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TEMPORARY SITE IN THE ANTI-TRYPTIC FRAGMENT FROM ADZUKI-BEAN PROTEINASE INHIBITOR II

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SUMMARY. The anti-tryptic fragment, derived from adzuki-bean proteinase inhibitor II, was subjected to limited proteolysis by trypsin at pH 2.9 for 48 h. Three peptide bonds, Lys-Ser, Arg-Cys and Arg-Asp, were split, inactivating the fragment. The temporary site, the point of inactivation against trypsin, was concluded to be Arg-Cys, since the Lys-Ser bond is the reactive site and the tripeptide $(Asp)_3$, released by the cleavage of the Arg-Asp bond, should not affect the inhibitory activity. This effective bond, corresponding to Arg^{32}-Cys^{33} of inhibitor II, was possibly more exposed to the enviromental solvent by cuting down the anti-chymotryptic domain from the parent inhibitor.

Temporary inhibition, the phenomenon of initial disappearance and later reappearance of enzymatic activity upon adding an inhibitor to an enzyme, has been observed on the system of trypsin and chicken ovomucoid or Kazal inhibitor: these inhibitors are inactivated by the cleavage of the peptide bond called "temporary site" (1-3).

We have proved that adzuki-bean proteinase inhibitor II (also II') is of the double-headed type, by dividing into two active-fragments, anti-tryptic and anti-chymotryptic. One of them, anti-tryptic, loses its own inhibitory activity upon prolonged incubation with trypsin, and the loss amounts to more than 80% (4). This inactivation is too serious to be attributed by the cleavage of the reactive site bond, because in the parent inhibitor, the cleavage of the reactive site, Lys-Ser bond, has little effect on the activity against trypsin (5). In this paper, we investigated the temporary site of the anti-tryptic fragment.

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MATERIALS AND METHODS

Preparation of anti-tryptic fragment. The anti-tryptic fragment was isolated from the peptic digest of adzuki-bean proteinase inhibitor II, by the reported procedure (4). The preparation was homogeneous as found by polyacrylamide gel electrophoresis and confirmed to be our object by the amino acid composition.

Modification of the fragment by trypsin. The fragment (10 μM) was treated with bovine TPCK-trypsin (Worthington Biochemicals) (molar ratio, 50:1) in acetic acid solution (pH 2.9) containing 0.05 M $CaCl_2$ at 30°C for 48 h. The modified fragment was freeze-dried after eliminating the enzyme by filtering through a Sephadex G-50 column (2 x 203 cm). This tryptic treatment drew down the anti-tryptic activity below 20%.

Reduction and S-carboxymethylation. The modified fragment was reduced and carboxymethylated according to Crestfield *et al.* (6) and followed by filtering through a Bio-gel P-2 column (2 x 200 cm), in 0.1 M NH_4HCO_3. Fractions containing peptides were pooled and freeze-dried.

Amino acid analyses and Edman degradation. Amino acid analyses were carried out on a Hitachi KLA-3B automatic amino acid analyzer, after hydrolyses with 5.7 N HCl in evacuated, sealed tubes at 105°C for 24 h.

Edman degradation was performed by the direct procedure of Edman as described by Iwanaga *et al.* (7). Detection of the ethyl acetate-soluble PTH-amino acids was performed by thin-layer chromatography on silica gel plates. The derivative of Cm-cysteine was identified by paper chromatography (8).

RESULTS

Isolation of peptides from the modified fragment. Figure 1 shows the result of DEAE-Sephadex A-25 chromatography of the trypsin-modified fragment after reduction and carboxymethylation. Six fractions indicated by bars, I, II,

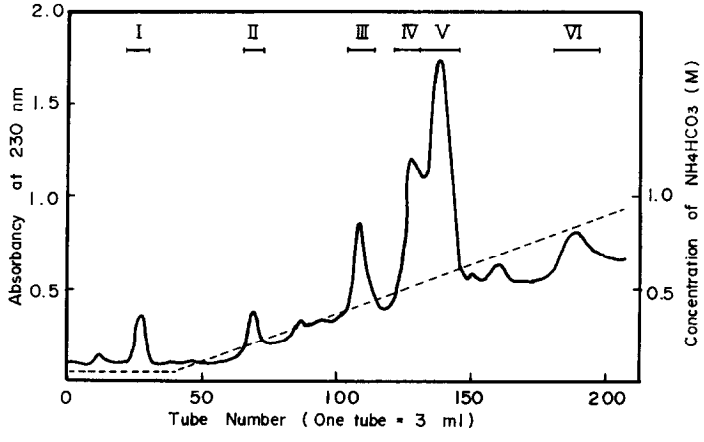

Fig. 1. Chromatography of the trypsin-modified fragment on DEAE-Sephadex A-25. The modified fragment was applied to a DEAE-Sephadex A-25 column (1.3 x 30 cm) equilibrated with 0.05 M NH_4HCO_3 solution, after reduction and carboxymethylation. Elution was performed with the same buffer and then with a linear gradient to 1 M NH_4HCO_3. ——, absorbancy at 230 nm; ---, NH_4HCO_3 concentration.

TABLE I. AMINO ACID COMPOSITIONS OF THE PEPTIDES FROM THE MODIFIED FRAGMENT.

Values shown are ratios with respect to the amino acid indicated by an asterisk in each column, and N-termini were determined by Edman degradation.

Amino acids	Peptides					
	I	II	III	IV	V	VI
Cm-Cysteine	0.74(1)	1.66(2)	1.06(1)	3.43(4)	1.94(2)	2.05(2)
Aspartic acid		1.23(1)	1.05(1)	0.98(1)		2.45(3)
Threonine				0.89(1)		
Serine	1.00*	1.84(2)	1.06(1)	2.34(2)	1.94(2)	2.01(2)
Glutamic acid				1.00*	1.09(1)	1.20(1)
Proline	1.88(2)	1.82(2)		1.00(1)	1.03(1)	1.08(1)
Methionine	0.51(1)	0.38(1)				
Isoleucine		1.02(1)	0.76(1)			
Leucine		1.00*	0.68(1)			
Tyrosine					0.97(1)	0.92(1)
Phenylalanine					1.00*	0.53(1)
Lysine	1.20(1)	1.16(1)		1.59(2)	0.97(1)	1.00*
Arginine	1.07(1)	1.96(2)	1.00*		1.08(1)	0.68(1)
Total	7	13	6	12	10	13
N-Termini	Ser-Met	Ser-Met	Cm-Cys	Ser-Ser	Phe	Phe

III, IV, V and VI, were freeze-dried, separately. Fractions IV and V were further purified by descending paper chromatography using *n*-butanol-acetic acid-water (3:1:1) as a solvent.

Amino acid compositions of the peptides. Table I shows the amino acid compositions of the peptides from the modified fragment, together with the N-terminals. Peptide IV corresponds to the region between N-terminal serine and the reactive site lysine residues in the fragment molecule, I is the subsequent peptide composed of 7 amino acid residues containing the reactive site serine, and the C-terminal region should be occupied by III. Peptide II is corresponding to the sum of I and III. Therefore, the order of these peptides in the N-terminal serine-containing chain of the fragment is: (IV)-(I)-(III) or (IV)-(II). Peptide VI is similar to the chain containing N-terminal phenylalanine residue of the original fragment, and peptide V corresponds to that losing 3 aspartic residues from C-terminal side. The elucidation is summarized in Fig. 2. It is obvious

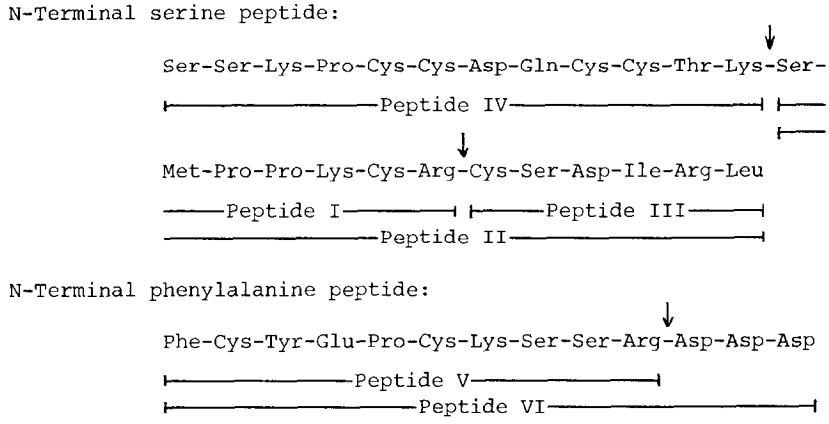

Fig. 2. Amino acid sequences of the anti-tryptic fragment and the peptides from the modified fragment.

that three peptide bonds, indicated by arrows in Fig. 2, have been cleaved by limited proteolysis by trypsin.

DISCUSSION

The initially-losing tryptic activity is found to reappear as time goes by, if the anti-tryptic fragment, derived from adzuki-bean proteinase inhibitor II (also II'), is added to trypsin at pH 7.6: the fragment is inactivated gradually (4). The temporary site, responsible for the inactivation, will be cleaved by trypsin at acidic pH as well as the reactive site, such as in the case of Kazal inhibitor (1-3).

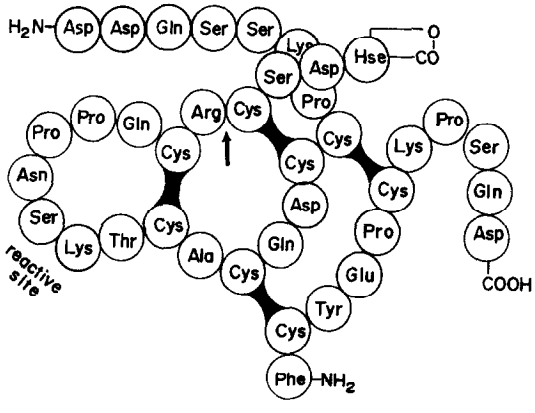

Fig. 3. Primary structure of the anti-tryptic fragment from Bowman-Birk soybean inhibitor, according to Odani and Ikenaka (9).

The three peptide bonds in the fragment molecule were split by trypsin at pH 2.9, as shown in Fig. 2. One of them, Arg-Asp, which lies in the peptide chain having N-terminal phenylalanine residue, should have no effect on the inhibitory activity since the C-terminal six residues of the parent inhibitor II are known not to be concerned with the action toward trypsin and chymotrypsin (data unpublished). The others are in the N-terminal serine peptide, and the bond, Lys-Ser, is the reactive site. Therefore, the last bond, corresponding Arg^{32}-Cys^{33} of the parent, should be the temporary site and its cleavage amounted to more than 70%, judging from the ratio between the yeild of peptides II and III. The cleavage must exclusively cause the inactivation and the remaining, low inhibitory activity of the trypsin-treated preparation will primarily depend on the fragment having the survival temporary site.

The parent inhibitor II also reduces its own inhibitory activity against trypsin upon prolonged incubation with the enzyme, but the inactivation proceeds very slowly (4). The difference in the instability against trypsin between the fragment and the parent inhibitor may be explained as follows: the temporary site, Arg-Cys bond, is more exposed to the environmental solvent by cutting down the anti-chymotryptic domain from the parent inhibitor.

Such temporary inhibition has been found in the case of the Bowman-Birk soybean inhibitor fragment (anti-tryptic) (9). The trypsin-susceptible bond, responsible for inactivation, should also exist in the molecule and be similar to that of our fragment since the amino acid sequences are highly homologous with each other. The corresponding peptide bond of Bowman-Birk inhibitor fragment lies in the loop adjacent to the reactive site loop (the bond, indicated by an arrow in Fig. 3). Thus, other leguminous inhibitors exhibit possibly such tryptic instability, if they have the similar peptide bond.

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