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ISOLATION AND CHARACTERIZATION OF POLYPEPTIDE PROTEINASE INHIBITORS FROM POTATO TUBERS.

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Abstract

Nine peptides having proteinase inhibitor activity were isolated from potato tubers by gel filtration through Sephadex G-50 and CM-cellulose chromatography. Five of them were designated as polypeptide trypsin inhibitors a, b, c, d and e (TIa, TIb, TIc, TId and TIe), and the others as polypeptide chymotrypsin inhibitors a, b, c and d (CIa, CIb, CIc and CId). TIc, CIa and CId were characterized in terms of their chemical and physicochemical properties and specificities against enzymes. Each inhibitor had a single polypeptide chain consisted of 50 amino acid residues. TIc and CId had arginine (mol/mol) at their amino-termini, and CIa proline (mol/mol). The carboxyl-terminal residues of the three inhibitors were arginine (mol/mol, each). TIc showed stoichiometric inhibition of trypsin, and it also inhibited chymotrypsin and subtilisin but weakly. Whereas, CIa and CId stoichiometrically inhibited chymotrypsin and subtilisin, but had little or no effect on trypsin.

Introduction

Many types of serine-proteinase inhibitors have been isolated from potato tubers.[2,3,9-11,13,20,21,23] In particular, the inhibitors with high contents of cystine residues (inhibitors of Type II) have been isolated in multiple forms by several workers.[2,3,9,11,13,23] This type of inhibitors can be classified into two groups in terms of their molecular weights, namely the inhibitors with molecular weights of about 10,000 and those with molecular weights of about 5,000. Four of the latter group of inhibitors (polypeptide inhibitors) have been first isolated by Sawada *et al.*[23] A polypeptide chymotrypsin inhibitor (MW=5,400) was next isolated and characterized by Hass *et al*,[9] We have also found nine polypeptide inhibitors in the course of the purification of potato proteinase inhibitors IIa and IIb (PPI-IIa and IIb, MW=10,500, each).[13,14] On the other hand, in our earlier studies, the active fragments of PPI-IIa and IIb(MW = 4,800 and 4,300, respectively) were prepared by prolonged incubation of the parent inhibitors with an excess quantity of trypsin.[15,16] Therefore, our interest has been focussed on the structural relationships among the artificially-prepared active fragments and the naturally-occurring inhibitors of type II with different molecular weights. In this connection, we have attempted to investigate the properties and structures of the nine naturally-occurring polypeptide inhibitors. In this paper, we will describe the purification procedure of the inhibitors and some properties of three main inhibitors.

Abbreviations: PTH, phenylthiohydantoin. SDS, sodium dodecyl sulfate.

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Experimental Procedure

Materials —— Japanese potatoes (Danshaku variety) were obtained from commercial source. Twice-crystallized bovine trypsin and α-chymotrypsin were purchased from Worthington Biochemical Co., and subtilisin BPN' from Sigma Chemical Co. Carboxypeptidases A and B were from Worthington. Hammarsten's casein was

obtained from E. Merck AG. Bacitracin and cytochrome c were from Schwarz/Mann Laboratories, Inc. All other chemicals used were of reagent or analytical grade.

Assay of Proteinase Inhibitor Activity —— This was determined by the casein-digestion method at 30°C and pH 7.6 according to Hagihara.[8] One unit of inhibitor activity was defined as the 50% inhibition of the activity of 2μg of enzyme.

Detection of Proteins and Polypeptide Inhibitors——Proteins and polypeptide inhibitors in column effluents were detected by measuring the absorbance at 278 nm by use of a Hitachi UV-VIS spectrophotometer, model 139.

DISC Electrophoresis —— This was carried out on polyacrylamide gel (7%) column at pH 8.3 and 5 mA per one column for 1 hr, according to Davis.[5] Coomassie Brilliant Blue R-250 was used as stain.

Determination of Molecular Weight —— Molecular weights of inhibitors were determined by SDS polyacrylamide gel electrophoresis.[25] The concentration of polyacrylamide was 15%, and gels contained 0.1% SDS and electrophoresis was performed at 8 mA per one column. The references used were cytochrome c (MW = 12,400), PPI-IIb (MW = 10,500)[14], the active fragment of PPI-IIb (MW = 4,300)[15] and bacitracin (MW = 1,450).

Amino Acid Analysis —— Samples were hydrolyzed with redistilled HCl *in vacuo* at 110°C for 24 hr. A Hitachi automatic amino acid analyzer, model KLA-3B, was used for the determination of amino acid according to Spackman *et al.*[24] Half-cystine content was analyzed as S-carboxymethylcysteine (CM-cysteine) after reduction and alkylation of samples by the method of Crestfield *et al.*[4] Tryptophan content was analyzed spectrophotometrically according to Goodwin and Morton.[7]

Calculation of the Dissociation Constants for Enzyme-Inhibitor Complexes——Approximate dissociation constants for the equilibrium EI = E+I (E=enzyme and I=inhibitor) were calculated according to Green and Work.[6]

Determination of Terminal Sequences —— Amino-terminal sequences were determined by the Edman degradation method.[12] The identification of PTH-derivatives of amino acids were performed by thin-layer chromatography using solvent II and V.[12] The PTH-derivative of arginine was identified by Sakaguchi reaction. Carboxyl-terminal residues were determined by the carboxypeptidase digestion method.[1] Asparagine and serine released by the digestion were finally identified as follows: Amberlite IR-120B was added into a tube containing the sample solution after carboxypeptidase digestion, and the tube was shaken for 20 min. The resin was washed with distilled water and then the amino acids adsorbed were extracted with 5N NH_4OH. The extract was next evaporated to dryness and subjected to hydrolysis with 2 N HCl at 110°C for 3 hr, and then to amino acid analysis.

Results

Preparation of Crude Inhibitors——Ten kg of potato tubers were cut to small pieces and ground by a juicer with 10 liters of 2% NaCl. The resulting extract was allowed to stand for one hr at room temperature. The sediments were filtered off using cheese cloth and the filtrate was heated at 80°C for 10 min. The precipitate formed was removed by filtrating through hyflosupercel and solid ammonium sulfate was added to the filtrate to give 60% saturation. After standing overnight, the precipitate formed was collected by centrifugation and dissolved in water and dialyzed aganst running water for 2 days. The insoluble materials occurred were removed by centrifugation and a sufficient amount of 2 % acrinol solution was added to the supernatant to precipitate some impurities. The precipitate formed was centrifuged off and a sufficient volume of cold acetone was added to the supernatant to precipitate crude inhibitors.

Isolation of Polypeptide Proteinase Inhibitors—— The crude inhibitors obtained above were collected by centrifugation, and dissolved in a small volume of 0.02 M sodium acetate buffer, pH 4.0. The insoluble materials were removed by centrifugation and then the supernatant was applied to a Sephadex G-50 column (2.8×135cm) previously equilibrated with the same buffer. Elution was carried out with the same buffer. A typical elution Profile is given

in Fig. 1, yielding four fractions (P1, P2, P3 and P4). Fraction P2 contained PPI IIa and IIb, and fraction P3 exhibited stronger activities than fraction P2 against both trypsin and chymotrypsin. This indicated that fraction P3 contained some inhibitors having lower molecular weights than those of PP-IIa and IIb. Fraction P4 contained acetone. Fraction P3, indicated by a bar, was collected and refiltered through Sephadex G-50 with 0.02 M sodium acetate buffer, pH 4.0 (Fig. 2).

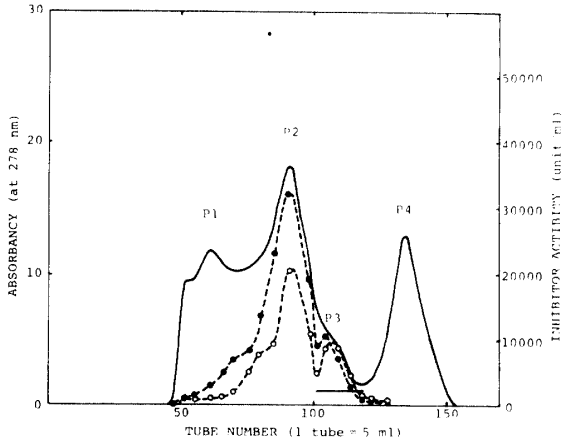

Fig. 1. Gel filtration of crude inhibitors through Sephadex G-50. Elution was carried out with 0.02M sodium acetate buffer, pH 4.0. Column size was 2.8×135 cm.
———, absorbancy at 278 nm:
○, inhibitor activity against trypsin:
●, inhibitor activity against chymotrypsin.

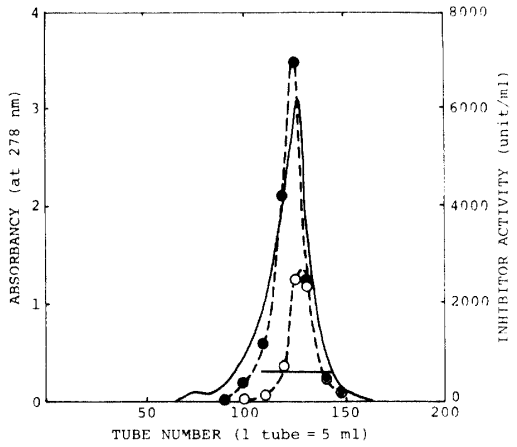

Fig. 2. Refiltration of the polypeptide proteinase inhibitors through Sephadex G-50.
Conditions were the same as in Fig. 1.
———, absorbancy at 278 nm:
○, inhibitor activity against trypsin:
●, inhibitor activity against chymotrypsin.

Purification of Polypeptide Proteinase Inhibitors by CM-Cellulose Chromatography ——The low molecular weight inhibitor fraction obtained above was applied to a CM-cellulose column (1.6×28cm) which had been previously equilibrated with 0.02 M sodium acetate buffer, pH 4.0. Elution was first carried out with the same buffer and subsequently with a linear gradient to 0.4 M sodium acetate. As given in Fig. 3, ten peaks with inhibitor activity were detected.

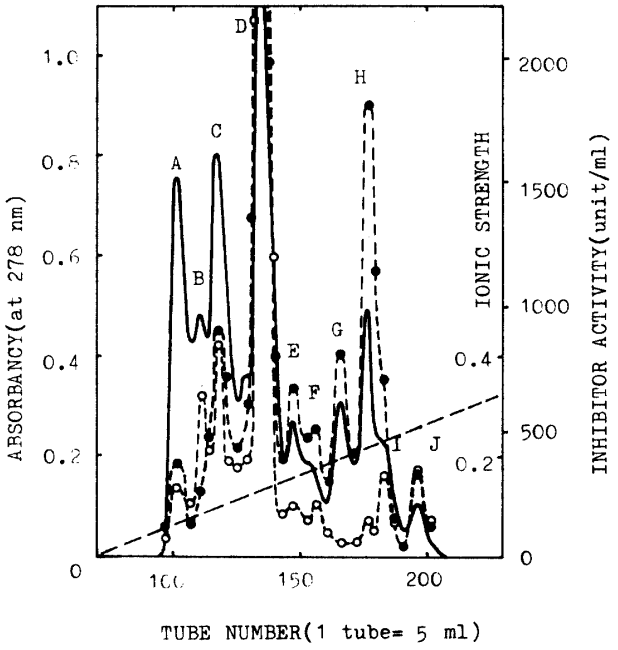

Fig. 3. CM-cellulose chromatography of the polypeptide proteinase inhibitors.
Elution was first carried out with 400 ml of 0.02 M sodium acetate buffer, pH 4.0, and then with a linear gradient to 0.4 M sodium acetate at a flow rate of 30 ml/hr. Column size was 1.6×28 cm.
———, absorbancy at 278 nm:
○, inhibitor activity against trypsin:
●, inhibitor activity against chymotrypsin.

The inhibitor activity of fraction A was lower than those of other fractions, indicating that this fraction contained some impurities and/or higher molecular weight of inhibitors. Fractions B, C, D, I and J had strong inhibitor activities against both trypsin and chymotrypsin, and fractions E, F, G and H showed strong activity against chymotrypsin but very weak or no activity against trypsin. Solid ammonium sulfate

was added to the solutions of these nine strong inhibitor fractions to give 60% saturation, respectively. Each precipitate was dissolved in 0.1 M acetic acid and desalted by gel filtration through Sephadex G-25 using 0.1 M acetic acid. The inhibitor fractions thus obtained were lyophilized. Preparations B, C, D, I and J were designated as polypeptide trypsin inhibitors a, b, c, d and e (TIa, TIb, TIc, TId and TIe), and preparations E, F, G and H as polypeptide chymotrypsin inhibitors a, b, c and d (CIa, CIb, CIc and CId), respectively.

Rechromatography of Polypeptide Proteinase Inhibitors on CM-Cellulose——The lyophilized preparations of TIc, CIa, and CId, which were obtained comparatively in good yields, were rechromatographed on CM-cellulose for further purification. The three preparations were dissolved in 0.02 M ammonium acetate buffer, pH 4.4, and applied to a CM-cellulose column (1.6 × 20 cm) equilibrated with the same buffer, respectively. Elutions were carried out with a linear gradient to 0.15 M NH_4OH (Fig. 4). The

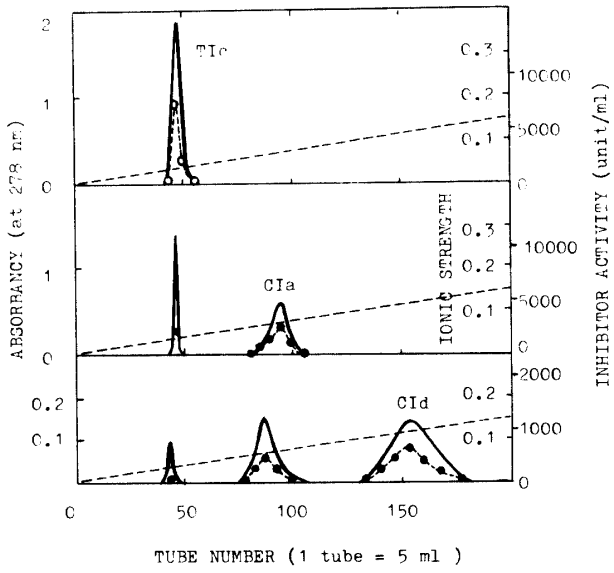

Fig. 4. Rechromatography on CM-cellulose of the polypeptide proteinase inhibitors.
Elution was first carried out with 300 ml of 0.02 M ammonium acetate buffer, pH 4.4, and then with a linear gradient to 0.15 M NH_4OH. Flow rate was 30 ml/hr and column size was 1.6 × 20 cm.
———, absorbancy at 278 nm:
○, inhibitor activity against trypsin;
●, inhibitor activity against chymotrypsin.

main fraction of each inhibitor was collected and lyophilized. The yields of TIc, CIa and CId were about 20 mg, 10 mg and 10 mg per 10 kg of potato tubers, respectively.

Homogeneities of Polypeptide Proteinase Inhibitor Preparations —— The three lyophilized inhibitor preparations obtained above were examined for the homogeneity by disc electrophoresis. Only a single band was observed in each case, indicating that each inhibitor preparation was essentially homogeneous (Fig. 5).

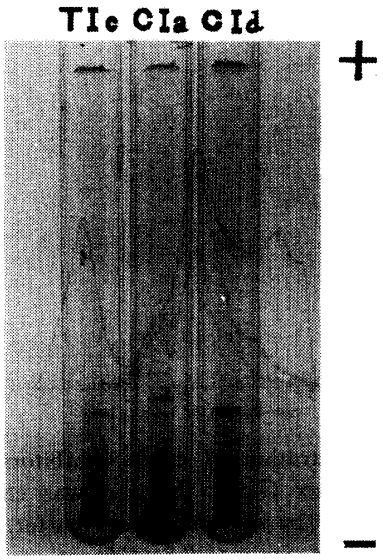

Fig. 5. Disc electrophoresis of the polypeptide proteinase inhibitors.
Conditions used are given in "EXPERIMENTAL PROCEDURE".

Molecular Weights of Polypeptide Proteinase Inhibitors —— The molecular weights of TIc, CIa and CId were determined by means of SDS polyacrylamide gel electrophoresis under the condition described in "EXPERIMENTAL PROCEDURE". The Rf values for reference proteins were plotted against the logarithms of their molecular weights. The Rf values for the three inhibitors all corresponded to a molecular weight of approximately 5,300 (Fig. 6). The homogeneities of the inhibitor preparations were again recognized by this procedure.

Amino Acid Compositions of Polypeptide Proteinase Inhibitors —— Table I shows the amino acid compositions of TIc, CIa and CId. The three inhibitors all consisted of 50 amino

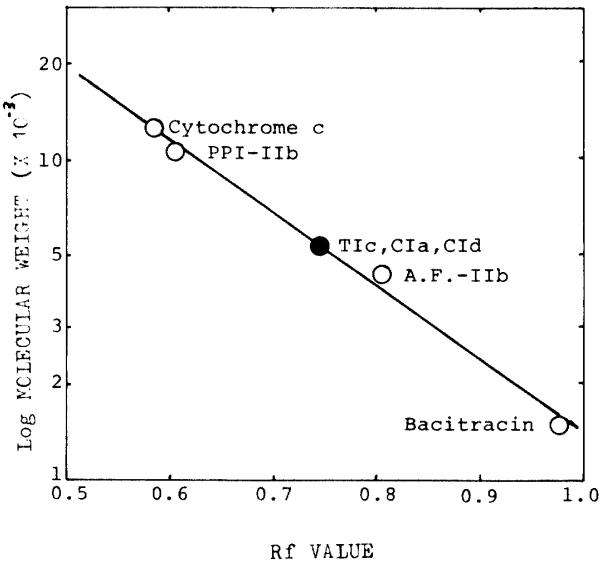

Fig. 6. Determination of the molecular weights of the polypeptide proteinase inhibitors by SDS polyacrylamide gel electrophoresis.
Conditions are given in "EXPERIMENTAL PROCEDURE".

Table I. Amino acid compositions[a] and molecular weights of polypeptide proteinase inhibitors.

Amino acid	TIc	CIa	CId
Aspartic acid	8.79 (9)	6.13 (6)	5.96 (6)
Threonine	1.62 (2)	0.89 (1)	0.91 (1)
Serine	2.42 (3)	2.79 (3)	2.58 (3)
Glutamic acid	2.27 (2)	2.06 (2)	2.08 (2)
Proline	3.43 (3)	6.12 (6)	5.25 (5)
Glycine	4.00 (4)	3.89 (4)	3.81 (4)
Alanine	3.72 (4)	4.84 (5)	4.73 (5)
Half-cystine[b]	6.93 (7)	7.43 (7)	6.80 (7)
Valine	1.13 (1)	0 (0)	0 (0)
Methionine	0 (0)	0 (0)	0 (0)
Isoleucine	2.79 (3)	3.02 (3)	2.86 (3)
Leucine	1.00 (1)	1.00 (1)	1.00 (1)
Tyrosine	3.64 (4)	3.76 (4)	3.69 (4)
Phenylalanine	0.90 (1)	0.96 (1)	0.95 (1)
Lysine	3.02 (3)	4.73 (5)	4.99 (5)
Histidine	0 (0)	0.96 (1)	0.98 (1)
Arginine	2.76 (3)	1.09 (1)	1.95 (2)
Tryptophan[c]	0 (0)	0 (0)	0 (0)
Total	50	50	50
Molecular weight	5,400	5,400	5,500

a) Expressed on the basis of 1 leucine.
b) Determined as S-carboxymethylcysteine.
c) Determined spectrophotometrically.[7]

acid residues. They did not contain any methionine and tryptophan residues, further the chymotrypsin inhibitors lacked valine and the trypsin inhibitor histidine. The high contents of aspartic acid and half-cystine residues were noticed in each inhibitor. Molecular weights of 5,400 for TIc and CIa, and 5,500 for CId were obtained when calculated from the amino acid compositions. These values are in good agreement with the values obtained by SDS electrophoresis.

Enzyme Inhibition Profiles of Polypeptide Proteinase Inhibitors——Fig. 7. shows the inhibitions of trypsin, α-chymotrypsin and subtilisin BPN' by increasing amounts of each inhibitor. One mol of TIc inhibited approximately one mol of trypsin, this inhibitor also weakly inhibited

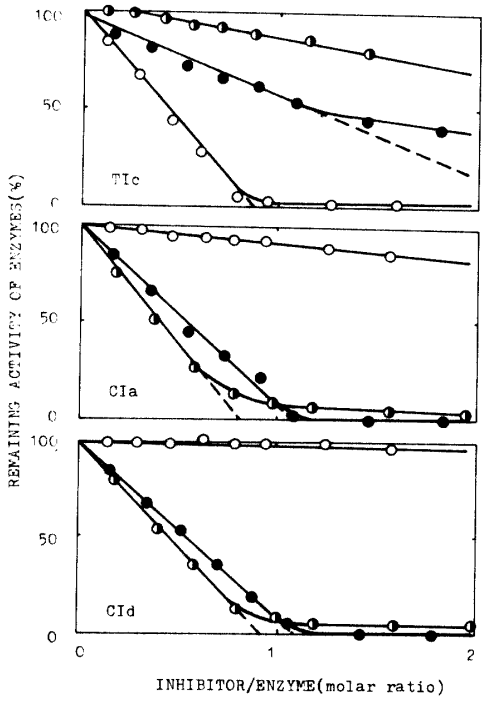

Fig. 7. Enzyme-inhibition profiles by the polypeptide proteinase inhibitors.
○, trypsin ; ●, chymotrypsin ;
◑, subtilisin.

Table II. Dissociation constants of the complexes of polypeptide proteinase inhibitors with enzymes.

Enzyme	TIc	CIa	CId
Trypsin	1.7×10^{-9}M	——	——
Chymotrypsin	1.7×10^{-7}M	3.9×10^{-10}M	4.0×10^{-10}M
Subtilisin	——	4.7×10^{-8}M	1.2×10^{-8}M

chymotrypsin and subtilisin. On the contrary, CIa and CId inhibited chymotrypsin and subtilisin at a molar ratio of 1/1, but had little or no effect on trypsin. The dissociation constants for the complexes of the inhibitors with enzymes are listed in Table II.

Terminal Sequences of Polypeptide Proteinase Inhibitors —— For the determination of carboxyl-terminal sequence, approximately 0.14 μmol of each inhibitor was digested with carboxypeptidases A and B at a molar ratio of 1/50 (enzyme/inhibitor). From TIc, 0.41 mol/mol of arginine and 0.17 mol/mol of leucine were released after digestion at 30°C and 3 hr. No detectable amino acid was released from CIa and CId under the condition. Next, the reduced and S-carboxymethylated preparation of each inhibitor was digested with carboxypeptidases A and B at a molar ratio of 1/100 (enzyme/inhibitor) at 30°C for 3 hr. Approximately 1 mol/mol of arginine and 0.6 mol/mol of leucine and smaller amounts of several amino acids were released from TIc after the digestion. This indicated that TIc had -Leu-Arg sequence at its carboxyl-terminus. From both of CIa and CId, arginine was released as the largest amount of amino acid, and serine as the next. This serine residue was identified by the procedure described in "EXPERIMENTAL PROCEDURE". Therefore, CIa and CId seemed to have Ser-Arg sequence at their carboxyl-termini. On the other hand, the amino-terminal sequences of the inhibitors were determined by Edman degradation. The reduced and S-carboxymethylated samples were used for the analysis. The amino-terminal sequence of TIc was determined tobe Arg-Ile-Cys-Thr-Asn-, and those of CIa and CId were Pro-Ile-Cys-Thr-Asn- and Arg-Ile-Cys-Thr-Asn-, respectively.

Discussion

The nine polypeptide proteinase inhibitors isolated in this study were found in the course of purification of PP-IIa and IIb. When the heat-stable protein fraction of potato tubers were passed through a Sephadex G-50 column, a small peak with strong inhibitor activity was obtained behind the peak containing PPI-IIa and IIb (Fig. 1). This fraction contained five polypeptide trypsin inhibitors (TIa, TIb, TIc, TId and TIe) and four polypeptide chymotrypsin inhibitors (CIa, CIb, CIc and CId), which were isolated by CM-cellulose chromatography (Fig. 3). In the present study, the yields of these inhibitors were very small, but larger yields could be expected if the dialysis procedure at an early step of purification was excluded. The purified preparations were found to be dialyzed out into water. This is in agreement with their low molecular weights which were about 5,500. The molecular weights of the polypeptide proteinase inhibitors were a little larger than those of the artificially-prepared active fragments of PPI-IIa and IIb (4,800 and 4,300, respectively),[15,16] but the enzyme inhibition profile of the purified TIc was similar to that of the active fragment of PPI-IIa, and also those of the purified CIa and CId to that of the active fragment of PPI-IIb. These similarities seem to suggest that the polypeptide proteinase inhibitors are closely related to PPI-IIa and IIb.

Judging from the chromatographic patterns and properties, the polypeptide inhibitors resemble the four inhibitors isolated by Sawada *et al*[23]., the polypeptide chymotrypsin inhibitor of Hass *et al.*,[9] inhibitor A5 of Belitz *et al.*[2] and the eggplant exocarp inhibitors isolated by Kanamori *et al.*[19,26] and Richardson.[22] In Table III, the amino acid compositions of TIc, CIa and CId are compared with those of the inhibitors mentioned above.

The amino acid composition of TIc somewhat resembles those of the active fragment of PPI-IIa, inhibitor A5 and the eggplant exocarp trypsin inhibitor. On the other hand, the amino acid compositions of CIa and CId are very similar to those of the chymotrypsin inhibitor of Hass *et al.* and the active fragment of PPI-IIb. CIa and CId consist of 50 amino acid residues, and CIa has one more proline residue and one less arginine residue than CId. The numbers of other amino acid residues of the two inhibitors are identical, therefore, it is probable that the amino-terminal proline residue of CIa is replaced by an arginine residue in CId molecule. The similarity among the amino acid compositions of the polypeptide inhibitors and the active

Table III. Comparison of the amino acid compositions of the polypeptide proteinase inhibitors with those of the active fragments of PPI-IIa and IIb, inhibitor A5, the inhibitor of eggplant exocarp and the inhibitor of Hass *et al.*

Amino acid	TIc	A. F. of PPI-IIa	inhibitor A5[a]	inhibitor of eggplant exocarp[b]	CIa	CId	A. F. of PPI-IIb	inhibitor of Hass *et al.*[c]
Aspartic acid	9	8	7	7(6)	6	6	6	6
Threonine	2	2	3	2	1	1	1	1
Serine	3	4	4	4	3	3	2	3
Glutamic acid	2	4	5	4	2	2	2	2
Proline	3	2	3	4	6	5	4	6
Glycine	4	4	7	6	4	4	3	4
Alanine	4	2	3	3	5	5	3	5
Half-cystine	7	6	7	7(8)	7	7	6	6
Valine	1	1	1	0	0	0	0	0
Methionine	0	0	0	0	0	0	0	0
Isoleucine	3	2	2	4	3	3	2	3
Leucine	1	1	2	1	1	1	1	1
Tyrosine	4	3	4	2	4	4	3	4
Phenylalanine	1	1	2	2	1	1	1	1
Lysine	3	3	5	3	5	5	4	5
Histidine	0	0	0	0	1	1	1	1
Arginine	3	2	2	3	1	2	1	1
Tryptophan	0	0	0	0	0	0	0	0
Total	50	45	57	52	50	50	40	49

a) Calculated from data of Belitz *et al.*[2]
b) Cited from data of Richardson.[22]
c) Cited from data of Hass *et al.*[9]

TIc	1 Arg—Ile—Cys—Thr—Asn.....	50 Leu—Arg
CIa	1 Pro —Ile—Cys—Thr—Asn.....	50 Ser—Arg
CId	1 Arg—Ile—Cys—Thr—Asn.....	50 Ser—Arg
Active fragment of PPI-IIa	8 -Arg—Ile—Cys—Thr—Asn.....	45 Leu—Arg
Active fragment of PPI-IIb	1 Ile—Cys—Thr—Asn.....	40 Pro—Arg
Inhibitor of Hass *et al.*	1 Pro—Ile—Cys—Thr—Asn.....	

Fig. 8. Comparison of the terminal sequences of the polypeptide proteinase inhibitors with those of the active fragments of PPI-IIa and IIb and the inhibitor of Hass *et al.*

fragments of PPI-IIa and IIb again indicates the homology of the polypeptide inhibitors to PPI-IIa and IIb.

In Fig. 8, the terminal amino acid sequences of TIc, CIa and CId are compared with those of the active fragments of PPI-IIa[17] and IIb[18] and that of the inhibitor of Hass *et al.*[9]

The amino-terminal sequences of TIc and CId (residues 1 through 5) are identical with the sequence of residues 8 through 12 of the active fragment of PPI-IIa, and the amino-terminal sequence (residues 1 through 5) of CIa is iden-

tical with that of the inhibitor of Hass *et al.* In consideration with the amino acid compositions (Table III), CIa and the inhibitor of Hass *et al.* are possibly identical. The sequences of residues 2 through 5 of all inhibitors compared (for the active fragment of PPI-IIb, residues 1 through 4) are identical with each other, indicating the close relationships of these inhibitors. Among them, TIc and the active fragment of PPI-IIa are strong trypsin inhibitors, and they have an identical carboxyl-terminal sequence (-Leu-Arg). TIc lacks the amino-terminal seven residues of the active fragment of PPI-IIa, though, as a whole, it has five more residues than the active fragment (Table III). These data indicate some differences between the inner sequences of TIc and the active fragment.

On the other hand, the strong chymotrypsin inhibitors, CIa, CId, the active fragment of PPI-IIb and the inhibitor of Hass *et al.*, have very similar amino-terminal sequences with each other, but the naturally-occurring polypeptide chymotrypsin inhibitors have ten or nine more amino acid residues than the active fragment of PPI-IIb. This indicates that the active fragment has a different inner sequence from those of the polypeptide inhibitors. For further elucidation of the relationships between the polypeptide proteinase inhibitors and PPI-IIa and IIb, it is required to determine the complete amino acid sequences and the reactive sites for enzymes of the polypeptide inhibitors to compare them with those of the active fragment of PPI-IIa[17] and IIb.[18]

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バレイショのポリペプチド性プロティナーゼインヒビターの分離と性質について

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要　約

バレイショの塊茎から，セファデックスG-50ゲル沪過とCM-セルロースクロマトグラフィーによって，5種のポリペプチド性トリプシンインヒビター（TI_a，TI_b，TI_c，TI_d 及び TI_e）と4種のポリペプチド性キモトリプシンインヒビター（CI_a，CI_b，CI_c 及び CI_d）を分離した。これらの中，TI_c，CI_a 及び CI_dについて化学的並びに物理化学的性質や酵素に対する阻害特異性を調べた。三者とも約50個のアミノ酸から成る1本のペプチド鎖を有し，TI_c と CI_d のN末端はアルギニン，又 CI_a のそれはプロリンであった。C 末端は三者ともアルギニンであった。TI_c はトリプシンを化学量論的に阻害したが，キモトリプシンやズブチリシンに対する阻害活性は弱かった。CI_a と CI_d はともにキモトリプシンのみならず，ズブチリシンも化学量論的に阻害したが，トリプシンは殆んど阻害しなかった。