinese gooseberry or kiwi fruit, *Actinidia chinensis*, is a thiol protease (*1,2*). This report shows the specificity of actinidin in the hydrolysis of polypeptides such as reduced and carboxymethylated insulin B-chain and N- and C-terminal fragment of hen egg-white lysozyme.

Experimental

Actinidin was isolated from the chinese gooseberry according to the procedure of McDowall (*2*). The proteinase activity of actinidin was determined by a modified Kunitz method (*3*) using amidated casein instead of native casein as a substrate. The assay was always performed in the presence of 1 mM cysteine and 1 mM EDTA. Reaction was carried out at pH 4.0 and stopped with 10% trichloroacetic acid. Amidated casein (pI 9～10) was prepared by amidation of casein with ethylenediamine.

Hen egg-white lysozyme consists of a single polypeptide chain and contains two methionine residues (residue No. 12 and 105) (*4*). The N- and C-terminal fragment of hen egg-white lysozyme were prepared by reduction and carboxymethylation and subsequent cleavage with cyanogen bromide. To 100 mg of the lyophilized hen egg-white lysozyme, were added 3.6 g of deionized urea, 3 ml of Tris-HCl 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

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in urea and 0.3% in EDTA. The above mixture was pre-incubated for 1 hour at 30°C under nitrogen stream. Then 0.1 ml of β-mercaptoethanol was added to the reaction mixture. The atmosphere 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 hours, 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 minutes 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 (*5*) was used. 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 hours in the dark room. The reaction mixture was diluted with 200 ml of water and lyophilized. To separate the individual peptide fragments, 20 mg lyophilized material was dissolved in a small volume of 1.0 M formic acid and applied to a column (2.0 × 100 cm) of Bio-Gel P-10 equilibrated 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 (*6,7*). Samples were hydrolyzed in constant-boiling hydrochloric acid at 110°C for 24 hours. The amino acid composition of the fragment eluted in the second peaks was identical to the N-terminal dodecapeptide of whole molecule of lysozyme. Finally, 6 mg of the N-terminal fragment was obtained. On the other hand, the third peak was the C-terminal fragment. Ten mg of this fragment was obtained.

The reduced and carboxymethylated insulin B-chain was prepared by the method of Crestfield *et al.* (*8*). To 50 mg of bovine insulin, was added 3.6 g of deionized urea, 3 ml of Tris-HCI buffer of pH 8.6, and 0.75 ml of 0.1 M EDTA. The mixture made up to 7.5 ml with water. The reaction mixture was treated with the procedure described in the above preparation of the N-terminal dodecapeptide of hen egg-white lysozyme. In order to isolate the reduced and carboxymethylated B-chain, the reaction mixture was applied to a column (2.0 × 100 cm) of Sephadex G-75 equilibrated with 50% of acetic acid and wrapped in aluminum foil. Fractions of 5 ml were collected at a flow rate of 25 ml per hour. The elution of the fragments was followed by analyzing 0.2 ml portion from each tube with the ninhydrin method after alkaline hydrolysis. The appropriate fractions were pooled and lyophilized after diluting the eluate about ten times with water. The amino acid composition of the product was in fair agreement with the values reported in the literature (*9*). From 50 mg of insulin 17 mg of B-chain was obtained.

Results and Discussion

Digestion of N-terminal fragment with actinidin-The digestion of the N-terminal dodecapeptide of hen egg-white lysozyme with actinidin was carried out at 30°C for 24 hours in 0.1 M acetate buffer, pH 5.0, using a weight ratio of enzyme to substrate of 1:30. The buffer was contained 1 mM EDTA and 1 mM β-mercaptoethanol. The digestion was stopped by freezing the solution and the lyophilized material was dissolved in the starting buffer used for column chromatography just before it was loaded onto the column. The digest was separated by ion-exchange 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°C. The column was stripped with 8.0 M pyridine. The elution of the peptides was followed by analyzing 0.2 ml aliquots of each tube after alkaline hydrolysis (Fig. 1).

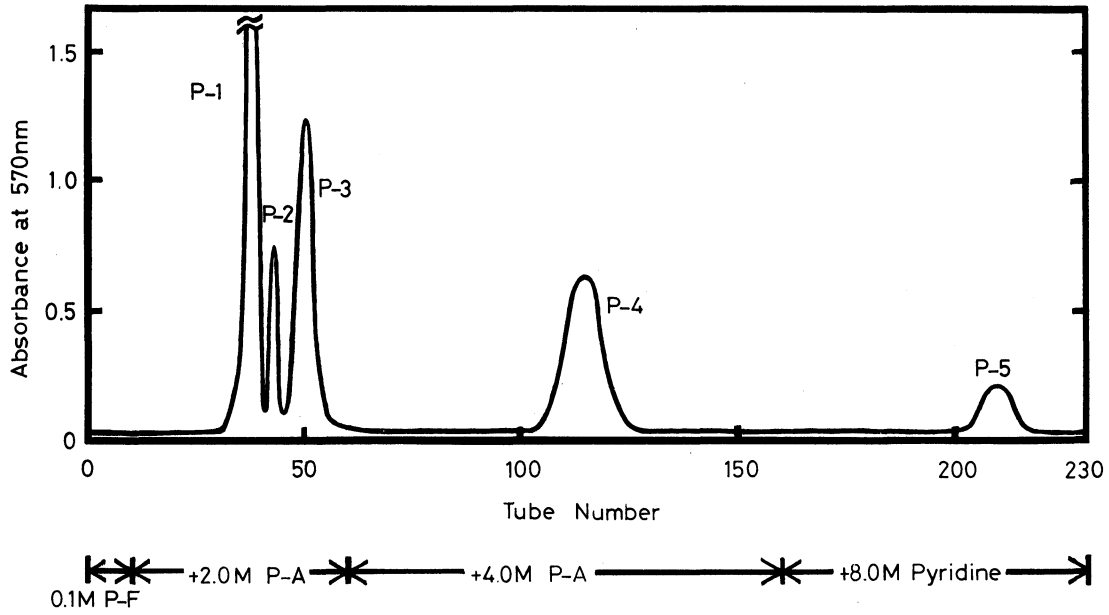

Fig. 1. Fractionation of actinidin peptides of N-terminal fragment on a column (0.9 × 50 cm) of Dowex 50-X2.
Elution of peptides was performed with pyridine-formic acid (P-F) and pyridine-acetic acid (P-A) buffer at 30°C at a flow rate of 20 ml per hour. The details are described in the text.

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 a electrolyte and 1,800 volts was applied for 70 minutes on a paper (Toyo No. 51) of 60 cm length. The paper was dried at room temperature and the peptide was stained with ninhydrin reagent. Peptides in peak- I were purified further by preparative paper

electrophoresis in the above system and named I-1 and I-2 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 were given in Table I. On the basis of the amino acid composition, it was possible to locate each peptide in the primary structure of N-terminal dodecapeptide of hen egg-white lysozyme (Fig. 2). It appeared that cleavage sites had occurred at Gly_4-Arg_5, Glu_7-Leu_8, and Ala_9-Ala_{10}.

Digestion of C-terminal fragment with actinidin-The C-terminal fragment was hydrolyzed with actinidin under similar conditions as the experiment for the N-terminal except weight ratio of enzyme to substrate of 1:20 for 27 hours. Column chromatography of the digest was accomplished under the same as those used to investigate the N-terminal fragment. The peptides were subjected to amino acid analysis. From the results it was possible to locate each peptide in the primary structure of the C-terminal fragment of hen

Table 1. Amino acid compositions of peptides obtained from N-terminal fragment of hen egg-white lysozyme by actinidin. The values in parentheses denote the assumed numbers of residues in the peptides.

Amino acid	Peptides			
	I-2	2	3	4
Lysine				0.9(1)
Arginine			1.0(1)	
CM-Cysteine			0.9(1)	
Glutamic acid			1.1(1)	
Glycine				0.9(1)
Alanine	2.0(2)	1.0(1)		
Valine				0.9(1)
Leucine		1.1(1)		
Phenylalanine				1.0(1)
H-Serine	1.0(1)			
Total	3	2	3	4
Yield (%)	50.6	31.3	60.4	22.7

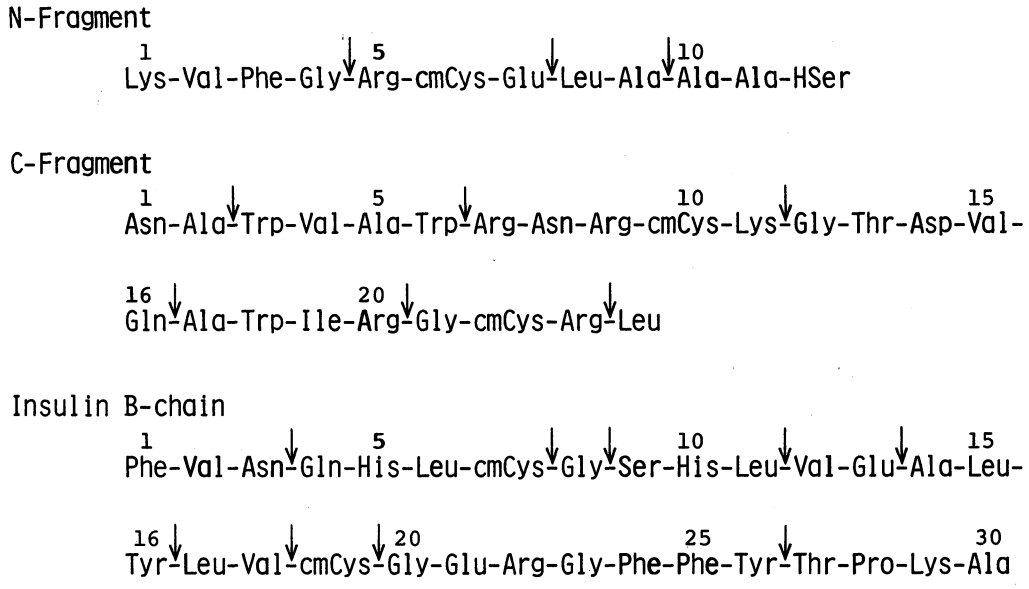

Fig. 2. Possible sites of cleavage of BrCN-fragments of hen egg-white lysozyme and reduced and carboxymethylated insulin B-chain by actinidin.
The arrows indicate the sites of splitting by actinidin.

lysozyme, as shown in Fig. 2. It appeared that cleavages had occurred at the peptide bonds of Ala_2-Trp_3, Trp_6-Arg_7, Lys_{11}-Gly_{12}, Gln_{16}-Ala_{17}, Arg_{20}-Gly_{21}, and Arg_{23}-Leu_{24}.

Digestion of insulin B-chain with actinidin-The digestion of insulin B-chain with actinidin was carried out at 30°C, 27 hours, pH 7.7, using a weight ratio of enzyme to substrate of 1:50. The buffer used here were 0.1 M citrate buffer, pH 5.0, containing 1 mM EDTA and 1 mM β-mercaptoethanol. The substrate did not dissolved in this buffer, then a solution of sodium hydroxide was added to the suspension until obtaining a clear solution. Actinidin was used after incubation in the above buffer for 20 min. The digestion was stopped by freezing the solution, and the lyophilized materials were dissolved in the starting buffer used for chromatography. The digest was separated by ion-exchange chromatography on a column (1.1 × 59 cm) of Dowex 1-X2 equilibrated with 3% pyridine. Elution was carried out at a flow rate of 14 ml/hr with exponential gradient using 0.2, 3.0, and 8.0 M acetic acid. Fractions of 7 ml were collected. The elution of the peptides was followed by analyzing 0.2 ml aliquots of each tube after alkaline hydrolysis. Homogeneity of the peptides fractionated by the column chromatography was examined by high-voltage paper electrophoresis. Pyridine-acetic acid-water (1:10:289) of pH 3.7 was used as the electrolyte and 1,800 volts was applied for 75 minutes on a paper (Toyo No. 51) of 60 cm length. The paper was dried at room temperature and the peptide was stained with ninhydrin reagent. The peptides were subjected to amino acid analysis. From the results it was possible to locate each peptide in the primary structure of insulin B-chain, as shown in Fig. 2. It appeared that cleavages had occurred at the peptide bonds of Asn_3-Gln_4,

$cmCys_7$-Gly_8, Gly_8-Ser_9, Leu_{11}-Val_{12}, Glu_{13}-Ala_{14}, Tyr_{16}-Leu_{17}, Val_{18}-$cmCys_{19}$, $cmCys_{19}$-Gly_{20}, and Tyr_{26}-Thr_{27}. Actinidin exhibited wide specificity in the hydrolysis of reduced and carboxymethylated insulin B-chain and hen lysozyme fragments. However, free amino acids were not liberated from these fragments by the enzyme except one leucine from the carboxyl terminal of the C-fragment.

References

1. Arcus, A.C. (1959) *Biochim. Biophys. Acta* **33**, 242.
2. McDowall, M.A. (1970) *Eur. J. Biochem.* **14**, 214.
3. Kaneda, M. and Tominaga, N. (1975) *J. Biochem.* **78**, 1287.
4. Canfield, R.E. and Liu, A.K (1965) *J. Biol. Chem.* **240**, 1997.
5. Gross, E. and Witkop, B. (1961) *J. Am. Chem. Soc.* **83**, 1510.
6. Moore, S., Spackman, D.H., and Stein, W.H. (1958) *Anal. Chem.* **30**, 1185.
7. Spackman, D.H., Stein, W.H., and Moore, S. (1958) *Anal. Chem.* **30**, 1190.
8. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) *J. Biol. Chem.* **238**, 622.
9. Sanger, F. and Tuppy, H. (1951) *Biochem. J.* **49**, 481.