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(Citation)
神戸大学農学部研究報告,15(2):367-377

(Issue Date)
1983-01-30

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(JaLCDOI)
https://doi.org/10.24546/00227296

(URL)
https://hdl.handle.net/20.500.14094/00227296

神大農研報 (Sci. Rept. Fac. Agr. Kobe Univ.) 15 : 367—377, 1983

AMINO ACID SEQUENCE AND REACTIVE SITE OF A POTATO POLYPEPTIDE TRYPSIN INHIBITOR (TIc).[1]

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(Received for publication on August 10, 1982)

Abstract

The amino acid sequence of potato polypeptide trypsin inhibitor c (TIc) was determined by means of Edman degradation and carboxypeptidase digestion. Sequence analyses were carried out on the reduced and S-carboxymethylated sample and its tryptic and chymotryptic peptides. The results revealed that TIc consisted of 51 amino acid residues. The amino-terminal sequence (residues 1 through 18) of TIc was homologous to that of the potato polypeptide chymotrypsin inhibitor of Hass et al. Furthermore, the overall sequence of the inhibitor was homologous to that of one of the polypeptide trypsin inhibitors of eggplant exocarp. It was not identical with the sequences of the artificially-prepared active fragments of potato proteinase inhibitors IIa and IIb, though strong homology was partially observed. Any Lys-Ser bond, the reactive site peptide bond of the active fragments, was not found in the sequence of this naturally-occurring polypeptide trypsin inhibitor. The inhibitor activities of TIc against trypsin and chymotrypsin were not greatly affected by the modification of amino groups but considerably decreased by the modification of guanidino groups in the molecule. This fact and the sequence study indicated that the reactive site peptide bond of TIc was Arg(38)-Asn(39), which was identical with that of the polypeptide trypsin inhibitor of eggplant exocarp.

Introduction

In our earlier studies,[7-10] it was noticed that the artificially-prepared active fragments of potato proteinase inhibitors IIa and IIb (PPI-IIa and IIb) were somewhat similar to the naturally-occurring potato polypeptide chymotrypsin inhibitor of Hass et al.[5] in terms of molecular weight, amino acid composition and partial amino acid sequence. Therefore, it is probable that the polypeptide chymotrypsin inhibitor of Hass *et al* is derived from its precursor by endogeneous proteolysis in potato tubers and also that PPI-IIa and IIb are endogeneously converted into some lower molecular weight inhibitors such as their artificially-prepared active fragments.[10] In a preceding paper,[17] we isolated five polypeptide trypsin inhibitors (TIa, TIb, TIc, TId and TIe) and four polypeptide chymotrypsin inhibitors (CIa, CIb, CIc, and CId). Among them, TIc, CIa and CId were characterized in terms of molecular weight, amino acid composition and specificity against enzymes. TIc was similar to the active fragment of PPI-IIa, and CIa and CId to the active fragment of PPI-IIb as to the properties. Moreover, the partial amino-terminal sequences of these three polypeptide inhibitors were homologous to the sequences of the amino-terminal regions of the active fragments of PPI-IIa and IIb.

In this paper, we will describe the results of sequence analysis and reactive site determination on TIc and discuss the structural correlation of TIc to the active fragment of PPI-IIa and

Abbreviations: PTH, phenylthiohydantoin; TNP, trinitrophenylated.

1. Abstract of this paper was presented at the annual meeting of the Japanese society of agricultural chemistry (April, 1981)

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other polypeptide inhibitors.

Experimental Procedure

Materials——TIc was purified by the procedure described in a preceding paper.[17] Reduced and S-carboxymethylated TIc (Cm-TIc) was prepared by the method of Crestfield *et al.*[2] Bovine trypsin (twice-crystallized) and α-chymotrypsin (crystallized three times) were purchased from Sigma Chemical Co. Carboxypeptidases A and B were from Worthington Biochemical Co. Thin-layer precoated sheets (DC-Fertigplatten Kieselgel 60 F-254, Merck) and ion exchange resin Dowex-50X2 were from Nakarai Chemicals Ltd. The "sequanal" grade preparations of the reagents for Edman degradation were from Wako Pure Chemical Industries, Ltd. All other chemicals used were of analytical grade or better.

Tryptic Digestion of Cm-TIc——Thirty seven mg of Cm-TIc (6.3 μmol) and 40 mg of $CaCl_2$ were dissolved in 3 ml of 0.1 M NH_4OH and the pH of the solution was adjusted to 8.0 with 0.1 M acetic acid. To this solution, trypsin dissolved in 0.0025 N HCl was added at a ratio of 1/50 to the substrate (W/W). After the pH of the mixture was readjusted to 8.0 with 0.1 N NaOH, reaction was allowed to proceed for 6 hr at 30°C and then terminated by adjusting the pH to 2 with 1 N HCl.

Chymotryptic Digestion of Cm-TIc——Twenty eight mg of Cm-TIc (4.7 μmol) was subjected to chymotryptic digestion. The digestion was carried out in a similar manner to the tryptic digestion, but without addition of $CaCl_2$. Reaction time was 1.5 hr and the ratio of enzyme to the substrate was 1/100 (W/W).

Fractionation of Tryptic and Chymotryptic Peptides——Each of the tryptic and chymotryptic digests was applied to a column (1.8×90cm) of Dowex-50X2 previously equilibrated with 0.2M pyridine-acetic acid buffer, pH 3.1, at 38°C. Elution was first carried out with 250 ml of the same buffer and then with a linear gradient to 2 M pyridine-acetic acid buffer, pH 5.0. The volumes of mixing and strage chambers were 500 ml. Finally, a stepwise elution with 2 M pyridine-acetic acid buffer, pH 5.0, was performed. The flow rate was 50 ml/hr. Detection of peptides in column effluents was performed by the ninhydrin reaction method after alkaline hydrolysis.[6]

Paper Chromatographic Separation of Peptides——This was carried out at room temperature using Toyo paper No. 50 (20×40 cm). The solvent systems employed were n-butanol-pyridine-water (1 : 1 : 1, V/V) and n-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, V/V). The paper was dried in the air and the parts with peptides were cut out with the aid of guide strips stained by ninhydrin-acetone solution (0.2%, W/V). Peptides were eluted from the paper with 10% pyridine and water.

Amino Acid Analysis——Samples (0.1-0.2 μ mol) were hydrolyzed with redistilled HCl *in vacuo* at 110°C for 20-24 hr. Amino acids were determined using a Hitachi amino acid analyzer, model KLA-3B, according to Spackman *et al.*[16]

Carboxypeptidase Digestion——Samples (0.1-0.2 μmol) were subjected to the digestion at pH 8.5 and 30°C in 0.2 M N-ethylmorphorine-acetic acid buffer. The molar ratio of enzyme to the sample was 1/50. The digestions were terminated by adding 30% acetic acid and the amino acids released were analyzed using an amino acid analyzer.

Edman Degradation——This was performed according to Iwanaga *et al.*[11] For the thin-layer chromatographic identification of PTH-derivatives of most of the amino acids, solvents II and V were used. The PTH-derivative of S-carboxymethylcysteine (Cm-cysteine) was identified by paper chromatography using n-heptane-ethylenedichloride-75% formic acid (6 : 12 : 1, V/V) as a solvent.[11]. The yields of PTH-derivatives were determined by measuring the absorbancy at 269 nm before thin-layer chromatographic identification of the derivatives.

Measurement of the Content of Sulfhydryl Groups——The content of free sulfhydryl groups of native TIc was measured by the titration with *p*-chloromercuribenzoate (*p*CMB).[1]

Modification of TIc by Limited Hydrolysis with Trypsin——Sample was incubated with trypsin (enzyme/substrate=1/30, W/W) in 0.1 M acetic acid-0.05 M $CaCl_2$ at pH's between 3 and 4.8 for 42-72 hr. The inhibitor was then separated from trypsin by gel filtration through

a Sephadex G-50 column (2.8×138 cm) equilibrated with 0.1 M acetic acid and lyophilized. This preparation was reduced and S-carboxymethylated and again passed through a Sephadex G-50 column under the same condition mentioned above.

Chemical Modification of Guanidino and Amino Groups——For the modification of guanidino groups, sample was treated with 0.05 M 1, 2-cyclohexanedione in 0.2 M sodium borate at pH 9.0 and 30°C for 18 hr.[13] The modification of amino groups was performed by the treatment with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) according to Plapp *et al.*[14] The inhibitor activities of these chemically-modified preparations were measured by the casein digestion method[4] The extent of modification of guanidino groups was determined by amino acid analysis, and that of amino groups was calculated from the absorbance of the reaction mixture at 367 nm, employing a molar extinction coefficient of 1.1×10^4/mol/cm for TNP-amino groups.[14]

Results

Amino Acid Composition and Terminal Sequences of Cm-TIc —— Table I shows the amino acid composition of Cm-TIc.

It consists of 51 amino acid residues including 8 Cm-cysteine residues. One μmol of Cm-TIc was subjected to Edman degradation. Twenty-three steps of degradation indicated the amino-terminal sequence of TIc as given in Table II. It should be noted that asparagine was identified together with Cm-cysteine at position 6 of Cm-TIc. To determine the carboxyl-terminal sequence, Cm-TIc (0.15μmol) was subjected to the digestion by carboxypeptidases A and B. After incubation for 20 min, arginine (0.30 mol/mol), leucine (0.29 mol/mol), Cm-cysteine (0.25 mol/mol) and several other amino acids (less than 0.20 mol/mol) were released. After 3 hr, the yields of the three main amino acids released were 0.63 mol/mol (arginine), 0.57 mol/mol (leucine) and 0.54 mol/mol (Cm-cysteine), respectively. These results indicated the carboxyl-terminal sequence of TIc as given in Table II.

Separation of Tryptic Peptides of Cm-TIc ——The tryptic digest of Cm-TIc was applied to a column of Dowex-50X2 under the conditions described in "EXPERIMENTAL PROCEDURE". The elution pattern of the tryptic peptides is given in Fig. 1.

Table I. Amino acid composition of Cm-TIc.

Amino acid	Molar ratio (Leucine=1.00)
S-carboxymethylcysteine	6.93 (8)[a]
Aspartic acid	8.79 (9)
Threonine	1.67 (2)
Serine	2.42 (3)[a]
Glutamic acid	2.27 (2)
Proline	3.43 (3)
Glycine	4.00 (4)
Alanine	3.72 (4)
Valine	1.13 (1)
Methionine	0 (0)
Isoleucine	2.79 (3)
Leucine	1.00 (1)
Tyrosine	3.64 (4)
Phenylalanine	0.90 (1)
Lysine	3.02 (3)
Histidine	0 (0)
Arginine	2.76 (3)
Tryptophan[b]	0 (0)
Total	51

a), This number of residues was obtained by sequence analysis.
b), Cited from the data of a previous paper.[17]

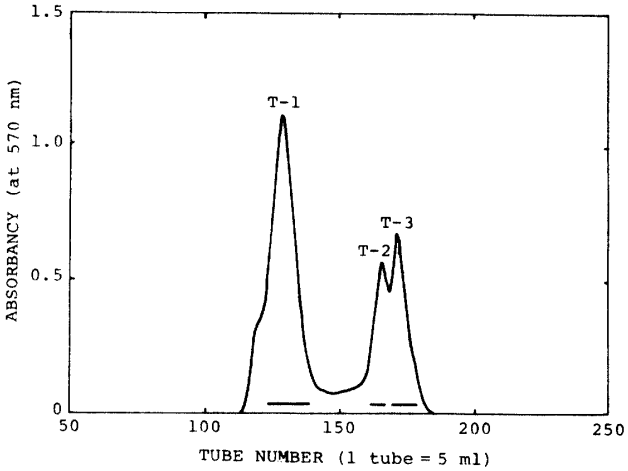

Fig. 1. Elution pattern of the tryptic peptides of Cm-TIc from Dowex-50X2. For conditions see "EXPERIMENTAL PROCEDURE." Aliquots (0.2 ml) of the effluents from alternate tubes were subjected to the ninhydrin reaction after alkaline hydrolysis. ——, absorbancy at 570 nm. The fractions indicated by bars were pooled.

Table II. Amino- and carboxyl-terminal sequences of Cm-TIc. The symbols used are: —→, Edman degradation; ←—, carboxypeptidase digestion. The yields of PTH-derivatives are shown in μmol under the arrows.

Arg	Ile	Cys(cm)	Thr	Asn	Cys(cm) (Asn)	Cys(cm)	Ala	Gly	Tyr	Lys	Gly
0.81	0.86	-0.50	0.62	0.36	0.22	0.25	0.39	0.39	0.33	0.22	0.28

Cys(cm)	Asn	Tyr	Tyr	Ser	Ala	Asn	Gly	Ala	Phe	Ile	(Cys (cm),
0.20	0.09	0.11	0.09	0.04	0.04	0.04	0.06	0.04	+	+	

Glu, Gly, Glu, Ser, Asn, Pro, Lys, Asn, Pro, Asn, Val, Cys(cm), Pro, Arg, Asn, Cys(cm), Asp, Thr, Asn, Ile, Ala, Tyr, Ser, Lys)—Cys(cm)—Leu—Arg

Three main peaks were obtained and designated as T-1, T-2 and T-3. The shoulder peak eluted before fraction T-1 contained only very low yield of peptide(s) (less than 2%), and was not analyzed. The fractions, indicated by bars, corresponding to each peak, were concentrated by evaporation at 40°C and next subjected to paper chromatography using the solvent systems described in "EXPERIMENTAL PROCEDURE". Fractions T-1 and T-2 yielded only one peptide each, but T-3 was separated into two peptides on the chromatogram. These two peptides from T-3 were designated as T-3a and T-3b. The four peptides thus purified were extracted with 10% pyridine and water, and the extracts were concentrated to make the volumes 10 ml by evaporating at 40°C. An appropriate volume of each peptide solution was taken for amino acid analysis. Table III shows the amino acid compositions of the purified tryptic peptides of Cm-Tlc. Peptides T-2 and T-3a exhibited similar amino acid compositions. This fact and the relatively low pields of these two peptides indicated that they were identical with each other. Thus, the total number of the amino acid residues contained in the three different tryptic peptides (T-1, T-2 or T-3a and T-3b) was 51, corresponding to the total of the intact Cm-TIc.

Amino Acid Sequences of Tryptic Peptides ——Table IV shows the amino acid sequences of the tryptic peptides of Cm-TIc, analyzed by Edman degradation and carboxypeptidase digestion.

T-1: This peptide contained 10 amino acid residues. Edman degradation indicated the sequence of 8 residues. Digestion with carboxyptidases A and B for 30 min released lysine (0.40 mol/mol) and serine (0.05 mol/mol), indicating the carboxyl-terminal sequence of this peptide to be -Ser-Lys.

T-2 and T-3a: These peptides contained 38 amino acid residues. The amino-terminal sequence (6 residues) of these peptides corresponded to that of the intact Cm-TIc (Table II). It should be added that asparagine was also identified besides Cm-cysteine at position 6 of the peptides. Only arginine (0.40 mol/mol) was released when these peptides were digested with carboxypeptidases A and B for 30 min.

T-3b: This contained 3 amino acid residues and its sequence corresponded to the carboxyl-terminal sequence of the intact Cm-TIc (Table II).

These data indicated a possible order of the tryptic peptic peptides as follows: (T-2 or T-3a)-(T-1)-(T-3b).

Separation of Chymotryptic Peptides of Cm-TIc —— Cm-TIc (4.7 μmol) was digested with chymotrypsin and the digest was applied to a column of Dowex-50X2 under the conditions described in "EXPERIMENTAL PROCEDURE". The elution pattern of the chymotryptic peptides is given in Fig. 2. Seven peaks were obtained and designated as C-1, C-2, C-3, C-4, C-5, C-6 and C-7. The fractions, indicated by bars, corresponding to each peak were concentrated by evaporating at 40°C and then subjected to paper

Table III. Amino acid compositions of the tryptic peptides of Cm-TIc. Values are expressed as molar ratio with respect to the amino acid denoted by an asterisk in each column.

Amino acid	T-1	T-2	T-3a	T-3b
S-Carboxymethylcysteine	0.80 (1)	4.86 (6)[a]	5.39 (6)[a]	0.62 (1)
Aspartic acid	3.14 (3)	6.33 (6)	7.12 (6)[a]	
Threonine	0.93 (1)	1.29 (1)	1.17 (1)	
Serine	1.07 (1)	2.34 (2)	2.29 (2)	
Glutamic acid		2.59 (2)[a]	2.66 (2)[a]	
Proline		3.02 (3)	3.12 (3)	
Glycine		4.63 (4)[a]	4.42 (4)	
Alanine	1.12 (1)	3.34 (3)	3.26 (3)	
Valine		1.00*(1)	1.00*(1)	
Isoleucine	1.00*(1)	1.71 (2)	2.00 (2)	
Leucine				1.00*(1)
Tyrosine	1.02 (1)	3.04 (3)	3.27 (3)	
Phenylalanine		1.04 (1)	1.08 (1)	
Lysine	1.06 (1)	2.40 (2)	2.27 (2)	
Arginine		2.00 (2)	2.08 (2)	1.05 (1)
Total	10	38	38	3
Yield(%)[b]	49	9	12	30

a), This number is obtained by sequence analysis.
b), The amount of peptide relative to starting material.

Table IV. Amino acid sequences of the tryptic peptides of Cm-TIc. The symbols used are the same as in Table II. The yields of PTH-derivatives are shown in μmol under the arrows.

Peptide	Sequence
T-1	Asn —(0.45)→ Cys(cm) —(0.52)→ Asp —(0.26)→ Thr —(0.41)→ Asn —(0.16)→ Ile —(0.18)→ Ala —(0.14)→ Tyr —(0.05)→ Ser ←— Lys
T-2 & T-3a	Arg —(0.19)→ Ile —(0.16)→ Cys(cm) —(0.16)→ Thr —(0.20)→ Asn —(0.10)→ Cys(cm) —(0.09)→ (Asn) —— (Cys(cm), Ala, Gly, Tyr, Lys, Gly, Cys(cm), Asn, Tyr, Tyr, Ser, Ala, Asn, Gly, Ala, Phe, Ile, Cys(cm), Glu, Gly, Glu, Ser, Asn, Pro Lys, Asn, Pro, Asn, Val, Cys (cm), Pro) ←— Arg
T-3b	Cys(cm) —(0.54)→ Leu —(0.44)→ Arg —(0.28)→

chromatography in the same way described for the tryptic peptides. No peptides were detected in fraction C-3. Fraction C-6 was separated into two peptides, which were referred to as C-6a and C-6b. The other fractions yielded one peptide on the chromatogram, respectively. Table V shows the amino acid compositions of the chymotryptic peptides. Peptides C-5 and C-6b had an identical amino acid composition and were regarded as an identical peptide.

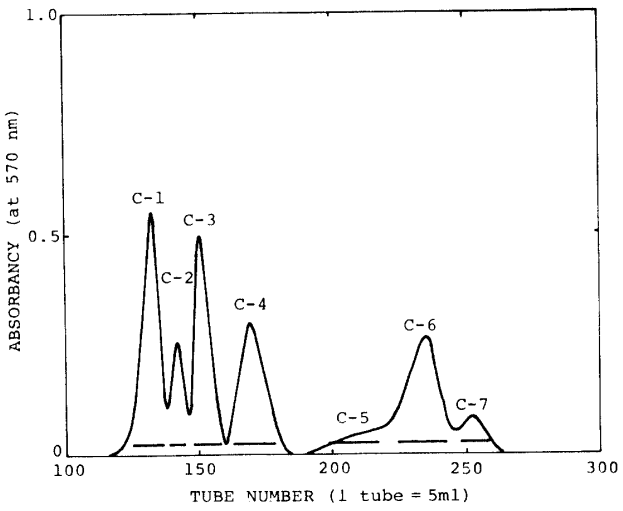

Fig. 2. Elution pattern of the chymotryptic peptides of Cm-TIc from Dowex-50X2. For conditions see "EXPERIMENTAL PROCEDURE." Aliquots (0.2 ml) of the effluents from alternate tubes were subjected to the ninhydrin reaction after alkaline hydrolysis. ——, absorbancy at 570 nm. The fractions indicated by bars were pooled.

C-6a also had the same amino acid composition as those of C-5 and C-6b, except that it had one more tyrosine. The relatively low yields of C-5, C-6a and C-6b indicated that these three peptides were derived from an identical region of TIc. The total number of amino acid residues contained in the five main peptides (C-1, C-2, C-4, C-6a and C-7) was consistent with the total of the intact Cm-TIc.

Amino Acid Sequences of Chymotryptic Peptides——The results of the sequence analyses are given in Table VI.

C-1: This peptide contained 24 amino acid residues. Twenty steps of Edman degradation indicated the amino-terminal sequence of this peptide. The sequence of residues 17 through 20 of this peptide overlapped the sequence of residues 1 through 4 of tryptic peptide T-1 (Table IV).

C-2: This peptide contained 6 residues. Five steps of Edman degradation established the entire sequence of this peptide. It overlapped the sequence of residues 17 through 22 of the

Table V. Amino acid compositions of the chymotryptic peptides of Cm-TIc. Values are expressed as molar ratios with respect to the amino acid denoted by an asterisk in each column.

Amino acid	C-1	C-2	C-4	C-5	C-6a	C-6b	C-7
S-carboxymethylcysteine	2.33 (3)[a]		2.21 (3)[a]	0.62 (1)	0.35 (1)	0.35 (1)	0.64 (1)
Aspartic acid	6.37 (6)	1.07 (1)	1.24 (1)	1.44 (1)	1.17 (1)	1.00*(1)	
Threonine	0.99 (1)		0.84 (1)				
Serine	1.16 (1)	0.77 (1)					0.87 (1)
Glutamic acid	2.17 (2)						
Proline	3.29 (3)						
Glycine	1.41 (1)	1.00*(1)	1.11 (1)	1.52 (1)[a]	1.29 (1)	1.21 (1)	
Alanine	1.46 (1)	1.64 (2)	1.08 (1)				
Valine	1.00*(1)						
Isoleucine	2.04 (2)		1.00*(1)				
Leucine							1.00*(1)
Tyrosine	0.93 (1)		0.95 (1)	0.91 (1)	2.07 (2)	1.29 (1)	
Phenylalanine		0.82 (1)					
Lysine	0.99 (1)			1.00*(1)	1.00*(1)	0.75 (1)	0.95 (1)
Arginine	0.99 (1)		0.86 (1)				0.91 (1)
Total	24	6	10	5	6	5	5
Yield(%)[b]	51	52	62	8	22	17	48

a), This number of residues was obtained by sequence analysis.
b), The amount of peptide relative to starting material.

Table VI. Amino acid sequences of the chymotryptic peptides of Cm-TIc. The symbols used are the same as in Table II. The yields of PTH-derivatives are shown in μmol under the arrows.

Peptide	Sequence
C-1	Ile —Cys(cm)— Glu —Gly— Glu —Ser —Asn— Pro —Lys —Asn— Pro — Asn 1.20 0.80 0.96 0.74 0.56 0.38 0.17 0.22 0.13 0.14 0.22 0.16
	Val —Cys(cm)— Pro —Arg— Asn —Cys(cm)— Asp — Thr — (Asn, Ile, Ala, Tyr) 0.14 0.14 0.15 0.15 0.07 0.07 0.10 0.07
C-2	Ser —Ala— Asn —Gly— Ala —Phe 0.44 0.62 0.44 0.34 0.06
C-4	Arg —Ile —Cys(cm)— Thr —Asn— Cys(cm) —Cys(cm)— Ala —Gly— Tyr 0.84 0.72 0.55 0.58 0.21 (Asn) 0.19 0.18 0.15 0.13
C-6a	Lys —Gly —Cys(cm)— Asn —Tyr— Tyr 0.62 0.55 0.26 0.25 0.14 0.04
C-7	Ser —Lys —Cys(cm)— Leu —Arg 0.53 0.63 0.76 0.71 0.66

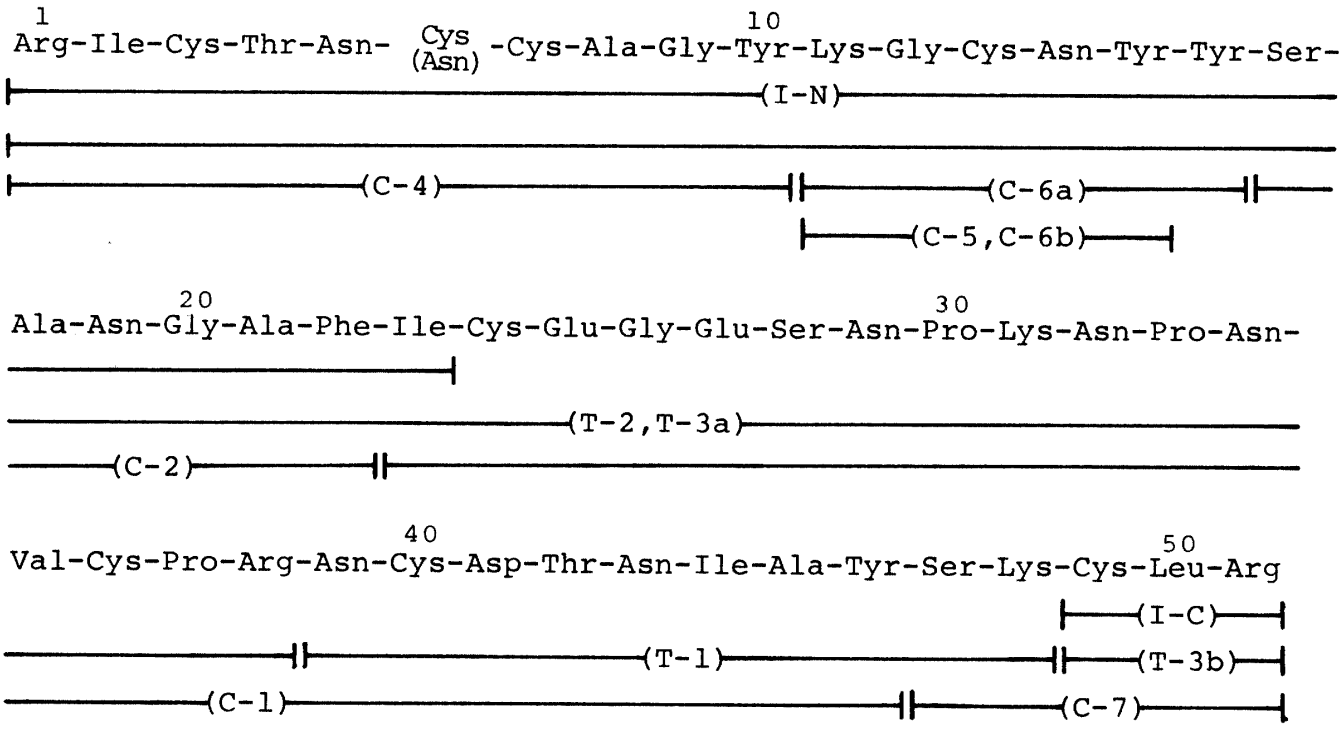

Fig. 3. Summary of the sequence analyses on TIc. I-N, amino-terminal sequence of the intact TIc; I-C, carboxyl-terminal sequence of the intact TIc; T, sequences of tryptic peptides; C, sequences of chymotryptic peptides.

intact Cm-TIc (Table II).

C-4 : This peptide consisted of 10 amino acid residues. Nine steps of Edman degradation established the sequence of this peptide. Asparagine was again identified besides Cm-cysteine at position 6 of this peptide. The sequence overlapped the amino-terminal sequence (residues 1 through 10) of the intact Cm-TIc (Table II).

C-6a, C-5 and C-6b : C-6a contained 6 residues. Six steps of Edman degradation indicated the entire sequence of this peptide. It overlapped the sequence of residues 11 through 16 of the intact Cm-TIc (Table II). C-5 and C-6b contained 5 residues and their sequences were identical with that of residues 1 through 5 of C-6a.

C-7 : This peptide consisted of 5 residues. Five steps of Edman degradation established the entire sequence of this peptide. The sequence of residues 1 and 2 of this peptide overlapped the carboxyl-terminal sequence (residues 9 and 10) of tryptic peptide T-1 and its sequence of residues 3 through 5 corresponded to the sequence of tryptic peptide T-3b and to the carboxyl-terminal sequence of the intact Cm-TIc (Tables II and IV).

The amino acid sequences of these chymotryptic peptides, together with the sequences of the tryptic peptides and the amino- and carboxyl-terminal sequences of the intact Cm-TIc, established the overall amino acid sequence of TIc as summarized in Fig. 3. The data shows that Cm-TIc contains 8 Cm-cysteine residues. Titration of native TIc with pCMB by the method of Boyer[1] indicated that TIc contained no sulfhydryl group, and consequently, it had 4 cystine residues in the molecule.

Modification of TIc by Trypsin at Acidic pH's ——This was carried out under the conditions described in "EXPERIMENTAL PROCEDURE". But no cleavages were observed by the hydrolysis of TIc with trypsin under the experimental conditions.

Effects of Chemical Modifications on Inhibitor Activity of TIc——Guanidino and amino groups of TIc were modified with 1, 2-cyclohexanedione and TNBS, respectively, under the conditions described in "EXPERIMENTAL PROCEDURE". The inhibitor activities of these chemically-modified TIc preparations against trypsin and chymotrypsin were determined (Table VII). The guanidino group-modified sample showed significant decrease in the inhibitor activities against both trypsin and chymotrypsin as compared with those of native TIc. The amino group-modified TIc also decreased its activities against the enzymes but to small extents.

Table VII. Inhibitor activities of guanidino group-modified and amino group-modified TIc.

Sample	Relative activity against	
	Trypsin	chymotrypsin
Native TIc	100(%)	100(%)
Guanidino group-modified TIc[a)]	8	29
Amino group-modified TIc[b)]	61	68

a), The extent of modification was 79%.
b), The extent of modification was 87%.

Discussion

Edman degradation and carboxypeptidase digestion of the intact Cm-TIc established the amino-terminal sequence (23 residues) and the carboxyl-terminal sequence (3 residues) of this polypeptide trypsin inhibitor (Table II). This made it possible to align the three tryptic peptides derived from the Cm-TIc (Fig. 3). Tryptic peptide T-2 (or T-3a) contained two arginine residues and two lysine residues, of which the arginine residues were found at the amino- and carboxyl-termini of this peptide. Therefore, the two lysyl bonds had remained intact in this tryptic peptide. This indicated that the tryptic digestion of Cm-TIc had been incomplete or that the complete tryptic digestion of T-2 or T-3a had been prevented because of its configuration. The overall sequence of TIc was established by the aids of the sequences of the chymotryptic peptides of Cm-TIc (Fig. 3). At position 6 in the sequence of TIc, asparagine was always detected besides Cm-cysteine. A similar result was also obtained by Richardson[15] on the sequence of one of the eggplant exocarp

inhibitors, which had strong homology with that of TIc as given in Fig. 4. These two inhibitors also exhibit strong similarity in their enzyme-inhibition profiles,[12] indicating that they are the isoinhibitors which are widely distributed in Solanaceae family of plants and that the genetic information for the synthesis of these inhibitors has been maintained in the course of evolution of Solanaceae. Several different types of isoinhibitors have been found in potatoes[17] and eggplants,[18] and it can be expected that these inhibitors are also homologous to each other as to their sequences. In Fig. 4, the amino acid sequence of TIc is also compared with those of the active fragments of PPI-IIa[9] and the polypeptide chymotrypsin inhibitor of Hass *et al.*[5] The amino-terminal sequence (residues 1 through 15, except residue 6) of TIc corresponds to the sequence of residues 8 through 22 of the active fragment of PPI-IIa, and the carboxyl-terminal sequeces (3 residues) of both inhibitors are identical with each other. But, the rest of the sequence of TIc differs from that of the active fragment, though strong homology is partially noticed between the sequences. Striking is that the sequence of residues 39 through 48 of TIc is very homologous to that of residues 23 through 32 of the active fragment of PPI-IIa. The sequence is also somewhat homologous to that of residues 15 through 26 of the active fragment of PPI-IIb, and yet the location of this region in the entire sequence of TIc is different from those of the respective regions in the sequences of the active fragments. Further, the positions of the only phenylalanine and valine residues in TIc are quite different from those in the active fragment of PPI-IIa. More important is that TIc does not contain any Lys-Ser bond which is the reactive site of the active fragments of PPI-IIa[9] and IIb.[10] The Arg(38)-Asn(39) bond in the sequence of the eggplant exocarp inhibitor of Richardson has been determined as the reactive site of the inhibitor by the limited hydrolysis with trypsin at a low pH.[15] An identical bond is located at the same position of the sequence of TIc. Judging from the strong homology between the sequences of the two polypeptide inhibitors, it is expected that the Arg-Asn bond is the reactive site of TIc. To elucidate this, the limited hydrolysis of TIc at low pH's were performed under various conditions. However, all the experiments were not successful. The attempt of determining the reactive site of the polypeptide chymotrypsin

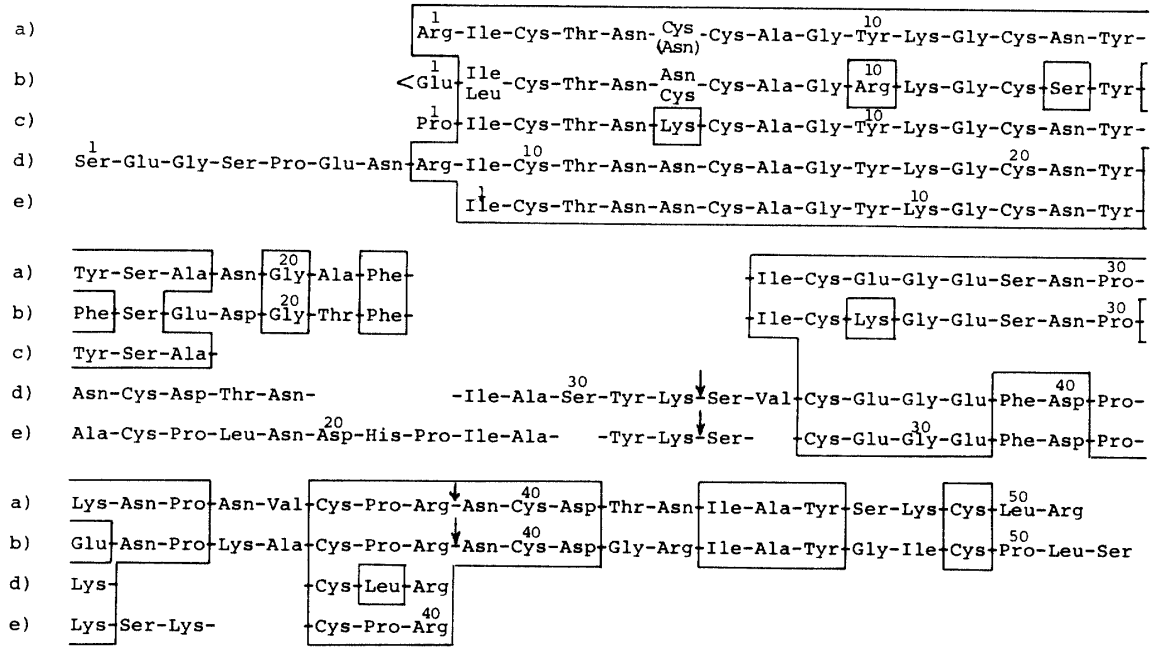

Fig. 4. Comparison of the amino acid sequence of TIc with those of other polypeptide inhibitors. a), TIc; b), inhibitor of eggplant exocarp[15]; c), chymotrypsin inhibitor of Hass *et al.*[5]; d), active fragment of PPI-IIa[9]; e) active fragment of PPI-IIb.[10] The arrows show the reactive sites. Segments of identical sequences in the inhibitors are enclosed in boxes.

inhibitor of Hass *et al.* also ended in failure.[3)]

These results may be ascribed to the small differences between the sequences of the eggplant exocarp inhibitor and the potato polypeptide inhibitors.

As shown in Table VII, the inhibitor activities of TIc against trypsin and chymotrypsin significantly decreased by the chemical modification of guanidino groups of the inhibitor. This indicates that an arginine residue is important for TIc to exhibit the inhibitor activities against the enzymes. Since two of the three arginine residues of TIc are located at the amino- and carboxyl-termini of the inhibitor (Fig. 4), the arginine residue at position 38 is supposed to be the important one, hence the arg(38)-Asn (39) is possibly the reactive site of TIc.

Thus, the present paper revealed that a polypeptide trypsin inhibitor, TIc, of potato tubers had a somewhat different amino acid sequence and a different reactive site from those of the active fragments of PPI-IIa and IIb. Therefore, it is probable that TIc is a derivative of a precursor which is different from PPI-IIa and IIb or is an independently synthesized inhibitor.

NOTE ADDED IN PROOF

While this manuscript was in preparation Hass et al. reported on the sequences of two low molecular weight proteinase inhibitors, which were homologous to that of TIc.[19)]

References

1) Boyer, P. D. : *J. Amer. Chem. Soc.*, **76**, 4331-4337, 1954.
2) Crestfield, A. M., S. Moore and W. H. Stein : *J. Biol. Chem.*, **238**, 622-627, 1963.
3) Eddy, J. L., J. E. Derr and G. M. Hass : *Phytochemistry*, **19**, 757-761, 1980.
4) Hagihara, B., H. Matsubara, M. Nakai and K. Okunuki : *J. Biochem.*, **45**, 185-194, 1958.
5) Hass, G. M., R. Venkatakrishnan and C. A. Ryan : *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1941-1944, 1976.
6) Hirs, C. H. W., S. Moore and W. H. Stein : *J. Biol. Chem.*, **219**, 623-642, 1956.
7) Iwasaki, T., T. Kiyohara and M. Yoshikawa : *J. Biochem.*, **75**, 843-851, 1974.
8) Iwasaki, T., J. Wada, T. Kiyohara and M. Yoshikawa : *J. Biochem.*, **78**, 1267-1274, 1975.
9) Iwasaki, T., T. Kiyohara and M. Yoshikawa : *J. Biochem.*, **79**, 381-391, 1976,
10) Iwasaki, T., J. Wada, T. Kiyohara and M. Yoshikawa : *J. Biochem.*, **82**, 991-1004, 1977.
11) Iwanaga, S. and Y. Samejima : *Tampakushitsu Kakusan Koso*, (in Japanese) **15**, 1037-1054, 1970.
12) Kanamori, M., F. Ibuki, M. Tashiro, M. Yamada and M. Miyoshi : *Biochim. Biophys. Acta*, **439**, 398-405, 1976.
13) Patthy, L. and E. L. Smith : *J. Biol. Chem.*, **250**, 565-569, 1975.
14) Plapp, B. V., S. Moore and W. H. Stein : *J. Biol. Chem.*, **246**, 939-945, 1971.
15) Richardson, M. : *FEBS Lett.*, **104**, 322-326, 1979.
16) Spackman, D. H., W. H. Stein and S. Moore : *Analt. Chem.*, **30**, 1190-1206, 1958.
17) Uchida, K., T. Iwasaki, T. Kiyohara and M. Yoshikawa : *Sci. Rept. Fac. Agr. Kobe Univ.*, **15**, 357-365, 1983.
18) Yamada, M. S., Santo, H, Yamaguchi, M. Tashiro, F. Ibuki and M. Kanamori : *Agric. Biol. Chem.*, **41**, 2343-2347, 1977.
19) Hass, G. M., M. A. Hermodson, C. A. Ryan and L. Gentry : *Biochemistry*, **21**, 752-756, 1982.

バレイショのポリペプチド性トリプシンインヒビターC (TIc)のアミノ酸配列と反応部位について

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要　　約

バレイショの塊茎から分離されたポリペプチド性トリプシンインヒビターC(TIc) の一次構造をエドマン分解法とカルボキシペプチダーゼ法を用いて分析した。TIc は51個のアミノ酸から成り，N末端から18番目までのアミノ酸配列は Hass らのポリペプチド性キモトリプシンインヒビターのそれと相同であった。TIc の全一次構造はナス外皮から分離されたポリペプチド性トリプシンインヒビターのそれと極めて相同性が高かった。また，われわれが前にバレイショのプロティナーゼインヒビター Ⅰa 及び Ⅰb から人工的に調製した2種の活性フラグメントの一次構造とも部分的に相同性が認められた。

しかし，TIc は全アミノ酸配列中に，活性フラグメントの反応部位である Lys-Ser 結合を欠いていた。化学修飾実験の結果から，TIc の反応部位は Lys-Ser 結合ではなく，ナス外皮のインヒビター同様 Arg(38)—Asn(39)結合であることが推定された。