Isolation and Partial Characterization of a Trypsin Inhibitor from Wheat Endosperm

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Summary Trypsin inhibitors were isolated from wheat endosperm, and a major inhibitor (wheat endosperm trypsin inhibitor-I, WETI-I) was purified by ion-exchange chromatographies on CM-Sephadex and SP-Sephadex, gel filtration on Sephadex G-75 and chromatofocusing on Polybuffer exchanger PBE 94. This inhibitor was a polypeptide composed solely of amino acids, and its *pI* value was 9.35. It was found to be homogeneous in gel electrophoresis and velocity sedimentation. It showed strong inhibition on bovine trypsin but weak inhibition on bovine α -chymotrypsin. The molecular weight of the inhibitor was approximately 7,800 as judged from SDS-gel electrophoresis. This finding, along with the trypsin inhibition data, suggested that the inhibitor bound trypsin in the molar ratio of 1:1. Certain other properties of the inhibitor, including amino acid composition and UV spectral characteristics are presented.

Key Words trypsin inhibitor, wheat endosperm, chromatofocusing, isoelectric point, gel electrophoresis, velocity sedimentation

The occurrence of protease inhibitors in wheat grain has been demonstrated by many workers (1-4). Shyamala *et al.* (5) isolated a heat-labile trypsin inhibitor from whole wheat flour by ammonium sulfate precipitation and chromatography on CM-cellulose. Hochstrasser and Werle (6) purified a trypsin inhibitor with a molecular weight of 17,000 from wheat germ and characterized it in terms of its chemical and physicochemical properties. Recently Chang *et al.* (7) isolated two trypsin inhibitors from red winter wheat and durum wheat, respectively, by affinity chromatography. They gave certain information on the characterization and heat stability of them (8). In our laboratory, several protease inhibitors have been detected in three portions of wheat grain, germ, bran and endosperm (9). Furthermore, two trypsin inhibitors (WGTI-I, WGTI-II) (10) and a trypsin inhibitor (WBTI-I) (11) have been purified from wheat germ and bran, respectively.

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In this paper, the isolation and certain properties of the trypsin inhibitor in wheat endosperm are described.

MATERIALS AND METHODS

1. Materials

Hard wheat endosperm of the Canada Western variety was kindly supplied by Nisshin Milling Ltd. Trypsin [EC 3.4.21.4] (2 × cryst., from bovine pancreas) and α -chymotrypsin [EC 3.4.21.1] (3 × cryst., from bovine pancreas) were the products of Sigma Chemical Co. α -N-Benzoyl-D,L-arginine-*p*-nitroanilide (BANA), a substrate of trypsin, was purchased from the Protein Research Foundation. Sephadex G-75, CM-Sephadex C-25, SP-Sephadex C-25, Polybuffer exchanger PBE 94 and Pharmalyte were purchased from Pharmacia Fine Chemicals Co. The molecular weight marker proteins and *p*-nitrophenyl-*p*'-guanidinobenzoate hydrochloride were obtained from Sigma Chemical Co. Carrier ampholyte was obtained from LKB. All other reagents used were of reagent grade.

2. Methods

Chemical analyses. Protein was determined by the Lowry-Folin method (12) using ovalbumin as a reference, or by the micro-Kjeldahl method (13). Carbohydrate was determined by the phenol-sulfuric acid method (14).

Amino acid analysis. The sample was hydrolyzed with constant-boiling HCl at 105°C in vacuum-sealed tubes for 24, 48 and 72 hr. Analyses were carried out using an automatic amino acid analyzer, Hitachi model KLA-835. Tryptophan and tyrosine contents were determined by the method of Goodwin and Morton (15).

Assay of protease activities and inhibitory activity. The hydrolytic activity of trypsin was measured by the modified method (10) of Erlanger *et al.* (16) using BANA as substrate.

The proteolytic activities of trypsin and α -chymotrypsin were measured by the casein digestion method (17).

The inhibitory activity was estimated from the residual protease activity in the reaction mixture incubated with the inhibitor, and expressed as percent inhibition (*I*) of the control activity using the following equation:

$$I(\%) = 100(T - T^*)/T$$

where T^* and T are the activities of protease with and without the inhibitor, respectively. The inhibitor unit was defined as the amount of inhibitor required for the complete inhibition of 1 μ g of active trypsin. The purity of the trypsin used was determined by active site titration using *p*-nitrophenyl-*p*'-guanidinobenzoate hydrochloride (18), and a value of 70% was obtained.

In routine assays BANA was used as substrate.

Velocity sedimentation. Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge equipped with Schlieren optics. The experiment was

run at 59,780 rpm in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl at 20°C. The sedimentation coefficient was calculated from the rate of movement of the maximum ordinate position of the Schlieren pattern.

Gel electrophoresis. Disc gel electrophoresis was carried out as described by Reinsfeld *et al.* (19) on 10% polyacrylamide gel in β -alanine-acetic acid buffer (pH 4.5). SDS-gel electrophoresis was performed, in the presence of 1% SDS, on 10% polyacrylamide gel in 0.1 M sodium phosphate buffer (pH 7.1). The procedure described by Shapiro *et al.* (20) was followed to prepare the gel column, and the electrophoresis was run at a constant current of 8 mA/column at room temperature. The gels were stained for protein with 1% Amido Black 10B in a 25% isopropyl alcohol solution containing 7% acetic acid.

For estimation of molecular weight, the samples and molecular weight marker proteins were incubated in 0.01 M sodium phosphate buffer (pH 7.1) containing 1% SDS and 1% 2-mercaptoethanol at 60°C for 2 hr, prior to SDS-gel electrophoresis (21).

Isoelectric focusing. The sample was subjected to isoelectric focusing in a thin layer of polyacrylamide gel, using LKB 2117 Multiphor equipment.

RESULTS

Extraction and separation of trypsin inhibitors

The endosperm powder was ground thoroughly with the aid of sea sand wetted with 0.1 M NaCl solution in a mortar. Then, 0.1 M NaCl solution (of a weight 10-fold that of the endosperm) was added to the resulting paste, the mixture was allowed to stand for 2 hr at room temperature, and was then filtered through glass wool to obtain a clear extract. The extract was heated at 60°C for 10 min to precipitate heat-

Fig. 1. Elution profile of crude trypsin inhibitor fraction on a CM-Sephadex C-25 column. The crude fraction (0.8 g) was applied to the column (4.3 $\times$ 100 cm). Elution was carried out first with 200 ml of 0.1 M acetate buffer, pH 4.7, and then with a linear gradient to 1.0 M NaCl at a flow rate of 73 ml/hr. —, absorbancy at 280 nm; ·····, trypsin inhibitory activity; ----, NaCl concentration.

labile proteins which were subsequently removed by centrifugation. Ammonium sulfate was added to the supernatant to 80% saturation to precipitate the fraction containing most of the inhibitory activity. The precipitate was dissolved in a minimum volume of 0.1 M acetate buffer (pH 4.7). The resultant solution was then dialyzed against the same buffer, using cellulose tubing (VT 351, cut-off molecular weight 3,500). The dialysate (crude trypsin inhibitor) was applied to a CM-Sephadex C-25 column (4.3×100 cm) previously equilibrated with 0.1 M acetate buffer (pH 4.7). The column was first washed with 200 ml of the same buffer and the trypsin inhibitory active fractions were then eluted with the acetate buffer using a linear gradient of NaCl (0–1.0 M), 10 g fractions being collected. The result of chromatographic fractionation of the crude trypsin inhibitor on a CM-Sephadex C-25 column is presented in Fig. 1 which shows the presence of four active fractions designated as fractions I, II, III and IV. Total activities of the fractions were in the order of IV, III, II and I.

It was impossible to collect fraction I in a workable amount. Fractions II and III were dialyzed, lyophilized and further fractionated by chromatography on Sephadex G-75 and SP-Sephadex C-25, and four inhibitors were obtained from these fractions (22).

Purification of fraction IV

Fraction IV was further purified by chromatography on a Sephadex G-75 column (Fig. 2), and the main fraction eluted between tube nos. 47 and 55 was chromatographed on an SP-Sephadex C-25 column. As shown in Fig. 3, a symmetrical protein peak was obtained and was clearly associated with the

Fig. 2. Elution profile of fraction IV in gel filtration on a Sephadex G-75 column. Fraction IV (140 mg) was dissolved in 7 ml of 0.1 M Tris-HCl buffer, pH 8.0. The solution was applied to the column (1.8×100 cm) and the column was eluted with 0.1 M Tris-HCl buffer (pH 8.0) at a flow rate of 12.7 ml/hr. —, absorbancy at 280 nm; -----, trypsin inhibitory activity.

Fig. 3. Elution profile of the active fraction from Fig. 2 on an SP-Sephadex C-25 column. One hundred milligrams of the active fraction was dissolved in 7 ml of 0.1 M acetate buffer, pH 4.7. The solution was applied to the column (1.6×25 cm). Elution was carried out first with 200 ml of 0.1 M acetate buffer, pH 4.7, and then with a linear gradient to 0.5 M sodium acetate at a flow rate of 10 ml/hr. —, absorbancy at 280 nm; -----, trypsin inhibitory activity; ----, pH.

Fig. 4. Elution profile of the active fraction from Fig. 3 on a Polybuffer exchanger PBE 94 column. The active fraction (10 mg) was dissolved in 5 ml of 0.025 M ethanolamine-HCl buffer, pH 9.5. The solution was applied to the column (1×21.5 cm). Elution condition: start buffer 0.025 M ethanolamine-HCl, pH 9.5, elution buffer 0.0075 mmol/pH unit/ml Pharmalyte pH 8–10.5 equilibrated to pH 8.0. Flow rate: 31.4 ml/hr.

inhibitory activity peak, but this fraction did not give a single band on disc electrophoresis on polyacrylamide gel. Further, the fraction was purified by chromatofocusing using Polybuffer exchanger PBE 94. The fractions indicated in Fig. 4 were combined, and ammonium sulfate was added to 100% saturation. The precipitate was collected by centrifugation and washed with saturated ammonium sulfate, dialyzed against water and lyophilized. The preparation was found to be

Table 1. Summary of purification of WETI-I (from endosperm 1 kg).

Step	Total protein ^a (mg)	Total unit	Specific activity (unit/mg protein)	Yield (%)
Extract	16,829.5	131,270	7.8	100.0
Dialysate	7,913.6	113,050	14.3	86.1
1st chromatography on CM-Sephadex C-25 column	1,124.3	46,837	41.7	35.7
2nd chromatography on Sephadex G-75 column	195.0	41,489	212.8	31.6
3rd chromatography on SP-Sephadex C-25 column	11.4	13,256	1,162.8	10.1
4th chromatography on Polybuffer exchanger PBE 94 column	3.2	11,429	3,571.6	8.7

^a Protein = nitrogen $\times$ 6.25.

Fig. 5. Gel electrophoresis and velocity sedimentation pattern of WETI-I. a: Electrophoresis of the inhibitor was run on 10% gel using a buffer system described by Reinsfeld *et al.* (19). b: The pattern was obtained with the inhibitor at a concentration of 0.4% at 20°C, the picture being taken 16 min after the reaching of a speed of 59,780 rpm.

electrophoretically homogeneous, giving a single band and hereafter denoted by "wheat endosperm trypsin inhibitor-I (WETI-I)." The summary of the purification is shown in Table 1.

Gel electrophoresis and velocity sedimentation of WETI-I

The homogeneity of the inhibitor was examined by disc electrophoresis and

Fig. 6. Concentration dependence of sedimentation coefficient of WETI-I. Data were from a series of experiments as shown in Fig. 5b.

Fig. 7. Estimation of molecular weight of WETI-I by SDS-gel electrophoresis. Gel electrophoresis was carried out on 10% gel in 0.1 M phosphate buffer (pH 7.1) containing 1% SDS. The samples and marker proteins were preincubated in phosphate buffer containing 1% SDS and 1.0% 2-mercaptoethanol at 60°C for 2 hr prior to electrophoresis.

velocity sedimentation. WETI-I exhibited a sharp single band in gel electrophoresis and a symmetric sharp boundary of 1.2S in velocity sedimentation (Figs. 5 and 6).

Isoelectric focusing of the inhibitor also gave only a single component, and the *pI* value was 9.35.

Molecular weight of WETI-I

The molecular weight was estimated by SDS-gel electrophoresis in 0.1 M phosphate buffer (pH 7.1) containing 1% SDS. Figure 7 shows the molecular weight vs. mobility plot from which the molecular weight of WETI-I approximates to 7,800.

Fig. 8. Proteases inhibition by WETI-I. Experimental procedures (casein method) are described in the text. ●, trypsin; ▲, α -chymotrypsin.

Inhibitory activity

The inhibitory activity of the inhibitor against trypsin and α -chymotrypsin is shown in Fig. 8. The inhibitor possessed strong inhibitory activity against trypsin. Extrapolation of the linear portion of the curve to zero trypsin activity revealed that one μg of the inhibitor completely inactivated 3.06 μg of trypsin when the activity of trypsin was assayed with casein as substrate and also inactivated 3.14 μg of trypsin when BANA was used as substrate. Thus, the molecular weight was estimated to be 7,610 or 7,420, assuming that 1 mol of the inhibitor inhibits 1 mol of trypsin (molecular weight 23,300). The values are in near agreement with the molecular weight determined by SDS-gel electrophoresis.

The inhibitor also inhibited α -chymotrypsin but the inhibition did not show stoichiometrical behavior.

Some other properties of WETI-I

The inhibitor is a polypeptide composed solely of amino acids. While neutral sugar was detected in the early stages of purification, the final preparation was found to be free from neutral sugar and hexosamine.

Figure 9 shows the UV absorption spectra of WETI-I in 0.05M sodium phosphate buffer (pH 6.0) and in 0.1M NaOH. At pH 6.0, the absorption maximum was 278 nm and the minimum at 247 nm. The absorption coefficient, $E_{1\%}^{1\text{cm}}$, was calculated to be 11.68 at 278 nm.

The amino acid composition of WETI-I is shown in Table 2. The inhibitor is rich in arginine and proline, and does not contain tryptophan.

Fig. 9. UV absorption spectra of WETI-I. The inhibitor concentration was 0.1%. —, in 0.05 M phosphate buffer (pH 6.0); ----, in 0.1 M NaOH solution.

Table 2. Amino acid composition^a of WETI-I.

Amino acid	No. of residues per molecule ^b	Nearest integer
Aspartic acid	5.83	6
Threonine ^c	6.32	6
Serine ^c	3.68	4
Glutamic acid	5.70	6
Glycine	4.93	5
Alanine	4.56	5
Half-cystine ^c	6.25	6
Valine	3.06	3
Methionine	4.08	4
Isoleucine	2.09	2
Leucine	2.49	2
Tyrosine ^d	1.94	2
Phenylalanine	2.09	2
Lysine	2.37	2
Histidine	2.02	2
Arginine	7.51	8
Proline	7.09	7
Tryptophan ^d	0	0
Total		72
Molecular weight		8,030

^a Average value from 24, 48 and 72 hr' hydrolysates. ^b Calculated for a molecular weight of 7,800. ^c Extrapolated value to zero hour. ^d Determined spectrophotometrically.

DISCUSSION

A trypsin inhibitor was isolated successively from wheat endosperm by extraction, heat-treatment, salting out and chromatographies. Chromatofocusing was used for the final purification, since a contaminated component could not be removed completely from the inhibitor purified by repeated ion-exchange chromatographies. The final purified inhibitor (WETI-I) was about 460 times more active than the crude extract. WETI-I was a polypeptide composed solely of amino acids, and amino acid analyses indicated that the inhibitor was rich in arginine and proline, and did not contain tryptophan. This composition is similar to the trypsin inhibitor isolated from wheat germ by Hochstrasser and Werle (6). However, WETI-I has a molecular weight of about 7,800, whereas the molecular weights of the trypsin inhibitors from wheat grain have been reported to be in the range of 10,000–38,500 (6, 7, 10, 11). Therefore, it is concluded that WETI-I has at present the lowest molecular weight among the trypsin inhibitors isolated from wheat endosperm, bran and germ. WETI-I inhibited trypsin and the inhibition showed stoichiometrical behavior, while it inhibited weakly α -chymotrypsin. Shyamala and Lyman (5) isolated a trypsin inhibitor from wheat flour, which appears to have a low specific activity and does not inhibit α -chymotrypsin. It should be noted that this inhibitor differs markedly from WETI-I in the chemical components, the specific activity for trypsin and the inhibition of α -chymotrypsin. The pI value (9.35) indicates that WETI-I may be the most basic inhibitor among trypsin inhibitors from plant origin, the pI value of them being 8.07 for rice bran (23), 4.7 for eggplant (17) and 8.9 and 9.1 for potato (24).

ADDENDUM

During the preparation of this manuscript, a paper by Boisen and Djurtoft on a similar subject appeared (25).

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