

Purification and Characterization of Three Trypsin Inhibitors from Beans, *Phaseolus vulgaris* 'Kintoki'

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ABSTRACT

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At least five trypsin inhibitors exist in kintoki beans, and we purified three of these by chromatofocusing. The three inhibitors, all of which lacked carbohydrate, were characterized by high contents of aspartic acid, serine, and cystine and by the absence of tryptophan. Inhibitors 2 and 4 formed a 1:1 enzyme-inhibitor complex with trypsin and chymotrypsin. Modification with trinitrobenzenesulfonic acid of the amino groups in inhibitors 2 and 4 caused nearly complete loss of trypsin inhibitory activity, but chymotrypsin inhibitory activity was not affected. The ternary complex

among trypsin, inhibitor, and chymotrypsin, demonstrated by chromatography, indicated two distinct sites for trypsin and chymotrypsin in both inhibitors 2 and 4. Inhibitor 5 inhibited only trypsin. The result of modifications with trinitrobenzenesulfonic acid and glyoxal indicated that arginine residue is essential for trypsin inhibitory activity of inhibitor 5. The activity of inhibitors 2, 4, and 5 was not affected by heating (100°C, 60 min), exposure to low pH (pH 2), or reaction with pepsin.

Since Kunitz first isolated trypsin inhibitor in soybeans (Kunitz 1947), proteinase inhibitors have been purified from several sources, particularly legume seeds (Liener et al 1980, Vogel et al 1968).

Legume proteinase inhibitors generally have low molecular weight (10,000 or less) and multiple forms. Soybeans, *Glycine max*, contain Kunitz, Bowman-Birk, F₁, and F₃ inhibitors, among others (Birk et al 1963, Frattali and Steiner 1968, Kunitz 1947). Six inhibitors have been purified from lima beans, *Phaseolus lunatus* (Haynes and Feeney 1967), three from green beans, *Phaseolus vulgaris* (Wilson and Laskowski 1973), and two from groundnuts, *Arachis hypogaea* (Hochstrasser et al 1969).

We previously reported the behavior of trypsin inhibitors in kintoki beans during heat treatment (Tsukamoto et al 1983). Kintoki (the Japanese term for red) beans are a subspecies of kidney beans (*Phaseolus vulgaris* L.). Additional information on the nutritional significance of trypsin inhibitors is needed, particularly in such important dietary components as legumes. We purified the iso-inhibitors of kintoki beans, utilizing chromatofocusing, and investigated how their molecular mechanisms encourage inhibitory function.

MATERIALS AND METHODS

Kintoki beans, *P. vulgaris* Kintoki, were obtained from Hokuren Co., Hokkaido, Japan. Trypsin (twice crystallized, type II) and α -chymotrypsin from bovine pancreas (crystallized three times) were purchased from Sigma Chemical Co. Sephadex G-25, Sephadex G-100, Polybuffer™74 and PBE™94 were purchased from Pharmacia Fine Chemicals. Bio-Gel P-200 and diethylaminoethyl (DEAE) cellulose were obtained from Bio-Rad Laboratories and Whatman Ltd., respectively. All other reagents were analytical grade.

Purification of the Inhibitors

All procedures were performed at 4–5°C.

Extraction of the Inhibitors. Finely ground beans were suspended in 10 volumes (w/v) of H₂O, and pH of the solution was adjusted to 5.3 with 6M HCl. After standing for 30 min, the suspension was centrifuged, and solid ammonium sulfate was added to the supernatant to 30% saturation at pH 6.8. After centrifugation, the supernatant was brought to 70% saturation with ammonium sulfate. The precipitate collected by centrifugation was dissolved in 40 ml of H₂O and dialyzed overnight against H₂O. Precipitation with 70% saturation of ammonium sulfate and dialysis were repeated after the inactive precipitate was removed by

centrifugation.

Gel Filtration on Sephadex G-100. The dialyze was chromatographed on a column of Sephadex G-100 (7×95 cm) that had been equilibrated with 50 mM Tris-HCl-buffer, pH 7.0. Fractions showing the inhibitory activity were pooled and brought to 70% saturation with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 ml of 5 mM sodium acetate buffer, pH 6.0. The solution was dialyzed overnight against the buffer.

Chromatography on DEAE Cellulose. The dialyze was applied to a column of DEAE cellulose (1.8×30 cm) equilibrated with 50 mM sodium acetate buffer, pH 6.0. The column was washed with 120 ml of the equilibration buffer, and the inhibitors were eluted by a linear gradient of NaCl from 0 to 0.2 M in the buffer of 300 ml each. The fractions of each inhibitor were pooled separately and brought to 70% saturation with ammonium sulfate.

Chromatofocusing. Each precipitate was collected by centrifugation and passed through a column of Sephadex G-25 (1.5×15 cm) equilibrated with 25 mM piperazine-HCl buffer, pH 5.5. Then the protein sample was applied to a column of PBE™94 (0.8×25 cm) equilibrated with the buffer. The inhibitor was eluted by Polybuffer™74 solution, pH 4.0–3.5, diluted 10 times. One-milliliter fractions were collected at a flow rate of 6 ml/hr. Absorbance at 280 nm, pH, and trypsin inhibitory activity on fractions were determined. Aliquots of the effluent fractions were analyzed by polyacrylamide gel electrophoresis (PAGE). The inhibitor fractions showing a single band on polyacrylamide gel were pooled and brought to 70% saturation with ammonium sulfate. The precipitate was passed through a column of Sephadex G-25 (1.5×15 cm) equilibrated with 50 mM ammonium bicarbonate and lyophilized.

Enzyme and Enzyme Inhibition Assays

Trypsin. A substrate of *N*-benzoyl-DL-arginine-*p*-nitroanilide was used to determine trypsin activity according to the method of Ehrlanger et al (1961). The concentration of trypsin on an active site was determined by titration with *p*-nitrophenyl-*p*'-guanidinobenzoate, according to the method of Chase and Shaw (1967). Inhibitor concentration was determined by the method of Lowry et al (1951). Inhibitory activities are expressed here as milligrams of active trypsin inhibited per milligram of inhibitor preparation.

Chymotrypsin. *N*-benzoyl-L-tyrosine-*p*-nitroanilide as substrate was used to measure chymotrypsin activity. The concentration of chymotrypsin was determined by titration with *N*-*trans*-cinnamoylimidazole according to the method of Schonbaum et al (1961). Chymotrypsin inhibitory activities were expressed as milligrams of inhibited active chymotrypsin per milligram of inhibitor preparation.

¹Deceased on August 16, 1981.

Amino Acid Analysis

The amino acid analysis was performed on an autoanalyzer according to a standard procedure (Spackman et al 1958). Values for threonine, serine, and tyrosine were corrected for destruction during hydrolysis, at 110°C for 24, 48, and 72 hr. Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs 1956).

Modification of Inhibitors 2, 4, and 5 with Trinitrobenzenesulfonic Acid (TNBS)

Inhibitors 2, 4, and 5 were modified with TNBS according to the method of Haynes et al (1967). We added 0.25 ml of 4% NaHCO₃, pH 8.5, and 0.25 ml of 0.1% TNBS in H₂O to 0.25 ml of protein solution (1.5 mg/ml). After the solution was incubated at 40°C for 5–120 min, 150 μl was withdrawn and used for the determinations of trypsin and chymotrypsin inhibitory activities. The remaining solution was used for the determination of the number of the modified amino groups after addition of 0.2 ml of 10% (w/v) sodium dodecyl sulfate and 0.1 ml of 1M HCl. The number of modified amino groups in a protein was calculated from the absorbance at 340 nm against a control treated as above but containing H₂O instead of the protein, using a molar extinction coefficient of $1.09 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ with lysozyme as a standard.

TABLE I
Initial Purification Procedure^a

| Steps | Protein (g) | Trypsin Inhibited (mg) | Trypsin Inhibited per Milligram of Protein (mg) | Recovery (%) |
|----------------------------------|-------------|------------------------|---|--------------|
| Extract | 5.6 | 950 | 0.17 | 100 |
| 30–70% Ammonium sulfate fraction | 2.0 | 675 | 0.34 | 71 |
| 70% Ammonium sulfate precipitate | 1.1 | 532 | 0.48 | 56 |
| Sephadex G-100 | 0.36 | 436 | 1.21 | 46 |
| DEAE-cellulose | 0.12 | 238 | 1.98 | 25 |
| Fractions | | | | |
| A | 0.029 | 60.9 | 2.10 | 6 |
| B | 0.026 | 55.8 | 2.13 | 6 |
| C | 0.037 | 86.0 | 2.33 | 9 |
| D | 0.029 | 35.3 | 1.21 | 4 |

^aStarting material was 150 g of bean powder.

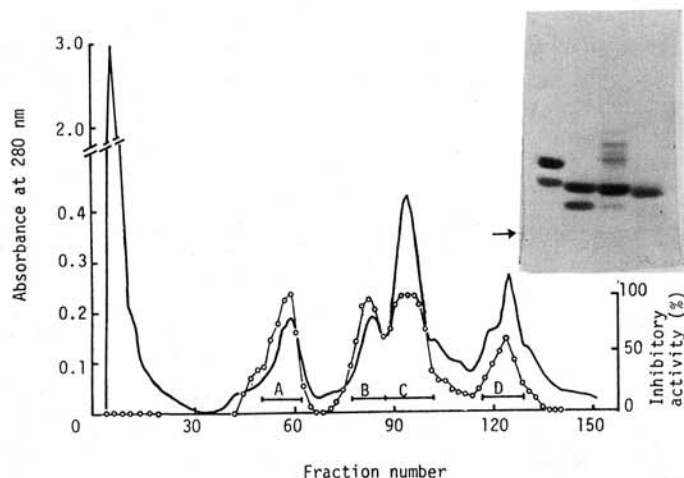


Fig. 1. Ion-exchange chromatography of the crude inhibitor from gel filtration on DEAE-cellulose. Each peak was separately pooled as indicated. Inset shows the PAGE patterns of 20-μl samples of the indicated pools A, B, C, and D (left to right). — = the position of the bromphenol blue tracking dye; — = absorbance at 280 nm; o—o = trypsin inhibitory activity.

Modification of Inhibitor 5 with Glyoxal

The protein solution (0.52 mg/ml) was incubated at 20°C for 30, 60, 120, and 180 min with 12.4 mM glyoxal in 0.3M bicarbonate buffer, pH 8.6. After each interval, 0.5-ml aliquots were withdrawn and applied on a Sephadex G-25 column (1 × 15 cm) equilibrated with 5 mM Tris-HCl buffer, pH 8.0. The protein fractions collected were used for the determinations of protein content, inhibitory activity against trypsin, and amount of modified arginine residue. The modification of arginine residue was quantitated by amino acid analysis after acid hydrolysis at 110°C for 24 hr.

Polyacrylamide Gel Electrophoresis

Electrophoresis of protein in slabs (0.2 × 0.9 × 13.5 cm) of 7.5% polyacrylamide gel at pH 8.2 was done according to the procedure of Reid and Bielecki (1968).

Gel Filtration on Bio-Gel P-200

A mixture of inhibitor (100 μg), trypsin (250 μg), and chymotrypsin (250 μg) was dissolved in 0.3 ml of 50 mM Tris-HCl buffer, pH 8.0, and incubated 5 min at 25°C. This solution was applied to a column of Bio-Gel P-200 (0.5 × 55 cm) equilibrated with the above buffer. The column was eluted with the buffer at 4°C at a flow rate of 1.5 ml/hr, and 1-ml fractions were collected. The absorbance at 280 nm of each fraction was determined. Similarly, a mixture of inhibitor (100 μg) and trypsin (250 μg) was chromatographed on Bio-Gel P-200. The absorbance at 280 nm and the chymotrypsin inhibitory activity were determined on fractions. In separate experiments, inhibitor (580 μg) was passed through the column to determine the elution pattern.

RESULTS

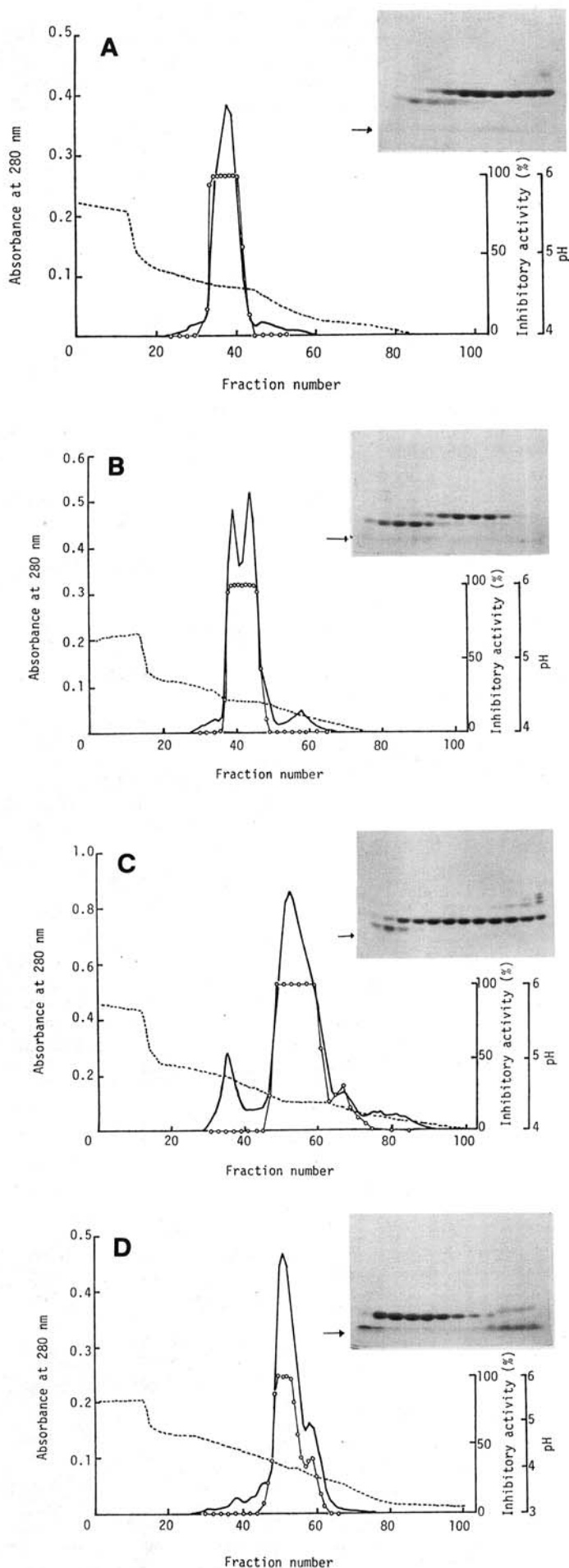
Purification of the Inhibitors

Table I summarizes the extraction and initial purification procedure. Ion-exchange chromatography on DEAE-cellulose of the crude inhibitor obtained from the gel filtration on Sephadex G-100 is shown in Fig. 1. Crude inhibitor (360 mg) obtained from gel filtration was applied on a column of DEAE-cellulose (1.8 × 30 cm) equilibrated with 50 mM acetate buffer, pH 6.0. After the column was eluted with 120 ml of the buffer, a linear gradient of NaCl in the buffer, 0–0.2M, was started (at fraction 30). The absorbance at 280 nm was measured, and 10-μl aliquots were withdrawn to determine the inhibitory activity against 20 μl of trypsin on each fraction. The activities of fractions A, B, C, and D separately pooled were 2.1, 2.1, 2.3, and 1.2 mg trypsin inhibited per milligram of protein, respectively. The PAGE patterns of these fractions were very similar under the conditions shown (Fig. 1, inset).

The inhibitor fractions from DEAE-cellulose chromatography were next subjected to chromatofocusing. The elution pattern of fraction A on chromatofocusing was shown in Fig. 2A. The main inhibitor peak was eluted at pH 4.6. The PAGE of the peak showed that fraction A contained two components that were different in the mobility on electrophoresis (Rf value). The component with a Rf value of 0.79 was designated inhibitor 1. Fractions 39–40, which showed single band with 0.70 of Rf value were pooled and designated inhibitor 2.

The chromatography of fraction B was shown in Fig. 2B. Two peaks that did not separate completely were eluted at pH 4.5 and 4.4. These proteins had almost same inhibitory activity but significantly different electrophoretic mobility. The component eluting at pH 4.5 had an Rf value of 0.90 and was designated inhibitor 3. Fractions 44–46, which were eluted at pH 4.4, showed a single band with an Rf value of 0.81. These fractions were pooled and designated inhibitor 4.

In the chromatofocusing of fraction C (Fig. 2C), the first peak eluted at pH 4.8 and had no trypsin inhibitory activity. The second major peak, however, eluted at pH 4.4 and did have inhibitory activity. Fractions 53–55 showed a single band with an Rf value of 0.81 on PAGE (Fig. 2C, inset). The Rf value and the pH eluted on chromatofocusing, including the position eluted on DEAE-



cellulose, indicated that this component was identical to inhibitor 4 obtained from fraction B. These fractions were pooled and combined with inhibitor 4.

The peak of fraction D (Fig. 2D) was eluted at pH 4.1 and had a shoulder at pH 3.8. The PAGE demonstrated that the peak (fractions 51–54) consisted of one component with 0.83 Rf value. These fractions were pooled and designated inhibitor 5.

At least five more trypsin inhibitors in kintoki bean had very similar properties under gel and ion-exchange chromatography. Three of these were easily separated by chromatofocusing. Further work on the inhibitors was done with chromatographically and electrophoretically homogeneous materials corresponding to inhibitors 2, 4, and 5.

Molecular Composition

Table II gives results of amino acid analyses of the inhibitors. All three inhibitors had high contents of aspartic acid, serine, and half-cystine. Tryptophan was absent from all three inhibitors, as judged from the ultraviolet absorption spectra (Beaven and Holiday 1952). Valine and methionine were also absent from inhibitors 2 and 4. Inhibitor 5 was devoid of phenylalanine.

Fig. 2. Chromatofocusing patterns of fractions A, B, C, and D from DEAE-cellulose chromatography. Fractions from DEAE-cellulose chromatography were applied on a column of PBE™94 (0.8 × 25 cm) equilibrated with 25 mM piperazine-HCl buffer, pH 5.5. The column was eluted with polybuffer. The pH and the absorbance at 280 nm were determined on each fraction. Then, 10-μl aliquots were withdrawn to determine the inhibitory activity against 40 μg of trypsin. The insets showed PAGE patterns. → = the position of the bromphenol blue tracking dye; --- = pH; — = absorbance at 280 nm; o—o = trypsin inhibitory activity. Fraction A (29 mg) applied and eluted with the polybuffer, pH 4.0. PAGE was performed on 15-μl aliquots of fraction tubes 33–42. Fraction B (26 mg) was applied and eluted with the polybuffer, pH 3.8. PAGE was performed on 15-μl aliquots of fraction tubes 37–48. Fraction C (37 mg) was applied and eluted with the polybuffer, pH 3.8. PAGE was performed on 10-μl aliquots of fraction tubes 48–59. Fraction D (29 mg) was applied and eluted with the polybuffer, pH 3.5. PAGE was performed on 20-μl aliquots of fraction tubes 48–61, omitting 56 and 58.

TABLE II
Amino Acid Composition of Inhibitors 2, 4, and 5^a

| Amino Acid ^b | Inhibitors | | |
|-------------------------------|------------|--------|--------|
| | 2 | 4 | 5 |
| Aspartic acid | 13 | 14 | 17 |
| Threonine | 6 | 6 | 8 |
| Serine | 15 | 14 | 20 |
| Glutamic acid | 9 | 9 | 8 |
| Proline | 9 | 9 | 9 |
| Glycine | 3 | 2 | 3 |
| Alanine | 4 | 4 | 3 |
| Half-cystine ^c | 18 | 16 | 18 |
| Valine | 0 | 0 | 3 |
| Methionine | 0 | 0 | 4 |
| Isoleucine | 6 | 5 | 5 |
| Leucine | 3 | 2 | 3 |
| Tyrosine | 2 | 1 | 3 |
| Phenylalanine | 3 | 2 | 0 |
| Lysine | 6 | 5 | 6 |
| Histidine | 6 | 6 | 4 |
| Arginine | 3 | 3 | 4 |
| Total | 106 | 98 | 118 |
| Molecular weight ^d | 11,802 | 10,896 | 13,016 |

^a Results expressed as residues per molecule.

^b Values for serine, threonine, and tyrosine obtained by extrapolation to zero time.

^c Determined as cysteic acid after performic acid oxidation.

^d Calculated from the amino acid analyses.

The phenol-sulfuric acid test (Dubois et al 1956) showed that total carbohydrate was less than 0.1% by weight in all three inhibitors.

The molecular weights calculated from the amino acid composition for inhibitors 2, 4, and 5 were approximately 12,000, 11,000, and 13,000, respectively.

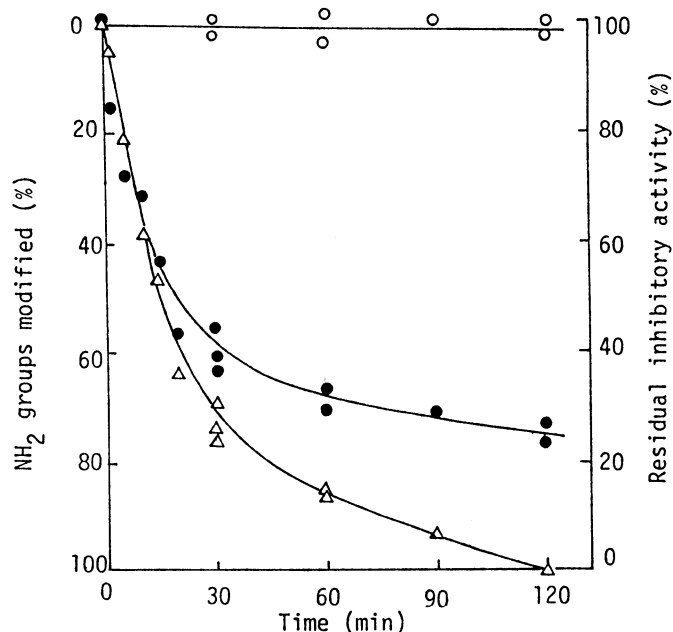


Fig. 3. Time course to modify amino groups of inhibitor 2 (375 µg) with trinitrobenzenesulfonic acid (TNBS) in a final volume of 0.75 ml and loss of inhibitory activities. The number of modified amino groups was expressed as percent detected in the sample reacted with TNBS for 2 hr. Residual inhibitory activities against trypsin and chymotrypsin were determined. Δ — Δ = modified amino groups; \bullet — \bullet = residual trypsin inhibitory activity; \circ — \circ = residual chymotrypsin inhibitory activity.

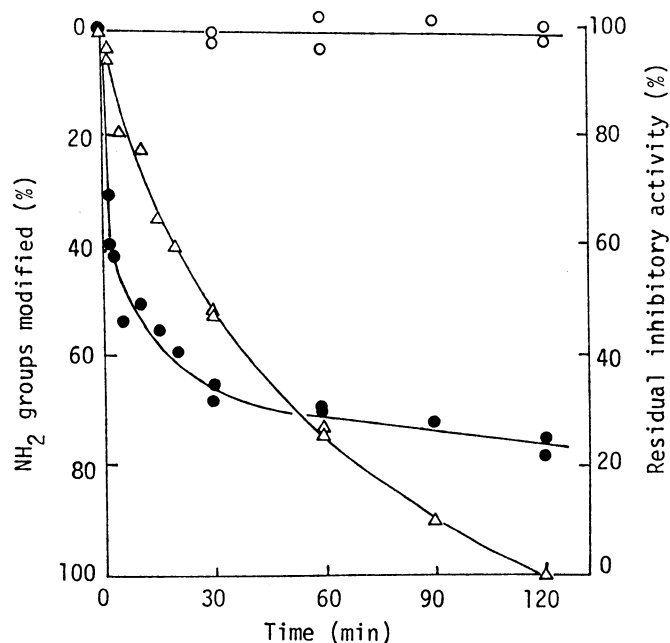


Fig. 4. Time course to modify amino groups of inhibitor 4 (375 µg) with trinitrobenzenesulfonic acid (TNBS) in a final volume of 0.75 ml and loss of the inhibitory activities. The number of the modified amino groups was expressed as percent detected in the sample reacted with TNBS for 2 hr. Residual inhibitory activities against trypsin and chymotrypsin were determined. Δ — Δ = modified amino groups; \bullet — \bullet = residual trypsin inhibitory activity; \circ — \circ = residual chymotrypsin inhibitory activity.

Enzyme Inhibitory Activity

Trypsin inhibition patterns of inhibitors 2, 4, and 5 are linear up to 80% inhibition. On the basis of calculated molecular weights, the moles of trypsin inhibited by one mole of these inhibitors were 1.18, 1.15, and 0.90, respectively. All three inhibitors thus seemed to have the same stoichiometry in the inhibition of trypsin and combined with trypsin in equimolar amount.

The inhibition of inhibitors 2 and 4 against chymotrypsin is linear up to 75%. Inhibitors 2 and 4 combined with chymotrypsin in equimolar amount. Inhibitor 5 exhibited little activity against chymotrypsin. Even after the addition of excess inhibitor 5 (eight times in weight), approximately 50% inhibition was observed, and the inhibition was linear only up to 50%.

Modification of Inhibitors 2, 4, and 5 with TNBS

Time required to modify and inactivate inhibitor 2 was investigated according to the method of Haynes et al (1967) (Fig. 3). The rate for loss of trypsin inhibitory activity (first order rate constant 0.037 min^{-1}) was nearly the same for that of the modification of the fast-reacting amino groups (0.04 min^{-1}). This suggests that one amino group was essential for the trypsin inhibitory activity of inhibitor 2.

In inhibitor 4 (Fig. 4), the rate constant for the inactivation (0.085 min^{-1}) was found to be approximately twice as fast as that for the modification of the amino groups ($0.038/\text{min}$) on the basis of the kinetic analysis as mentioned above. This may indicate that modification of either of the two amino groups in the active site may have caused inactivation.

The complete TNBS reaction resulted in no loss of the chymotrypsin inhibitory activity of both inhibitors. This observation suggested that inhibitors 2 and 4 were double-headed.

Extensive modification of inhibitor 5 with TNBS (six amino groups per mole) caused no loss of the trypsin inhibitory activity.

Modification of Inhibitor 5 with Glyoxal

Modification of 1.1 arginine residues per mole of inhibitor 5 with glyoxal caused 80% loss of the trypsin inhibitory activity (Table III). This suggested that one arginine residue was essential for the activity of inhibitor 5.

Ternary Complex Formation in Inhibitors 2 and 4

The complex formation between inhibitor 4 and the enzymes is shown in Fig. 5. When the mixture of trypsin and inhibitor 4 was subjected to a column of Bio-Gel P-200, a peak with maximal protein value occurred at an elution volume of 27 ml. Inhibitor 4 itself was eluted with a peak at 30 ml. The fractions of the inhibitor-trypsin complex (26–30) displayed chymotrypsin inhibitory activity. When the mixture of trypsin, chymotrypsin, and inhibitor 4 was subjected to gel filtration, a protein peak with a shoulder at 27

TABLE III
Modification of Inhibitor 5 with Trinitrobenzenesulfonic Acid (TNBS) and Glyoxal

| Modification with | Reaction Time (min) | Trypsin Inhibitory Activity (%) | Modified | | |
|-------------------|---------------------|---------------------------------|---|---|-----|
| | | | Amino Groups ^a (residue per mole, %) | Arginine ^b (residue per mole, %) | |
| TNBS | 0 | 100 | 0 | 0 | ... |
| | 30 | 94 | 4.0 | 64 | ... |
| | 60 | 98 | 4.8 | 77 | ... |
| | 120 | 92 | 6.2 | 100 | ... |
| Glyoxal | 0 | 100 | ... | 0 | 0 |
| | 30 | 87 | ... | 0.1 | 3 |
| | 60 | 58 | ... | 0.3 | 8 |
| | 120 | 21 | ... | 1.1 | 28 |
| | 180 | 13 | ... | 1.7 | 43 |

^aQuantitated by TNBS reaction.

^bQuantitated by amino acid analysis assuming four residues of histidine per mole.

ml was observed at an elution volume of 23 ml. The peak should represent the ternary complex, and the shoulder may represent the mixture of the inhibitor-trypsin and the inhibitor-chymotrypsin complexes. For inhibitor 2, a similar result was obtained. These results indicated that both inhibitors 2 and 4 could inhibit trypsin and chymotrypsin at different sites and combine both enzymes simultaneously.

Stability to Heating and Pepsin Treatment

Inhibitory properties of 2, 4, and 5 dissolved in 0.15M NaCl solution were unaffected by heating at 100°C for periods up to 60 min. The iso-inhibitors, singly and in combinations, were heat-stable and did not exert a denaturing effect mutually even at 100°C. This point is very interesting in view of the fact that a variety of proteins (such as cytochrome C, bovine serum albumin, or high molecular weight proteins of bean extract) caused inactivation of the inhibitors (Tsukamoto et al 1983).

The three inhibitors had no inhibitory activity against pepsin, when they were treated at pH 2, for 1–3 hr at 37°C. This exposure of the inhibitors did not affect their inhibitory activities against trypsin and chymotrypsin.

On treatment with pepsin in a 1:1 weight ratio the inhibitory activities of all three inhibitors did not change. When inhibitor 5 was treated with a large excess of pepsin (1:10, w/w), it gradually lost activity as treatment time increased. Treatment for 0.5, 1, or 2 hr caused 50, 65, or 80% loss of the inhibitory activity against trypsin, respectively. Inhibitory activities against trypsin and chymotrypsin in inhibitors 2 and 4, however, did not occur under the same conditions.

DISCUSSION

Five or more trypsin inhibitors in kintoki beans had similar properties under gel filtration and ion-exchange chromatography. Three of the inhibitors were easily separated by chromatofocusing. They resemble inhibitors from lima beans (Haynes and Feeney 1967, Jones et al 1963), kidney beans (Pusztai 1966), and garden beans (Wilson and Laskowski 1973) in amino acid compositions characterized by high contents of aspartic acid, serine and half-cystine, and the absence of tryptophan and carbohydrate.

Although these three inhibitors resemble legume inhibitors in general, they vary significantly among themselves. Both inhibitors 2 and 4 are double-headed, as are those of Bowman-Birk soybean

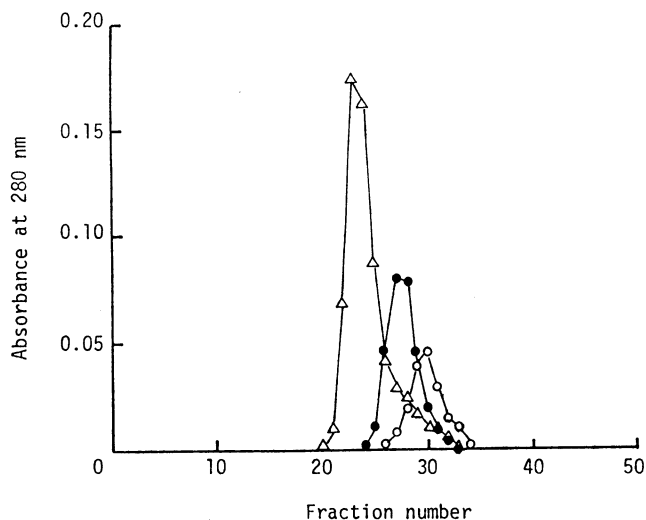


Fig. 5. Gel filtration of the inhibitor 4 and enzymes on Bio-Gel P-200. Inhibitor 4 (580 μ g), or mixtures of inhibitor 4 (100 μ g) with 250 μ g trypsin or with 250 μ g trypsin and 250 μ g chymotrypsin was applied on a column of Bio-Gel P-200 (0.8 \times 55 cm) and eluted with 50 mM Tris-HCl buffer, pH 8.0. \circ — \circ = the inhibitor only; \bullet — \bullet = the mixture of inhibitor and trypsin; and \triangle — \triangle = the mixture of the inhibitor, trypsin, and chymotrypsin.

(Birk et al 1967, Frattali and Steiner 1969), lima bean (Haynes and Feeney 1967, Krahn and Stevens 1970), and garden bean III_b (Wilson and Laskowski 1973). Inhibitor 5, however, is inactive towards chymotrypsin and inhibits one mole of trypsin per mole of inhibitor.

The inhibitors also differ in the reactive-site residue involved in trypsin inhibition. Inhibitors 2 and 4 have lysine residue as their reactive site, as do the inhibitors in Bowman-Birk soybean (Seidl and Liener 1972), lima bean (Krahn and Stevens 1970, 1972), and garden bean I (Wilson and Laskowski 1973). But inhibitor 5 involves arginine as its reactive-site residue, as does Kunitz soybean inhibitor (Ozawa and Laskowski 1966).

These are examples of both arginine- and lysine-type inhibitors and the presence of both single- and double-headed inhibitors within the same plant. The biological meaning of these extensive heterogeneities of trypsin inhibitors has not been explained. Inter- and intraspecific comparisons of the legume trypsin inhibitors may provide answers to questions about evolution and structure-function relationships in proteins.

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