Purification and Characterization of Four Trypsin Inhibitors from Seeds of Okra, *Abelmoschus esculentus* L.

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Four trypsin inhibitors were isolated from seeds of okra (*Abelmoschus esculentus* L.) and named okra trypsin inhibitors (OTI) A, B, C, and D. The molecular weights of all the inhibitors were estimated to be about 20,000 by gel filtration on Sephadex G-100 and SDS-polyacrylamide gel electrophoresis. All of these inhibitors inhibited bovine trypsin strongly with the formation of complexes at a 1:1 molar ratio and three (OTI-A, OTI-B, and OTI-C) of them also weakly inhibited bovine chymotrypsin, but not simultaneously. Subtilisin Carlsberg, pronase, papain, and porcine pepsin were not inhibited by these inhibitors. The trypsin inhibitory activity of all four inhibitors was suppressed by treatment with 1,2-cyclohexanedione but not with 2,4,6-trinitrobenzenesulfonate, suggesting that each of the inhibitors contains an arginine residue at the reactive site.

Okra (*Abelmoschus esculentus* L.) is a botanical species cultivated for its pod for more than 2000 years and grows in many parts of the earth.¹⁾ It belongs to the Malvaceae, as does cotton. In Japan, okra has been used as a vegetable for its green pods, in the fresh, raw, unmaturing state, and no attempt has been made to use its seeds as a source of protein. In 1975, Karakoltsidis and Constantinides reported that the amino acid composition of whole mature seeds of okra is similar to that of soybeans, yet the protein efficiency ratio is higher for okra.²⁾ They suggested that antinutritional factors are absent in okra seeds because of the lack of difference in the protein efficiency ratio value between heated and non-heated okra flour.

During the course of our studies on proteases and their inhibitors in plants, several protease inhibitors were detected in seeds of a variety of okra that is usually cultivated in

Japan. They inhibited both bovine trypsin and chymotrypsin which are typical digestive enzymes. Although it is uncertain whether the okra protease inhibitors are antinutrients for humans and other animals or not, the isolation of these inhibitors in pure form and the characterization of them will help in establishing their nutritional importance.

One of the major component of these okra protease inhibitors has been purified by a seven-step procedure using trypsin-Sepharose affinity chromatography as described previously.³⁾ In this paper, we describe the purification without the use of affinity chromatography and properties of the four trypsin inhibitors.

MATERIALS AND METHODS

Materials. One variety "Ichiban-boshi" of okra seeds was used throughout this study. Bovine trypsin [3.4.21.4]

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Abbreviations: OTI, okra trypsin inhibitor; BAPA, *N*-benzoyl-L-arginine *p*-nitroanilide; BTPA, *N*-benzoyl-L-tyrosine *p*-nitroanilide; SDS, sodium dodecyl sulfate; CHD, 1,2-cyclohexanedione; TNBS, 2,4,6-trinitrobenzenesulfonate; IU, inhibitor unit.

and chymotrypsin [3.4.21.1] were purchased from Boehringer Mannheim. Porcine pepsin was from Worthington Biochemical Corp. Subtilisin Carlsberg was from the Sigma Chemical Co. Pronase was from Kaken Kagaku Co., Ltd. and papain was from Merck. Hammersten casein was a product of Wako Pure Chemical Industries Ltd. *N*-Benzoyl-L-arginine *p*-nitroanilide (BAPA) and *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPA) were the products of the Protein Research Foundation. Sephadex G-100 and CM-Sepharose were from Pharmacia Fine Chemicals. DEAE-cellulose (Brown) was from Nakarai Chemical Ltd. All other chemicals were of reagent grade.

Purities of trypsin and chymotrypsin. Active site titration of trypsin with *p*-nitrophenyl *p*'-guanidinobenzoate was done by the method of Chase and Shaw,⁴⁾ and that of chymotrypsin with *p*-nitrophenyl-*N*²-acetyl-*N*¹-benzyl-carbazate was by the method of Elmore and Smith.⁵⁾ By these procedures, trypsin and chymotrypsin used in this study were found to contain 56% and 95% active enzyme, respectively.

Activity assays. Inhibition of trypsin was measured by the method described previously, using BAPA as a substrate.⁶⁾ Inhibition of chymotrypsin was measured by a method based on that of Kassell⁷⁾ using BTPA as a substrate. A mixture of chymotrypsin (50 µg) and inhibitor in 5.0 ml of 0.1 M Tris-HCl, pH 8.0, containing 0.01 M CaCl₂ was incubated for 5 min at 37°C, and then 0.5 ml of 2 mM BTPA in dimethylsulfoxide was added. After incubation for 30 min at 37°C, one ml of 30% acetic acid solution was added and the absorbance of the reaction mixture at 410 nm was measured. One inhibitor unit (1 IU) is defined as the amount of inhibitor which suppressed the liberation of one mole of *p*-nitroaniline per min by the active enzymes.

Proteolysis by other proteases was measured by the methods of Hagihara⁸⁾ for subtilisin Carlsberg, Arnon⁹⁾ for papain, Narahashi¹⁰⁾ for pronase, and Tang¹¹⁾ for pepsin.

Measurement of protein concentration. The concentration of protein was measured by the method of Lowry *et al.*¹²⁾ with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was done by the method of Davis¹³⁾ in 7.5% polyacrylamide gels at pH 9.4. Proteins were stained with 0.1% Coomassie brilliant blue G-250 in 50% trichloroacetic acid. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done essentially by the method of Weber and Osborn¹⁴⁾ in 14% gels.

Measurement of molecular weights. The molecular weights of the inhibitors and the inhibitor-enzyme complexes were estimated using a Sephadex G-100 column

(1.4 × 90 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. Standard proteins used were bovine serum albumin (MW = 66,000), ovalbumin (45,000), chymotrypsinogen (25,000), and lysozyme (14,000). The void volume was identified with blue dextran.

Measurement of isoelectric points and hexose contents. Isoelectric points of the purified inhibitors were measured at 20°C by polyacrylamide gel electrofocusing¹⁵⁾ using 2% Pharmalyte of pH 3~10. Bovine chymotrypsinogen (pI 9.5), bovine hemoglobin (pI 7.6), and ovalbumin (pI 4.6) were used as standard proteins.

The hexose of the purified inhibitors were measured by the phenol-sulfuric acid method.¹⁶⁾

Measurement of inhibitor constants (*K_i*). The approximate *K_i* values of the inhibitor-enzyme complexes were measured by the method of Green and Work¹⁷⁾ at pH 8.0, using BAPA for trypsin, and the method of Dixon¹⁸⁾ at pH 8.0, using BTPA for chymotrypsin.

Amino acid analysis. Protein samples (1.0 mg) were hydrolyzed with 1.0 ml of 6 M HCl in evacuated, sealed tubes for 24, 48, and 72 hr at 110°C, and analyzed with a JEOL model JLC-6AS amino acid analyzer. Cysteine was measured as cysteic acid after performic acid oxidation.¹⁹⁾ Tryptophan was measured spectrophotometrically.²⁰⁾ The absence of free sulfhydryl groups was confirmed by the method of Ellman.²¹⁾

Identification of amino-terminal residue. The amino-terminal residue was identified by the dansylation method of Gray.²²⁾ Dansyl-amino acids were separated by polyamide layer chromatography.²³⁾

Chemical modification. Arginine residues in the inhibitors were modified by the method of Patthy and Smith²⁴⁾ using 1,2-cyclohexanedione (CHD). A 0.3-ml sample of 0.5 M CHD in distilled water was added to 2.7 ml of 0.2 M borate buffer, pH 9.0, containing 1.2 mg of inhibitor. After various periods of incubation at 37°C, 0.1-ml samples were withdrawn and assayed for residual inhibitory activities against trypsin and chymotrypsin.

Amino groups were modified by the method of Plapp *et al.*²⁵⁾ using 2,4,6-trinitrobenzenesulfonate (TNBS). A 0.3-ml sample of 0.8% TNBS in distilled water was added to 3.0 ml of 0.05 M borate buffer, pH 9.5, containing 1.5 mg of inhibitor. After various periods of incubation at 25°C in the dark, the absorbance at 367 nm of the reaction mixture was measured and residual trypsin inhibitory activity was assayed. The extent of trinitrophenylation of amino groups was calculated on the basis of an $\epsilon_{367} = 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

RESULTS

Purification of the inhibitors

All chromatography was done at room temperature.

Okra seeds (100 g) were homogenized in one liter of distilled water and the homogenate was filtered through cotton cloth. Cetavlon (cetyltrimethylammonium bromide) was added to the filtrate until the concentration was 0.2% (wt/v). The mixture solution was centrifuged at $5800 \times g$ for 30 min to remove the resulting precipitate. The supernatant was brought to 80% saturation with solid ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation at $18,000 \times g$ for 20 min and then it was dissolved

in 0.05 M Tris-HCl buffer, pH 8.0. The solution was dialyzed against distilled water at 4°C. After centrifugation, the supernatant was lyophilized. The lyophilized sample (1800 mg) was dissolved in 200 ml of 0.05 M Tris-HCl buffer, pH 8.0 and put on a DEAE-cellulose column (2.8×60 cm) equilibrated with the same buffer. The column was washed with the same buffer and eluted with a linearly increasing concentration of NaCl from zero to 0.4 M in the same buffer (Fig. 1). Two main peaks containing inhibitory activity appeared, and they were designated as peaks I and II. Peaks I and II were separately pooled, dialyzed, and lyophilized. The results of the preparation of peaks I and II are summarized in Table I.

The lyophilized sample of peak I (185 mg)

FIG. 1. DEAE-cellulose Column Chromatography of the Lyophilized Sample Obtained by Ammonium Sulfate Precipitation.

Details are described in the text. Flow rate, 40 ml/hr and fraction volume, 10 ml; —, absorbance at 280 nm; ---●---, trypsin inhibitory activity; ---○---, chymotrypsin inhibitory activity; —·—, NaCl concentration.

TABLE I. PURIFICATION OF PEAK I AND PEAK II FROM SEEDS OF OKRA

Step	Total protein (mg)	Total activity (IU) ^c		Recovery (%)	
		T ^a	CT ^b	T ^a	CT ^b
Extraction	9640	1370	252	100	100
Cetavlon treatment	3290	1250	173	91	67
(NH ₄) ₂ SO ₄ precipitation	1890	838	110	61	44
DEAE-cellulose Peak I	662	622	87	45	35
Peak II	510	366	53	27	21

^a T, trypsin inhibitory activity.

^b CT, chymotrypsin inhibitory activity.

^c One inhibitor unit (1 IU) is defined as the amount of inhibitor which suppressed liberation of one μ mol *p*-nitroaniline per min by active enzymes.

FIG. 2. DEAE-Toyopearl Column Chromatography of Peak I.

Details are described in the text. Flow rate, 40 ml/hr and fraction volume, 8 ml: The symbols are the same as in Fig. 1.

was dissolved in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and put on a DEAE-Toyopearl column (1.8×60 cm) equilibrated with the same buffer. The column was eluted with a linearly increasing concentration of NaCl from 0.05 to 0.15 M (800 ml each) in the same buffer (Fig. 2). Two major peaks appeared and both of the components inhibited trypsin and chymotrypsin. They were separately pooled, dialyzed, lyophilized, and designated as OTI-A for the first peak and as OTI-B for the second peak. The yields of OTI-A and OTI-B were 64 mg and 99 mg, respectively. Each of them was purified more by rechromatography on DEAE-Toyopearl under the same conditions. The yields of rechromatographed OTI-A and OTI-B were 36 mg and 72 mg, respectively.

The lyophilized sample of peak II (255 mg) was put on a CM-Sephacrose CL-6B column (3.5×30 cm) equilibrated with 0.1 M acetate buffer, pH 4.0 (Fig. 3), and eluted with a pH gradient formed from 1 liter each of 0.1 M acetate buffers, pH 4.0 and 5.7. As shown in Fig. 3, proteins appeared as two major peaks with inhibitory activity. They were named peak D for the former and peak C for the latter because the elution position of the two peaks was reversed in the DEAE-cellulose column chromatography.³⁾ Peak D inhibited trypsin

FIG. 3. CM-Sephacrose CL-6B Column Chromatography of Peak II.

Peak II (255 mg) was dissolved in 30 ml of 0.1 M acetic acid, pH 2.9. After removal of insoluble materials by centrifugation, the supernatant was put on a CM-Sephacrose column (3.5×30 cm) equilibrated with 0.1 M acetate buffer, pH 4.0. Elution was done with a pH gradient formed from 1 liter each of 0.1 M acetate buffers, pH 4.0 and 5.7. Flow rate, 40 ml/hr and fraction volume, 10 ml. The symbols are the same as in Fig. 1. —x—, pH.

FIG. 4. DEAE-Toyopearl Column Chromatography of Peak C.

Peak C (34 mg) was dissolved in 0.05 M phosphate buffer, pH 6.5, and put on a DEAE-Toyopearl column (1.4×60 cm) equilibrated with the same buffer. The column was developed with a linear gradient formed from zero to 0.2 M of NaCl in the same buffer. Flow rate, 40 ml/hr and fraction volume, 6 ml. The symbols are the same as in Fig. 1.

but not chymotrypsin and was almost homogeneous by polyacrylamide gel electrophoresis (Fig. 5). Therefore, peak D fractions indicated by the bar in Fig. 3 were pooled, dialyzed, lyophilized, and named OTI-D (35 mg). The

unsymmetrical peak C was further purified by chromatography on DEAE-Toyopearl.

The lyophilized peak C fraction (34 mg) was dissolved in 0.05 M phosphate buffer, pH 6.5, and put on a DEAE-Toyopearl column (1.4 × 60 cm) equilibrated with the same buffer (Fig. 4). Proteins appeared as two peaks, a minor peak and a main symmetrical peak, and both peaks inhibited trypsin and chymotrypsin. The latter main peak was designated as OTI-C. After dialysis and lyophilization, the yield of OTI-C was 14 mg. The minor peak was not purified further, although it contained some components which inhibited trypsin and chymotrypsin.

Homogeneity of the purified inhibitors

Each of the purified inhibitors was contaminated by minute amounts of electrophoretically distinguishable components on an analytical polyacrylamide gel in the presence or absence of SDS (Fig. 5).

Molecular weights

The molecular weights of the four inhibitors were all estimated to be approximately 20,000 by SDS-polyacrylamide gel electrophoresis and also by gel filtration on a Sephadex G-100 column. The results of SDS-polyacrylamide gel electrophoresis in the presence of a reducing agent suggested that all inhibitors were composed of a single polypeptide chain.

Isoelectric points and hexose contents

The isoelectric points were estimated to be pH 4.7 for OTI-A, 4.6 for OTI-B, 4.3 for OTI-C, and 4.2 for OTI-D by gel electrofocusing.

Hexose in the inhibitors was measured by the phenol-sulfuric acid method.²⁵⁾ OTI-A, OTI-B, OTI-C, and OTI-D contained 1.6%, 1.4%, 3.9%, and 0.7% hexose (w/w), respectively.

Stability

On standing at room temperature, the inhibitors were stable throughout the pH range of 2 to 12. After incubating the inhibitors in buffers, pH 3 and pH 8, for 10 min at various

FIG. 5. Polyacrylamide Gel Electrophoresis of the Purified Inhibitors.

(A) Polyacrylamide gel electrophoresis in 7.5% gels, pH 9.4. The four inhibitors (30 μ g each) were electrophoresed at a current of 2 mA per gel. (b) SDS-polyacrylamide gel electrophoresis in 14% gels. Protein samples were treated with 1% SDS in the presence of 5% 2-mercaptoethanol and electrophoresed at a current of 8 mA per gel for 6 hr. 1, OTI-A; 2, OTI-B; 3, OTI-C; 4, OTI-D; 5, standard proteins, from top to bottom, bovine serum albumin (66,000); ovalbumin (45,000); chymotrypsinogen (25,000); cytochrome *c* (12,400).

temperatures, they were stable up to about 60°C but they were rapidly inactivated at 80°C.

Amino acid compositions

In Table II the amino acid compositions of the four inhibitors are summarized. These inhibitors contained more tryptophan than the inhibitors from legume seeds such as soybean²⁶⁾ and winged bean.²⁷⁾ The half-cystine contents were 6 residues per molecule for all inhibitors. No free sulfhydryl groups were detected by the method of Ellman,²¹⁾ so all half-cystine residues appear to be in the form of disulfide bonds.

Amino-terminal residue

The amino-terminal residue was analyzed by the dansylation method. Only dansyl-alanine was detected by polyamide layer chromatography. All inhibitors have alanine as the amino-terminal residue.

Inhibition of proteases

When the inhibitory activities of the purified

TABLE II. AMINO ACID COMPOSITIONS OF THE PURIFIED OKRA TRYPSIN INHIBITORS

Values are molar ratios with respect to glutamic acid and the values of parentheses are the nearest integers.

Amino acid	Residues per molecule			
	OTI-A	OTI-B	OTI-C	OTI-D
Asp	22.3 (22)	17.2 (17)	23.2 (23)	21.4 (21)
Thr ^a	7.2 (7)	6.4 (6)	8.5 (9)	6.9 (7)
Ser ^a	15.3 (16)	16.7 (17)	16.4 (17)	19.5 (20)
Glu	14.0 (14)	17.0 (17)	16.0 (16)	14.0 (14)
Pro	10.2 (10)	10.3 (10)	7.3 (7)	10.2 (10)
Gly	20.8 (21)	22.8 (23)	22.3 (22)	21.4 (21)
Ala	9.2 (9)	10.9 (11)	9.8 (10)	9.3 (9)
1/2Cys ^b	5.6 (6)	5.5 (6)	5.7 (6)	5.9 (6)
Val	19.7 (20)	18.9 (19)	21.2 (21)	21.6 (22)
Met	2.8 (3)	2.6 (3)	3.2 (3)	2.6 (3)
Ile	4.8 (5)	6.1 (6)	4.4 (5)	7.3 (7)
Leu	8.2 (8)	9.1 (9)	9.5 (10)	10.9 (11)
Tyr	4.6 (5)	3.7 (4)	2.8 (3)	3.9 (4)
Phe	5.8 (6)	6.3 (6)	5.0 (5)	5.6 (6)
Lys	11.9 (12)	8.3 (8)	6.5 (7)	6.9 (7)
His	1.8 (2)	1.0 (1)	0.8 (1)	1.2 (1)
Arg	9.7 (10)	10.3 (10)	9.4 (9)	6.6 (7)
Trp ^c	7.1 (7)	5.7 (6)	10.1 (10)	9.3 (9)
Total	183	179	185	186

^a Values of 24 hr hydrolysates.

^b Measured as cysteic acid.⁽¹⁹⁾

^c Measured spectrophotometrically.⁽²⁰⁾

FIG. 6. Titration of Bovine Trypsin with OTI-C.

Fixed amount of trypsin (1.8×10^{-7} M) was titrated with increasing amounts of OTI-C at pH 8.0. BAPA was used as a substrate for trypsin.

inhibitors toward several proteases were tested, these inhibitors had no effect on porcine pepsin, papain, subtilisin, or pronase. The four inhibitors inhibited trypsin strongly and stoi-

TABLE III. INHIBITOR CONSTANTS OF THE FOUR OKRA TRYPSIN INHIBITORS

Inhibitor	Inhibitor constant (K_i)	
	Trypsin ^a	Chymotrypsin ^b
OTI-A	7.3×10^{-9} M	5.4×10^{-7} M
OTI-B	1.5×10^{-9} M	2.1×10^{-7} M
OTI-C	2.4×10^{-10} M	9.6×10^{-8} M
OTI-D	2.7×10^{-9} M	ND ^c

^a Calculated by the method of Green and Work.⁽¹⁷⁾

^b Calculated by the method of Dixon.⁽¹⁸⁾

^c ND, not determined.

chiometrically. A representative titration curve of trypsin with OTI-C is shown in Fig. 6. All of the four inhibitors reacted with trypsin at a molar ratio of 1:1. Three of the four inhibitors (OTI-A, OTI-B, and OTI-C) were weaker and nonstoichiometric inhibitors of chymotrypsin under the conditions we used but OTI-D did not inhibit the enzyme.

The inhibitor constants (K_i 's) of each inhibitor toward trypsin and chymotrypsin calculated by the methods of Green and Work⁽¹⁷⁾ and Dixon⁽¹⁸⁾ are shown in the Table III.

Gel filtration of trypsin-OTI complex

When a mixture of trypsin and an excess of molar equivalent OTI (each of OTI-A, OTI-B, and OTI-C) was filtered on a Sephadex G-100 gel column, two major protein peaks appeared (Fig. 7). The molecular weights of the first and second major peaks were about 43,000 and 20,000, respectively. The 1:1 molar inhibitor-trypsin complex which appeared as the first peak (MW 43,000) no longer inhibited either trypsin or chymotrypsin. The second major peak (MW 20,000) was that of excess inhibitor. The three inhibitors, OTI-A, OTI-B, and OTI-C, inhibited both the enzymes but not simultaneously.

Modification of lysine and arginine residues

The effects of CHD on the inhibitory activity of the four inhibitors are shown in Fig. 8. For OTI-A, the trypsin and chymotrypsin inhibition were similarly lost with time of incubation. For OTI-B, about 80% of the

FIG. 7. Gel Filtration of the Inhibitor-Trypsin Complex.

Trypsin (1 mg) and 2 mg each of OTI-A, OTI-B, and OTI-C were dissolved in 1.0 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. After incubation for 5 min at room temperature, the mixture was put on a Sephadex G-100 column (1.4 × 90 cm) equilibrated with the same buffer. Flow rate, 13 ml/hr and fraction volume, 2 ml; —, absorbance at 280 nm; ---○---, chymotrypsin inhibitory activity.

FIG. 8. Modification of Arginine Residues in the Inhibitors with CHD.

Experimental conditions are described in the text. ●, trypsin inhibitory activity; ○, chymotrypsin inhibitory activity.

original trypsin inhibition was lost after 90 min, while chymotrypsin inhibition remained without change. For OTI-C, the decrease of chymotrypsin inhibition was slower

than that of trypsin inhibition. For OTI-D, after 1 hr of incubation, almost all of the trypsin inhibition was lost. The trypsin inhibition of all the four OTI's was not suppressed by modification with 2,4,6-trinitrobenzenesulfonate (data not shown). We concluded that these inhibitors contain arginine residues in a reactive site toward trypsin. However, it is uncertain whether the reactive site against chymotrypsin of OTI-A and OTI-C contains arginine or not.

DISCUSSION

The four okra trypsin inhibitors have a molecular weight of about 20,000 and a low half-cystine content. In these respects the inhibitors resemble soybean trypsin inhibitor (Kunitz),²⁶⁾ winged bean chymotrypsin inhibitor,²⁷⁾ and silk tree inhibitor.²⁸⁾ The legume seed inhibitors having high molecular weights generally contain two cystines, but the four inhibitors from okra contain three cystine residues. The difference in cystine content seems to reflect the botanical family specificities. Relatively high content of tryptophan is one of the unusual characteristics of the okra inhibitors.

To avoid the formation of so-called modified inhibitors,²⁹⁾ the four inhibitors were purified by the usual procedure without the use of affinity chromatography. Therefore, the four inhibitors purified from okra seeds are isoinhibitors which have much the same molecular weight (20,000), the identical amino-terminal amino acid, alanine, and closely related amino acid compositions (Table II). Distinct differences are observed in the values such as hexose content, isoelectric points, and inhibitor constants (see RESULTS).

The results of the titration experiments and the gel filtration of inhibitor-trypsin complex on Sephadex G-100 show that one molecule of these inhibitors could bind one molecule of trypsin. The inhibitor constants of the four inhibitors suggest that affinity of these inhibitors toward trypsin is stronger than that toward chymotrypsin. OTI-C showed the

strongest affinity to trypsin and chymotrypsin among the four inhibitors.

The four inhibitors showed a variety of behaviors upon the chemical modification of arginine residues by CHD. It is clear that one or more arginine residues of OTI-A participate in binding of this inhibitor with both trypsin and chymotrypsin. OTI-B appears to have two independent reactive sites, one for trypsin and the other for chymotrypsin, and this inhibitor has an arginine residue as the reactive site for trypsin. OTI-C appears to have two independent reactive sites for the two enzymes as OTI-B dose. However, approximately 40% of the chymotrypsin inhibitory activity of OTI-C was lost upon the modification of arginine residues. Therefore, the reactive site for chymotrypsin in OTI-C may be near an arginine residue. The arginine residue is almost certain to be the reactive site for trypsin of OTI-C. The independent reactive sites for trypsin and chymotrypsin of OTI-B or OTI-C may be very close to each other, because chymotrypsin was not inhibited by the inhibitor-trypsin complexes form OTI-B and OTI-C which were isolated by the gel filtration (Fig. 7). If the two reactive site are very far apart, the inhibitor-trypsin complex could inhibit chymotrypsin through the formation of the ternary complex of chymotrypsin-inhibitor-trypsin. Further investigation is necessary to elucidate the interaction of two reactive site for the two enzymes with these inhibitors, especially the reactive site for chymotrypsin in these inhibitors.

The nutritional significance of okra trypsin inhibitors is still unclear. The inhibitors are presumably not antinutrients since they are relatively unstable over 80°C and have no effect toward porcine pepsin. The high protein efficiency ratio of okra seeds reported by Karakoltsidis and Constantinides also supports the above speculation. In our preliminary experiments, however, the trypsin inhibitory activity was detected in the unmaturing seeds of eatable young pods. When the young raw pods are eaten by animals, it is unclear at present if the inhibitors act as antinutrients

or not.

The physiological function of protease inhibitors in the plant is not clear. The protease inhibitors in seeds or tubers may exist as stored proteins that will play a role in germination. Therefore the stored protein may be present in a stable form against attack of proteases of invaders, insects, and microorganisms.

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