SOYBEAN TRYPSIN INHIBITOR-C: AN ACTIVE DERIVATIVE OF SOYBEAN TRYPSIN INHIBITOR COMPOSED OF TWO NONCOVALENTLY BONDED PEPTIDE FRAGMENTS

Ikunoshin KATO and Naotomo TOMINAGA
Department of Chemistry, Faculty of Science, Kagoshima University, Kamoike, Kagoshima, Japan

Received 24 August 1970

1. Introduction

Soybean Kunitz trypsin inhibitor (STI) has been shown to contain 197 amino acid residues (M.W. 21,500) and to have two disulfide bonds and two methionine residues (104 and 135), (fig. 1), [1]. Since one methionine residue (135) is located in the position between the two disulfide-bond loops, cyanogen bromide treatment [2] of the native STI yields two peptide fragments.

The present communication describes experiments on the cleavage of native STI by cyanogen bromide treatment to yield two fragments: STI-L (residues 1 to 135) and STI-S (residues 136 to 197). None of the fragments showed the trypsin-inhibitory activity. However, the two fragments can associate to form an active, noncovalently bonded, derivative designated STI-C (STI-CNBr) which possesses more than 80% of the native trypsin-inhibitory activity.

2. Experimental

Three times crystallized STI (Worthington Biochemical Corp.) was purified on a DEAE-cellulose column and followed by gel filtration on Sephadex G-75 [3]. Twice crystallized trypsin (Worthington) was used after dialysis against 2 mM HCl containing 5 mM $CaCl_2$.

The proteolytic activity of trypsin was determined at 35° by the method of Kunitz [4] using casein (Hammarsten, Merk). The esterase activity was measured at 22° by the spectrophotometric method of Hummel [5] using toluensulfonyl-L-arginine methyl ester HCl (TAME), (Miles Laboratories, Inc.). The trypsin-inhibitory activity of native STI and its derivatives was established after preincubation of trypsin with inhibitors for 10 min at room temperature. In gel filtrations, the trypsin-inhibitory activities of fractions were assayed using casein as substrate.

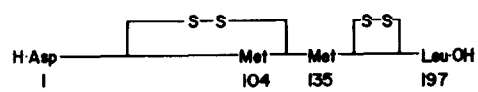

Fig. 1. Topological representation of soybean trypsin inhibitor. The assignment of residues is based on [1].

Aliquots (25–50 μl) of fractions, dissolved in 0.2 ml of 0.1 M phosphate buffer (pH 7.8) after lyophilization, were incubated with trypsin (20 μg). The reaction was allowed to proceed at 35° by adding 2 ml of 1% casein in 0.1 M phosphate buffer (pH 7.8), and stopped after 10 min by the addition of 2 ml of 5% trichloroacetic acid. The mixture was allowed to stand for 1 hr at room temperature and then filtered through Toyo Roshi no. 5A filter paper. The absorbancies of the filtrates were measured at 275 nm. The inhibitory activity was expressed by the difference of the absorbancy in the absence and the presence of inhibitor.

The concentrations of trypsin and native STI were estimated using the extinction coefficients at 280 nm, $E^{1\%}_{1cm} = 17.1$ [4] and 9.1 [4], respectively. The concentrations of fragments were determined by the amino acid analyses [6].

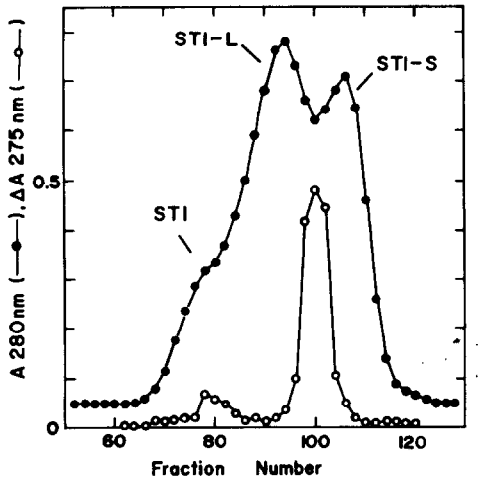

Fig. 2. Separation of STI-L and STI-S. 150 mg native STI, 150 mg CNBr, dissolved in 15 ml of 70% HCOOH, was incubated 30° for 24 hr and lyophilized. The dried material, dissolved in 4 ml of 70% HCOOH, was applied to a Sephadex G-100 (3.6 × 50 cm) column equilibrated with 50% CH_3COOH.

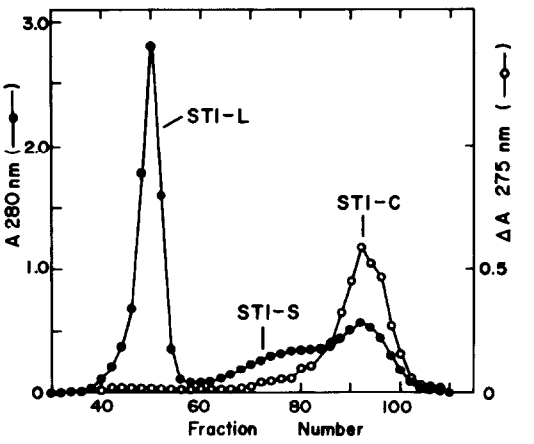

Fig. 3. Association of STI-L and STI-S. 135 mg STI-L and 50 mg STI-S, dissolved in 10 ml of 0.1 tris-HCl (pH 8.0), were dialyzed at room temperature against 50 mM CH_3COONH_4 (pH 6.1) for 15 hr. Then, the mixture of fragments was applied to a column of Sephadex G-75 (2.8 × 90 cm) equilibrated with 0.1 M CH_3COONH_4 (pH 6.1).

All chromatographic procedures were carried out at room temperature. DEAE-cellulose (Serva), Sephadex G-100 and G-75 (Pharmacia) and Bio-Gel P-10 (Bio Rad) were employed. The absorbancies of fractions (3 ml) were read at 280 nm. The materials of gel filtration peaks were identified by their amino acid analyses.

Polyacrylamide electrophoresis of native STI and its derivatives at pH 9.4 gel was carried out essentially according to Davis [7].

3. Results and discussion

Upon separation of STI-L and STI-S on Sephadex G-100 equilibrated with 50% acetic acid, trypsin-inhibitory activities were observed at two positions (fig. 2). Similar results were also obtained when Bio-Gel P-10, equilibrated with 20% acetic acid, was employed for separating the fragments. The first activity may be due to intact STI or to a derivative whose methionyl bond between residues 135 and 136 was not cleaved. The second activity was usually observed between the STI-L and STI-S peaks. A second gel filtration through the same column of the second active fractions (95–105), pooled and lyophilized, resulted in no active material but STI-L and STI-S, suggesting that the association of these two fragments may yield an active material.

The fragments were applied repeatedly to gel filtration in acetic acid to remove the contaminating fragments of each other, respectively, and used for the following experiments.

The association of the two fragments to yield an active complex, STI-C, with a yield of about 30%, occurred in a medium of neutral pH (fig. 3). The position of STI-C peak of the elution pattern corresponds exactly to that of native STI. However, the retarded position of STI-C compared with the fragments may indicate that a rearrangement of the conformation of the fragments was induced on complex formation.

Polyacrylamide electrophoresis of the mixture of both fragments on pH 9.4 gel yielded three bands at the positions occupied by STI-L, STI-S and native STI and the order of the positions of bands from anode to cathode was the same as the elution pattern of gel filtration on Sephadex G-75 (see fig. 3).

The active fractions (85–100) of the two experiments shown in fig. 3 were pooled and dialyzed against distilled water. The sample was then concen-

Table 1
Amino acid compositions of STI and its derivatives[a].

Amino acid	STI-L	STI-S	STI-C	STI	STI[b]
Lys	5.1 (7)	4.4 (5)	10.5	11.4 (12)	11
His	1.1 (1)	0.7 (1)	1.9	2.1 (2)	2
Arg	6.7 (7)	3.3 (3)	9.6	9.4 (10)	9
Asp	17.0 (17)	12.0 (12)	29.0	29.0 (29)	29
Thr	6.9 (7)	0.7 (1)	7.0	6.4 (8)	8
Ser	7.6 (8)	3.0 (4)	10.3	9.1 (12)	13
Glu	13.3 (11)	7.1 (8)	20.0	21.0 (19)	21
Pro	9.0 (8)	1.3 (3)	9.4	10.3 (11)	10
Gly	15.7 (13)	3.6 (4)	18.1	17.1 (17)	18
Ala	8.6 (8)	0.9 (1)	8.5	8.2 (9)	9
Half-Cys	+ (2)	+ (2)	2.6	3.4 (4)	4
Val	8.9 (11)	5.0 (4)	14.3	13.8 (15)	12
Met	– (2)	– (0)	–	1.9 (2)	3
Ile	13.3 (12)	2.5 (3)	14.3	14.2 (15)	14
Leu	10.8 (10)	4.6 (5)	15.7	15.2 (15)	16
Tyr	3.1 (3)	0.6 (1)	2.0	3.7 (4)	4
Phe	5.9 (6)	3.1 (4)	9.7	8.8 (10)	9
Trp[c]	– (2)	– (0)	–	– (2)	0

[a] Values are expressed moles/mole of aspartic acid and obtained from the analyses of the duplicate samples hydrolyzed for 20 hr, respectively. Values are not corrected. Homoserine lactone was observed in STI-L and STI-C. Theoretical values from [1] are given in parentheses.

[b] Reported by Wu and Scheraga [8].

[c] Not determined.

trated to approximately 10 ml using Sartorius-Membranfilter (Cat.No.SM 13200) and subjected to a second gel filtration under the condition described in fig. 3. The STI-C was recovered with about 90% yield, suggesting once formed, the active derivative is very stable at neutral pH. STI-C thus obtained was lyophilized and used for the next experiments.

50 mg STI-C, dissolved in 5 ml of 20% acetic acid, was dissociated into two fragments by gel filtration on a Bio-Gel P-10 (2.8 X 97 cm) column equilibrated with 20% acetic acid. The dissociated fragments were applied to Sephadex G-75, under the condition described in fig. 3, to remove small amounts of STI-C and aliquots were subjected to amino acid analyses; good agreement with reference values was obtained (table 1).

The reconstitution of STI-C was performed in alkaline medium, 0.1 M tris-HCl (pH 8.0), and the reconstituted STI-C was isolated by gel filtration on Sephadex G-75 equilibrated with 0.1 M ammonium acetate (pH 6.1) with about 90% yield (fig. 4). The yield of the reconstituted STI-C was high compared with that of the constituted STI-C (about 30% yield, see fig. 3). This discrepancy would suggest that some unidentified molecule, unable to form a complex might be present in the starting fragments. However, their amino acid compositions showed no detectable difference from the dissociated fragments from STI-C. After a second gel filtration under the same condition as described in fig. 4, the reconstituted STI-C was subjected to amino acid analyses.

The amino acid compositions of native STI and its derivatives are summarized in table 1. No significant amount of methionine residues was found in any STI derivatives, indicating that STI-L is composed of the

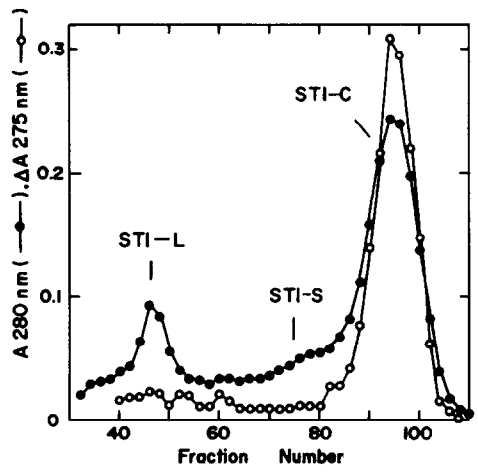

Fig. 4. Reconstitution of STI-C. 12.8 mg STI-L and 6.0 mg STI-S (mole ratio of L/S, about 1), which were obtained by the dissociation of STI-C in acidic condition as described in text, dissolved in 7 ml of 0.1 M tris-HCl (pH 8.0) was let stand for 18 hr at room temperature. The mixture then applied to a Sephadex G-75 (2.8 X 90 cm) column equilibrated with 0.1 M CH_3COONH_4 (pH 6.1).

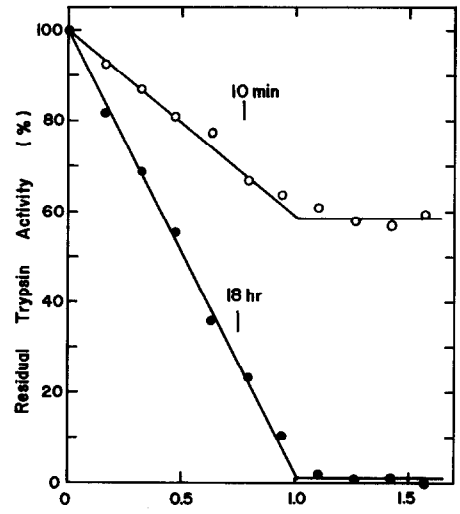

Fig. 5. Trypsin-inhibitory activity as a function of the ratio of STI-S to STI-L. Both fragments used were obtained by the dissociation of STI-C in acid condition as described in text. The amounts of STI-L and trypsin were constant in all assays, 0.65 nmole and 0.77 nmole, respectively. Solutions of both fragments dissolved in 0.1 M tris-HCl (pH 8.0) were mixed adjusting the volume to 0.2 ml with the same buffer and incubated for 10 min and 18 hr at room temperature before the addition of trypsin. The residual trypsin activity was measured using casein as substrate. The assay procedures are the same as described in the text.

two peptide fragments linked with a disulfide bond. The amino acid composition of the reconstituted STI-C indicates that equimolar amounts of STI-L and STI-S associate to form an active derivative, STI-C.

The reconstituted STI-C inhibited the proteolytic and esterase activities of trypsin to 93% and 83% of native STI, respectively.

The inhibitory activity as a function of the ratio of STI-S to STI-L is shown in fig. 5. The data indicate the equimolar binding of the two fragments to form STI-C and the remarkable time dependence of the active complex formation suggests that the comformational changes required to have the inhibitory activity proceed slowly. The conformational rearrangement of the fragments on association was also supported by the tryptic susceptibility of the dissociated fragments. When STI-L or STI-S and trypsin were incubated in 0.1 M phosphate buffer (pH 7.8) for 1 hr, at 35°, the trypsin-inhibitory activity, assayed with the addition of another fragment, was not detected.

References

[1] T.Ikenaka, T.Koido and S.Tsunazawa, in: Symposium of Protein Structure (Japan Chem. Soc., Tokyo, 1968) p. 25.
[2] E.Gross and B.Witkop, J. Am. Chem. Soc. 83 (1961) 1510.
[3] M.Yamamoto and T.Ikenaka, J. Biochem. 62 (1967) 141.
[4] M.Kunitz, J. Gen. Physiol. 30 (1947) 291.
[5] B.C.W.Hummel, Can. J. Biochem. Physiol. 37 (1959) 1393.
[6] D.H.Spackman, W.H.Stein and S.Moore, Anal. Chem. 30 (1959) 1190.
[7] B.J.Davis, Ann. N.Y. Acad. Sci. 121 (1964) 404.
[8] Y.V.Wu and H.A.Scheraga, Biochemistry 1 (1962) 698.