ISOLATION AND CHARACTERIZATION OF TRYPsin INHIBITORS FROM WHEAT GERM

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(Received November 10, 1978)

Summary Trypsin inhibitors were isolated from wheat germ and two major inhibitors (trypsin inhibitors I and II) were purified by various chromatographies including ion-exchange chromatographies on DEAE-Sephadex and CM-Sephadex as well as gel filtration on Bio-gel and Sephadex. Both inhibitors were polypeptides composed solely of amino acids. In the presence of 1% SDS, inhibitor I showed a single symmetrical sedimentation boundary of 1.6 S and a single band in SDS-gel electrophoresis, but in the absence of SDS, it tended to aggregate. Inhibitor II was found to be homogeneous in gel electrophoresis and velocity sedimentation with or without SDS in the solutions. The molecular weights of inhibitors I and II were approximately 16,000 and 10,000, respectively, by SDS-gel electrophoresis. Some other properties of the two inhibitors, including specific inhibitory activities, amino acid compositions and UV spectral properties are presented.

Keywords trypsin inhibitors, wheat germ, ion-exchange chromatography, gel-filtration, velocity sedimentation, SDS-gel electrophoresis

It is known that wheat germ contains several protease inhibitors, some of which have been isolated and fully characterized. HOCHSTRASSER and WERLE (1) reported on trypsin inhibitors isolated from wheat germ by means of selective binding on trypsin resin. They found that the obtained preparation comprises four trypsin inhibitors, one having an approximate molecular weight of 17,000. The remaining three inhibitors with lower molecular weights (ca. 12,000 as estimated by gel filtration) were not further studied. They suggested that hydrolytic cleavage of a peptide bond (or bonds) occurred during the selective binding on trypsin resin, and accordingly, they used the term “modified inhibitors” to denote their inhibitor preparations. It is, therefore, quite possible that inhibitors, once exposed to trypsin resin, no longer retain their natural, intact forms. In the present study for comparative investigations, wheat germ inhibitors were isolated by a different

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procedure, in which a series of chromatographic fractionations was employed. It was found that all three inhibitors isolated and purified were entirely different from that of Höchstrasser and Werle in amino acid composition and susceptibility to reduction with 2-mercaptoethanol. Accordingly, further investigations were undertaken on these inhibitor preparations to clarify their general properties.

EXPERIMENTAL

1. Materials
Hard wheat germ of the Canada Western variety harvested in 1975 was kindly supplied from Nisshin Milling Ltd. The sample was defatted with petroleum ether, and ground. Trypsin [EC 3.4.21.4] (2 × cryst., from bovine pancreas) was the product of Sigma Chemical Co. \(\alpha\)-N-Benzoyl-D,L-arginine-p-nitroanilide (BApNA), a substrate of trypsin, was purchased from the Protein Research Foundation. Sephadex G-75, DEAE-Sephadex A-25 and CM-Sephadex C-25 were obtained from Pharmacia Fine Chemicals Co. and Bio-gel P-30 from Bio-Rad Laboratories. The molecular weight marker proteins were purchased from Boehringer Mannheim GmbH. All other reagents used were of reagent grade.

2. Methods
Isolation and purification of trypsin inhibitors. The defatted germ 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 (10-fold the germ weight) was added to the resulting paste, the mixture was allowed to stand for 2 hr at room temperature, and filtered through glass wool to obtain a clear extract. The extract was heated at 60°C for 30 min to precipitate heat-labile proteins, which were subsequently removed by centrifugation. Ammonium sulfate was added to the supernatant to 80% saturation to precipitate the fraction containing most inhibitory activity. The precipitate thus obtained was dissolved in a large quantity of water, and any insoluble material was removed by centrifugation. The resulting supernatant was pale brown. This supernatant was adjusted to pH 8.0 with 0.1 M NaOH and diluted with one volume of 0.1 M Tris-HCl buffer (pH 8.0). The solution was then applied to a DEAE-Sephadex A-25 column, previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0), to remove the colored substance of polyphenol type which was irreversibly absorbed to the column medium. The eluate was desalted by ultrafiltration through a Diafilter (membrane: G-01T, fractionation range of molecular weight 1,000) and lyophilized. No trypsin inhibitory activity was detected in the filtrate. Approximately 1.3 g of the crude fraction was obtained from 100 g of defatted germ.

The crude inhibitor was fractionated by gel filtration using a Bio-gel column. One gram of the crude fraction was dissolved in 10 ml of 0.01 M HCl containing 0.1 M NaCl, and the solution was applied to a Bio-gel P-30 column (5 × 92 cm) and chromatographed using 0.01 M HCl-0.1 M NaCl solution as the effluent, a fraction collector being actuated to collect 10-ml fractions. The fractions containing inhibitory activity were lyophilized and subjected to further chromatographic
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fractionation on a CM-Sephadex C-25 column.

The active fractions from the Bio-gel P-30 column (150–200 mg) were dissolved in 0.05 M acetate buffer (pH 4.0) and applied to a CM-Sephadex C-25 column (2 × 55 cm) previously equilibrated with the same buffer. The column was first washed with 100 ml of the 0.05 M acetate buffer and the inhibitors were then eluted using the acetate buffer with a linear gradient of NaCl (0–1.0 M), 5-ml fractions being collected. Hence, the obtained fractions were dialyzed against water and lyophilized. Before proceeding to further analytical work, all the inhibitor fractions obtained from chromatography on CM-Sephadex were passed through a column of Sephadex G-75 (2 × 65 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) for preliminary examination of apparent homogeneity.

Chemical analyses. Protein was determined by the LOWRY-FOLIN method (3) using ovalbumin as a reference, or by the micro-Kjeldahl method (4). The amino acid compositions of inhibitors were obtained using an Hitachi KLA-5A amino acid analyzer. The samples were hydrolyzed in 6 M HCl in vacuum-sealed tubes at 110°C for 24 hr.

Assay of trypsin activity and inhibitory activity. The hydrolytic activity of trypsin was measured by the modified method of ERLANGER et al. (5) using BApNA as the substrate. The activities of the inhibitors were assayed as follows: An aliquot of sample solution (0.38 ml) was mixed with 0.02 ml of trypsin solution. After preincubation for 5 min at 37°C, a substrate solution (2 ml) was added and the remaining activity of trypsin was measured. The inhibitory activity was expressed in terms of percent inhibition (I) relative to the control value, i.e., $I(\%) = 100(T - T^*)/T$ where $T^*$ and $T$ are the activities of protease with and without the inhibitor (6). The inhibitor unit was defined as the amount of inhibitor required for the complete inhibition of 1 µg of crystalline trypsin.

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-glycine buffer (pH 8.0) with or without SDS (1.0%) 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 ORNSTEIN and DAVIS (7) on 7% polyacrylamide gel in Tris-glycine buffer (pH 8.4). SDS-gel electrophoresis was performed in the presence of 1% SDS on 7% polyacrylamide gel in 0.1 M sodium phosphate buffer (pH 7.1). The procedure described by SHAPIRO et al. (8) was followed to prepare the gel column, and the electrophoresis was run at a constant current of 8 mA per column at room temperature. The gels were stained for protein with 1% Amido Black 10B in a 20% methanol solution containing 7% acetic acid.

For estimation of molecular weight, the samples and molecular weight marker proteins were incubated in 0.1 M phosphate buffer containing 1% SDS and 0.1% 2-mercaptoethanol at 37°C for 7 hr, prior to SDS-gel electrophoresis (9).
RESULTS AND DISCUSSION

Isolation of wheat germ inhibitors and inhibitory activities of the purified inhibitors

Ground wheat germ was first extracted with 0.1 M NaCl, and the extract was subjected to a series of preliminary fractionation procedures including heating at 60°C, ammonium sulfate precipitation and chromatography on DEAE-Sephadex. Thus, the obtained crude preparation, in which 85% of the original activity was recovered, was found to contain at least five inhibitors (refer to Fig. 1–Fig. 3).

![Graph](image-url1)

**Fig. 1.** Elution profile of crude inhibitor fraction in gel filtration on a Bio-gel P-30 column. One gram of the crude fraction was dissolved in 10 ml of 0.01 M HCl containing 0.1 M NaCl. The solution was applied to the column (5 × 95 cm) and the column was eluted with 0.01 M HCl containing 0.1 M NaCl. —— Absorbancy at 280 nm; ‒‒‒, trypsin inhibitory activity.

![Graph](image-url2)

**Fig. 2.** Elution profile of fraction I on a CM-Sephadex C-25 column. Elution was carried out first with 100 ml of 0.05 M acetate buffer, pH 4.0, and then with a linear gradient to 1.0 M NaCl at a flow rate of 17 ml/hr.
The crude inhibitor fraction was first fractionated on a Bio-gel P-30 column to obtain three active fractions designated as fraction I, II and III in Fig. 1. The relative proportion of fraction III was less than the other two fractions, but the specific activities were in the order of III > II > I. Fractions I and II were ultrafiltered, lyophilized and further fractionated by chromatography on CM-Sephadex and Sephadex G-75, but it was not possible to collect fraction III in a workable amount.

The result of chromatographic fractionation of fraction I on a CM-Sephadex column is presented in Fig. 2 which shows the presence of the main inhibitor fraction well separated from the two minor components. The main fraction was desalted and

| Step                                      | Total protein (mg) | Total unit (unit/mg protein) | Specific activity (unit/mg protein) | Yield (%) |
|-------------------------------------------|--------------------|------------------------------|------------------------------------|-----------|
| Crude fraction                            | 6,500              | 55,705                       | 8.6                                | 100       |
| (Inhibitor I) 1st chromatography on Bio-gel P-30 column | 660                | 15,597                       | 23.6                               | 28        |
| (Inhibitor II) 1st chromatography on Bio-gel P-30 column | 180                | 8,913                        | 49.5                               | 16        |
| 2nd chromatography on CM-Sephadex C-25 column | 180                | 8,913                        | 49.5                               | 16        |
| 2nd chromatography on CM-Sephadex C-25 column | 5.9                | 14,483                       | 2,439.0                            | 26        |

* Protein = nitrogen × 6.25.
concentrated by ultrafiltration, and subjected to gel filtration on a Sephadex G-75 column (2 × 65 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The result showed that this fraction was chromatographically homogeneous, giving a single symmetrical peak and hence hereafter denoted by "trypsin inhibitor I."

Fraction II was similarly purified by chromatography on a CM-Sephadex C-25 column (Fig. 3) and the main fraction eluted between 150 ml and 200 ml, after ultrafiltration, was chromatographed on a Sephadex G-75 column. This fraction was also found to be chromatographically homogeneous and likewise denoted by "trypsin inhibitor II." The purification procedures are summarized in Table 1. It should be noted that while trypsin inhibitor II showed stoichiometric inhibition, 30 mole equivalents of inhibitor I were required for complete inhibition, indicating that the association constant of the latter is low.

**Gel electrophoresis and velocity sedimentation of inhibitors I and II**

The homogeneity of the two inhibitors was examined by disc electrophoresis and velocity sedimentation. Since inhibitor I tended to aggregate in the absence of SDS, only the results obtained in the presence of SDS are presented. As shown in Fig. 4, inhibitor I showed a single band on 7% gel in the presence of 1% SDS. In the absence of SDS, however, a blurred band appeared indicative of reversible polymerization. This is in agreement with the result of velocity sedimentation, in which inhibitor I exhibited a sharp single boundary of 1.6 S in the presence of SDS.
Fig. 5. Velocity sedimentation patterns of trypsin inhibitors I and II. a: Inhibitor I at concentrations of 0.2% (the upper pattern) and 0.4% (the lower pattern) in the presence of 1% SDS at 20°C, the picture being taken at 32 min after reaching a speed of 59,780 rpm; b: Inhibitor II at a concentration of 0.4% in the absence of SDS at 20°C, the picture being taken at 16 min after reaching a speed of 59,780 rpm.

(Fig. 5) but a heterogeneous boundary with the sedimentation coefficient ranging from 2 S to 5 S in the absence of SDS. All the above results apparently indicate that in the absence of SDS inhibitor I exists in oligomeric forms which dissociate upon exposure to 1% SDS giving a single subunit. Further details on the reversible aggregation of inhibitor I will be published elsewhere.

Inhibitor II, on the other hand, exhibited a sharp single band in gel electrophoresis and a symmetric sharp boundary in velocity sedimentation with and without SDS in the solution. For inhibitor II, only the results (gel electrophoresis and velocity sedimentation) obtained in the absence of SDS are presented in Figs. 4 and 5.

Molecular weights of inhibitors I and II

Since inhibitor I completely disaggregated in the presence of 1% SDS, the molecular weight was estimated by SDS-gel electrophoresis in 0.1 M phosphate buffer containing 1% SDS. Figure 6 shows the molecular weight vs. mobility plot from which the molecular weights of inhibitors I and II were approximated to 16,000 and 10,000, respectively. Particularly noteworthy is that the SDS-gel electrophoretic patterns of inhibitors I and II were not affected by preincubation with 0.1% 2-mercaptoethanol. This presents a striking contrast to the inhibitor preparation (W-I fraction) of Hochstrasser and Werle (1) which was susceptible to reduction with 2-mercaptoethanol giving two polypeptide fragments. While the inhibitor I and W-I fractions have similar molecular weights (16,000 and 17,000 respectively), they apparently represent distinct entities.

Some other properties of inhibitors I and II

Figure 7 shows the UV absorption spectra of inhibitors I and II in 0.05 M
Fig. 6. Estimation of molecular weights of inhibitor I and II by SDS-Gel electrophoresis. Gel electrophoresis was carried out on 7% 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 0.1% 2-mercaptoethanol at room temperature for 7 hr prior to electrophoresis.

Fig. 7. UV Absorption spectra of inhibitors I and II. The absorption of a 0.1% solution of each inhibitor was measured. ———, in 0.05 M phosphate buffer (pH 6.0); ———, in 0.1 M NaOH solution.

sodium phosphate buffer (pH 6.0) and in 0.1 M NaOH. At pH 6.0, the absorption maximum was at 271 nm for inhibitor I and 274 nm for inhibitor II, and the minimum was at 250 nm and at 252 nm, respectively. The extinction coefficients,
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$E_{1\%cm}$ were calculated to be 1.52 at 271 nm for inhibitor I and 4.05 at 274 nm for inhibitor II. The tryptophan content of inhibitor II as estimated from the spectrophotometric data was 0.8 (mole %), but inhibitor I contained only a trace amount of tryptophan ($l0$).

Both inhibitors are polypeptides composed solely of amino acids. While neutral sugar was detected in the early stages of purification, the final preparations (inhibitors I and II) were found to be free of neutral sugar and hexosamine. In Table 2 the amino acid compositions of the two inhibitors are shown. It should be noted that these compositions differ markedly from that reported for the wheat germ inhibitor (W-I fraction) isolated by HOCHSTRASSER and WERLE ($l$) using selective binding on trypsin resin, particularly in the contents of cystine and proline. This strongly supports the foregoing argument that inhibitors I, II and the W-I fraction are entirely different entities. As HOCHSTRASSER and WERLE ($l$) suggested in their report, their preparation may have suffered hydrolytic cleavage of the peptide bond.

**Table 2.** Amino acid compositions of trypsin inhibitors I and II.

| Amino acid   | Trypsin inhibitor I | Trypsin inhibitor II |
|--------------|---------------------|---------------------|
|              | No. of residues per molecule$^a$ | Nearest integer | No. of residues per molecule$^a$ | Nearest integer |
| Lysine       | 11.40               | 11                  | 5.26                  | 5                  |
| Histidine    | 1.39                | 1                   | 1.69                  | 2                   |
| Arginine     | 9.24                | 9                   | 6.51                  | 7                   |
| Aspartic acid| 12.01               | 12                  | 8.27                  | 8                   |
| Threonine    | 9.09                | 9                   | 4.61                  | 5                   |
| Serine       | 9.55                | 10                  | 6.20                  | 6                   |
| Glutamic acid| 24.64               | 25                  | 14.29                 | 14                  |
| Proline      | 4.31                | 4                   | 5.52                  | 6                   |
| Glycine      | 17.86               | 18                  | 11.09                 | 11                  |
| Alanine      | 20.02               | 20                  | 9.51                  | 10                  |
| Half-cystine | 4.16                | 4                   | 2.73                  | 3                   |
| Valine       | 7.08                | 7                   | 4.70                  | 5                   |
| Methionine   | 2.31                | 2                   | 1.50                  | 2                   |
| Isoleucine   | 4.31                | 4                   | 3.01                  | 3                   |
| Leucine      | 8.62                | 9                   | 3.95                  | 4                   |
| Tyrosine$^b$ | 0.94                | 1                   | 0.92                  | 1                   |
| Phenylalanine| 4.93                | 5                   | 1.88                  | 2                   |
| Tryptophan$^b$ | 0                    | 0                   | 0.79                  | 1                   |
| **Total**    | **151.86**          | **151**             | **92.43**             | **95**              |

Molecular weight 15,846 10,163

$^a$ Calculations were based on the molecular weights of 16,000 and 10,000 for inhibitors I and II, respectively.

$^b$ Determined spectrophotometrically.
Although the molecular weights of the W-I fraction and inhibitor I are similar, the striking difference in the amino acid compositions eliminate the possibility that the W-I fraction is derived from, or related to inhibitors I and II. However, since wheat germ contains at least five trypsin inhibitors, it is necessary to characterize the remaining three inhibitors before a conclusive explanation may be given.

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