Chymotrypsin Inhibitor I from Potatoes

LARGE SCALE PREPARATION AND CHARACTERIZATION OF ITS SUBUNIT COMPONENTS*

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SUMMARY

A method for the large scale preparation of chymotrypsin Inhibitor I from potato tubers is described. The method utilizes the inhibitor's stability to acid at elevated temperatures and its dissociation in 4 M guanidine hydrochloride into subunits (protomers) to produce the pure protein with a 47% recovery and a 40-fold purification.

Tetrameric Inhibitor I in potatoes is therefore a heterogeneous mixture of isoinhibitors whose properties reflect those of its individual protomers.

All four purified protomers resolved from Inhibitor I can be reassociated either individually or hybridized with each other to form tetrameric isoinhibitors.

Tetrameric Inhibitor I species prepared from each of the four protomeric types all have an NH₂-terminal glutamic acid. However, they differ quantitatively from each other in amino acid composition, reactivity with chymotrypsin and trypsin, digestibility by pepsin, and electrophoretic mobility.

Tetrameric Inhibitor I in potatoes is therefore a heterogeneous mixture of isoinhibitors whose properties reflect those of its individual protomers.

The molecular weights of tetrameric Inhibitor I and its complex with chymotrypsin were re-evaluated. The molecular weight of Inhibitor I was found to be 39,000 ± 2,000, and that of its complex saturated with chymotrypsin, 140,000 ± 4,600.

Several recent reports from our laboratory have been concerned with the characterization (1–3) and function (4, 5) of a potent chymotrypsin inhibitor from potato tubers that is composed of subunits (3). We recorded earlier a method for purifying this protein (called Inhibitor I) that involved a final step of crystallization (1). This method, however, was tedious, and it often produced poor yields of crystals even from preparations which contained high concentrations (1 to 2%) of Inhibitor I protein. In this communication we describe a large scale, improved procedure for the preparation of Inhibitor I and its individual subunits. The properties of the subunits and their relationship to the over-all properties of Inhibitor I are described.

EXPERIMENTAL PROCEDURE

Bovine α-chymotrypsin (trichryd crystalized) was purchased from Worthington Biochemical Corporation. BTEE and TAME were purchased from the Nutritional Biochemical Corporation. Sephadex G-25 and G-75 were purchased from Pharmacon and sulfoethylcellulose from Bio-Rad Laboratories. Russet Burbank potatoes were obtained through the Horticulture Department of Washington State University, from experimental plots in Central Washington.

Esterase activities of chymotrypsin and trypsin were determined with BTEE and TAME, respectively, by the method of HurnuI (6) with a recording Beckman model DB spectrophotometer. Protein concentrations of extracts were determined by the method of Lowry (7). The protein concentration of purified inhibitor preparations was determined either spectrophotometrically at 280 nm employing an optical factor (O.F.) or immunologically by the method of Immunochemistry (1). The quantitative method of radial diffusion in agar gels containing anti-Inhibitor I serum (8). In this method radial diffusion of a protein antigen (Inhibitor I) through a gel containing anti-Inhibitor I antibodies results in precipitin rings. The diameter of the rings is a log-log function of antigen concentration and can be measured with a precision of ±10%.

Rabbit anti-Inhibitor I serum was obtained by injecting young rabbits subcutaneously with 1 mg of pure preparations of Inhibitor I emulsified in complete Freund's adjuvant, twice weekly for 3 weeks. The rabbits were rested for approximately 3 months and injected again twice weekly for 2 weeks. After 10 days the rabbits were bled through an ear vein. Thirty milliliters of blood were collected twice weekly for 3 weeks.

The abbreviations used are: BTEE, N-benzoyl-L-tyrosine ethyl ester; TAME, p-toluenesulfonyl-L-arginine methyl ester; dansyl, 1-dimethylaminonaphthalene-5-sulfonyle.
The clear serum was drawn off and stored frozen. The frozen serum was immediately after collection the blood was chilled and stored overnight in a refrigerator and centrifuged at 2000 x g for 15 min. The clear serum was drawn off and stored frozen. The frozen serum was fully active after several months of storage.

Standard proteins for calibrating Sephadex columns for molecular weight determination (9) were chymotrypsinogen, lima bean inhibitor, and ribonuclease, purchased from Worthington Biochemical Corporation; bacitracin, colaminulin, human transferrin, and γ-globulin, purchased from Nutritional Biochemical Corporation; and Kunitz trypsin inhibitor which was a gift of the Laboratory Chasy, Paris, France.

Ultracentrifuge analyses were made with a Spinco model E analytical ultracentrifuge and a standard six-cell Yphantis centerpiece having sapphire windows. An An-D rotor was utilized at a speed of 32,000 rpm. A computer program, kindly furnished by Dr. R. W. Brosemer of the Chemistry Department of Washington State University, was employed for the analysis of the sedimentation equilibrium experiments.

Sedimentation velocity experiments were performed with the An-D rotor with a double sectored centerpiece at a rotational speed of both 56,000 and 59,787 rpm at 20°C. Sedimentation rates were measured with a toolmakers microscope and all sedimentation coefficients were corrected to a solvent density of 1.0.

Amino acid analyses were performed with a Beckman 120C automatic amino acid analyzer. Total analysis time per sample was 4 hours. Duplicate samples for each protein were hydrolyzed in constant boiling hydrochloric acid under nitrogen at 105°C for 20 hours. Samples were dried under vacuum to remove hydrochloric acid and dissolved in 0.2 N sodium citrate, pH 2.2 for analysis. The values for each amino acid were the average of duplicate analyses.

Analysis of NH₂-terminal end groups were performed by the dansylation method of Grey (10). Thin layer chromatographic analyses of the hydrolyzed dansyl amino acids were carried out employing the solvent systems of (a) Morse and Horecker (11); (b) Gross and Labouesse (12); and (c) System B of Neldov and Genov (13). Glass plates were coated with a 1:2 (w/v) slurry of Silica Gel G and water. The plates were activated by drying at 110°C for 1 hour. After chromatography the dansyl amino acids were localized under an ultraviolet light.

Immunoelectrophoresis was performed as described previously (14) on glass slides (2.5 x 7.5 cm) with 0.55% ionagar in 0.1 M sodium barbital buffer, pH 8.6. The precipitin lines were stained with 1% Amido black dye.

Preparation of Chymotrypsin Inhibitor

Stage I: Extraction, Ammonium Sulfate Fractionation, and Heat Treatment—New Russet Burbank potatoes (Solanum tuberosum) (100 pounds) were cut into small pieces with peels intact, and soaked in 5 gallon buckets containing a sodium dithionite solution (7 g per liter). The potato pieces were homogenized in a 4-liter blender with about 500 ml of dithionite solution. The pulp (approximately 3 liters per homogenization) was collected in buckets and expressed through nylon cloth (Style A, W. G. Rununkles Machinery Co., Trenton, New Jersey) with a rack and cloth press at approximately 1000 p.s.i. The expressed juice (about 15 liters) was adjusted to pH 3.0 with 6 N hydrochloric acid and centrifuged at 1000 x g for 15 min at 5°C.

To the clear supernatant solid ammonium sulfate was added slowly with stirring at 5°C to give 70% saturation (472 g per liter). The mixture was stirred for 1 hour, the precipitate collected by centrifugation at 6000 rpm in the Sorvall RC3 centrifuge at 5°C, dispersed in 2 liters of 70% ammonium sulfate, and filtered with suction at room temperature through Whatman No. 1 filter paper and Celite No. 545 as a filter aid. The filter cake was washed with 1 liter of 70% saturated ammonium sulfate to remove residual sodium dithionite. The precipitate was dissolved at room temperature in 2 liters of distilled water by stirring for 1 hour, and the Celite was then removed by suction filtration.

The clear filtrate was divided into 500-ml portions and heated in a 2-liter Erlenmeyer flask in a steam bath, with stirring, to a temperature of 80°C for 5 to 6 min. At approximately 60°C precipitation was evident, and by 80°C a voluminous precipitate had appeared. Inhibitor I survived this step almost quantitatively. The hot solution was suction-filtered through Whatman No. 1 filter paper coated with Celite No. 545. The clear filtrates from each heated fraction were pooled and lyophilized.

The dry powder was dispersed in 500 ml of distilled water and dialyzed against several changes of distilled water for 48 hours. The suspension in the dialysis tubing cleared as salts were removed, and some protein precipitated again as the salt concentration diminished further. The final clear filtrate was lyophilized and stored at 4°C. This preparation was termed crude inhibitor.

Stage II: Gel Filtration—Two grams of crude inhibitor were dissolved in 140 ml of 0.05 M Tris-0.1 M KCl, pH 8.2 buffer, centrifuged at 10,000 x g for 10 min to remove insoluble material and applied to a column (100 x 10.0 cm) of Sephadex G-75 equilibrated with the same buffer. An upward flow of about 450 ml per hour was established with a peristaltic pump. The effluent was collected at 280 nm by a flow through cell in a Beckman model DB spectrophotometer connected to a recorder, and 25-ml fractions were collected. Collections were begun just before the void volume of the column, previously determined with blue dextran (Pharmacia). Inhibitor I was assayed by the radial diffusion method. The pooled solutions containing Inhibitor I were reduced in volume to 300 ml by vacuum evaporation and were desalted by passing through a column (100 x 5.0 cm) of Sephadex G-25 using 0.05 M ammonium bicarbonate as eluant. The breakthrough peak (the only peak) was collected in its entirety and lyophilized. The resulting salt-free product, termed gel-filtered inhibitor, was approximately 90% Inhibitor I protein by weight with respect to crystalline Inhibitor I as determined by either chymotrypsin inhibitory activity or immuno- logical reactivity in the radial diffusion assay.

Stage III: Purification by Dissociation—Gel-filtered inhibitor (99 mg) was dissolved in 10 ml of 4 M guanidine-HCl, pH 8.0 and applied to a Sephadex G-75 column (100 x 2.5 cm) equilibrated with the same solution. An upward flow of 60 ml per hour was maintained. Only the second peak contained Inhibitor I. This peak eluted with a V/V₀ of 1.82, corresponding to a molecular weight of 10,000. Bacitracin, Kunitz inhibitor, lima bean inhibitor, and chymotrypsin were the standard proteins. This peak was diluted 4-fold with water and desalted on a column of Sephadex G-25 (100 x 2.5 cm) with 0.05 M ammonium bicarbonate as eluant. The breakthrough peak consisting of Inhibitor I was lyophilized and stored in the cold. This preparation was called dissociation purified inhibitor. The lyophilized protein was readily soluble when dissolved in buffers containing ammonium ion, but solution in buffers containing potassium or sodium ions resulted in slight turbidity.

Stage IV: Chromatography of Gel-filtered Inhibitor on Sulfenyl-
Fig. 1. The effect of temperature on the stability of Inhibitor I. Ammonium sulfate-precipitated proteins (0 to 70%) from potato juice were dissolved in water, diluted with 0.1 M NaCl, and adjusted to pH 4.3 with 1 N hydrochloride to a concentration of 8 mg per ml. Two-milliliter samples were heated separately to the appropriate temperature and after exactly 5 min of immersion were transferred to an ice bath, centrifuged, and the supernatant assayed for protein by the Lowry method (7) and Inhibitor I concentration immunologically (8). The open circles represent the percentage of the original protein remaining in the supernatants; closed circles represent the percentage of original Inhibitor I activity remaining in the supernatants.

Cellulose—Sulfoethylcellulose was equilibrated with water for at least 1 day prior to use, and the fines were removed by decantation. The exchanger was washed successively with approximately 20 volumes of 0.1 N NaOH, 0.1 N hydrochloride, distilled water, and then the buffer to be used in the column. The washed exchanger was added to about 5 volumes of initial column buffer of 0.2 M formic acid in 8 M urea, pH 2.8, and the column was poured as a thick slurry. To equilibrate at least 10 void volumes of buffer were allowed to flow through the column before the protein sample was applied. In all cases samples were lyophilized desalted proteins, dissolved in the column buffer at least 1 hour prior to application to the column. A linear salt gradient of 0 to 0.4 M KCl was used to elute Inhibitor I fractions. Fractions of about 1.5 ml were collected at a flow rate of 18 ml per hour. Protein concentration in the effluents was monitored using an Isco model UA-2 ultraviolet analyzer, and subsequently the absorbances were quantitated at 280 nm, determined with a Beckman model D.U. spectrophotometer.

RESULTS

The preparation of Inhibitor I was facilitated by its stability to heat. Fig. 1 shows the effect of heating solutions of the proteins precipitated from potato tuber juice at 0 to 70% saturation with ammonium sulfate. Inhibitor I is stable above 80°, whereas over 75% of the total protein present precipitates at that temperature. The stability of Inhibitor I to the treatment at 80° extends over a pH range from 3.0 to 10 (Fig. 2), but at pH 11.3 it begins to deteriorate considerably. At pH 3.0 the inhibitor is stable at 80° for at least 30 min. For purification, a 5-min heating step at 80° was found to be suitable.

The utilization of a large column (100 X 10 cm) of Sephadex G-75 having upward flow further facilitated purification. This allowed the separation of over 2 g of crude protein per run as shown by the elution profile in Fig. 3. The second peak to elute contained nearly all of the Inhibitor I. A small amount of antigenically cross-reactive protein eluted in Peak I. This high molecular weight material was not further investigated. The protein in Peak II usually contained over 90% Inhibitor I and seldom less than 80%. This indicated that the previous heating step was remarkably thorough in precipitating other proteins in this molecular size range.

The final isolation step for preparing highly purified Inhibitor I from gel-filtered inhibitor was based on a previous observation...
that Inhibitor I could be dissociated into small molecular weight (9,000 to 10,000) subunits when dissolved in guanidine HCl (3). In order to select concentration of guanidine HCl that ensured complete dissociation, the effect of guanidine HCl concentration on the sedimentation velocity of Inhibitor I was studied utilizing the Spinco model L ultracentrifuge. For these experiments four times crystallized Inhibitor I was used, prepared by the method of Balls and Ryan (1). Fig. 4 shows that the sedimentation coefficient of Inhibitor I decreased abruptly from an $S_{20W}$ of 3.4 to 1.0, when it was dissolved in 2 M or a higher concentration of guanidine HCl. Thus, 4 M guanidine HCl, pH 3.0, was selected as the dissociating solvent for gel filtration to separate Inhibitor I subunits from nondissociable impurities.

In Fig. 5 is shown the gel filtration profile of gel-filtered inhibitor on Sephadex G-75 equilibrated with 4 M guanidine HCl in 0.5 M Tris-HCl buffer, pH 8.0. Gel-filtered inhibitor (99 mg) in 10 ml of this buffer was applied to the column ($87 \times 2.5$ cm). Fractions of 2.5 ml were collected at the rate of 1 ml per min. $O$, absorbance at 280 nm; $\bullet$, Inhibitor I concentration as determined by the radial diffusion assay (8).

![Fig. 4. The effect of guanidine hydrochloride concentration on the sedimentation coefficient of Inhibitor I. The concentration of Inhibitor I was 1 mg per ml at pH 7.0. At zero concentration of guanidine hydrochloride, the solvent was 1% NaCl. Sedimentation coefficients were corrected to a solvent density of 1.0.](image)

![Fig. 5. Chromatography of gel-filtered inhibitor on Sephadex G-75 in 4 M guanidine hydrochloride in 0.55 M Tris-HCl buffer, pH 8.0. Gel-filtered inhibitor (99 mg) in 10 ml of this buffer was applied to the column ($87 \times 2.5$ cm). Fractions of 2.5 ml were collected at the rate of 1 ml per min. $O$, absorbance at 280 nm; $\bullet$, Inhibitor I concentration as determined by the radial diffusion assay (8).](image)

**Table I**

| Step | Description                                      | Total Protein | Total Inhibitor I | Fold Purification | Recovery |
|------|--------------------------------------------------|---------------|------------------|-------------------|----------|
| 1.   | Original juice, filtered at pH 3.0               | 8940          | 212              | 0                 | 100      |
| 2.   | 0-70% Ammonium sulfate precipitate               | 5975          | 156              | 1.8               | 73       |
| 3.   | Heat, 80° 5 min, filter                          | 771           | 145              | 17.0              | 70       |
| 4.   | Sephadex G-75 chromatography, Tris buffer, pH 8.2|               |                  |                   |          |
| 5.   | Sephadex G-75 chromatography, guanidine hydrochlo-| 100           | 100              | 39.6              | 47       |

*Lowry method of protein determination (7).

*Radial diffusion assay (8), with four times crystallized Inhibitor I as a standard.

A summary of the purification of Inhibitor I from potato tuber juice is given in Table I. The final product represents a 47% yield of protein and a 40-fold purification from potato juice, based on the radial diffusion immunological assay. Dissociation-purified Inhibitor I neutralized 2.8 mg of chymotrypsin per mg, equalling that of the highest purity preparations of crystallized Inhibitor I (2).

Inhibitor I subunits were resolved into four types. The four subunit components were obtained from either the gel-filtered or the dissociation-purified inhibitor preparations by chromatography on sulfoethylcellulose using the dissociating solvent of 0.2 M formic acid in 8 M urea, pH 2.8. With this buffer all of the

![Fig. 6. Chromatography of gel-filtered inhibitor on a sulfoethylcellulose column (1 X 10 cm) equilibrated with 8 M urea, 0.2 M formic acid, pH 2.8, and eluted with a linear KC1 gradient, represented by —. The column was charged with 102 mg of the preparation. $O$, Inhibitor I as determined by radical diffusion assays (8); $O$, absorbance at 280 nm. Fractions of 1.4 ml were collected at 3-min intervals.](image)
Conditions were identical with those in Fig. 3. The substrate for chymotrypsin was BTEE, and for trypsin, TAME, using the assay system of Hummel (6).

The similarities between the electrophoretic mobilities of the protomer of Peak C and crystalline Inhibitor I, together with the identical inhibitory capacities of their reassociated tetramers toward chymotrypsin and trypsin, led us to conclude that crystalline Inhibitor I closely resembles Protomer C. In attempts to crystallize the tetrameric forms of B and C protomers by the procedure used for crystallizing Inhibitor I (1), 30% magnesium sulfate in 0.1 M sodium acetate, pH 5.5, only the tetrameric C component would crystallize. The crystals obtained from tetrameric C had many of the same general sharp edged hexagonal crystals as the original crystalline Inhibitor I, but many of the crystals were not well formed hexagons and did not recrystallize as readily as the original crystallized Inhibitor I. On the other hand, the tetrameric B component from several different preparations would not crystallize at all. The reasons for the selective crystallization from gel-filtered inhibitor preparations of only one form of Inhibitor I is not known, but must be a result of the population of Inhibitor I tetramers present.

Properties of Inhibitor I and of Its Individual Protomers

Inhibitory Activities—The inhibitory activities of tetramers reconstituted from the four protomer types are compared in Table II. These activities are from titration curves of chymotrypsin and trypsin and provide a convenient means of directly comparing inhibitory activities under identical conditions. Protomers B and C have identical inhibitory activities against chymotrypsin, whereas Protomers A and D under these conditions inhibited only 80% and 25% as much chymotrypsin activity as Protomers B and C.

Protomer B is the most effective inhibitor of trypsin. Protomers A, C, and D are 40%, 18%, and 18% as reactive as Protomer B.

Molar combining ratios of the most effective inhibitors, B and C, based on a molecular weight of 39,000 for inhibitor and 25,000 for chymotrypsin, show 4 moles of chymotrypsin inhibited per mole of inhibitor.

Electrophoresis—Electrophoresis of the four individual protomers on cellulose acetate strips in 8 M urea is shown in Fig. 8, compared with gel-filtered inhibitor, dissociation-purified inhibitor, and four times crystallized Inhibitor I. The four protomers represent the four electrophoretically identifiable components of these two proteins. Only the protomer from Peak C is similar to crystalline Inhibitor I in its electrophoretic mobility. Components of Peaks A and D were not rechromatographed before electrophoresis and the electrophoretic pattern of Peak D noticeably contains impurities of each of the other three peak components. As anticipated, the gel-filtered and dissociation-purified preparations are quite similar in their electrophoretic components and both are comprised of all four of the protomers.

The inhibitory activity of the gel-filtered preparation was absorbed by the column. A breakthrough peak, representing about 15% of the applied protein, did not contain Inhibitor I by radial diffusion immunological assay. The application of a KCl gradient to the column, as shown in Fig. 6, resulted in the elution of four subunit components of Inhibitor I. Two different samples prepared from different batches of potatoes gave the same four peaks.

The components of the two major peaks, B and C, when isolated and separately rechromatographed under the same conditions, eluted at the same salt concentrations as in the original chromatographic isolation (Fig. 7). Minor amounts of other proteins eluted during rechromatography, probably representing some overlap during the first chromatographic separation. However, the second small peak to elute in the upper curve of Fig. 7 at tube 65 is too large for a simple overlap, and its presence is as yet unexplained.

Native Inhibitor I is evidently a heterogeneous mixture of tetramers composed of subunits that, in turn, consist of at least four separable components. Being interchangeable subunits, despite their variability of properties, the term protomer to describe the subunits seem appropriate. The term protomer is used in the sense of Monod et al. (15) being identical subunits with respect to their ability to form the tetramer. However, it is proposed from the data in this report that the four subunit types isolated from Inhibitor I are from homologous genes and are thus protomers with respect to oligomer formation but not truly identical by having evolved different primary structures that account for their different inhibitory, electrophoretic, and chromatographic properties. Oligomers (tetramers) that differ in their complement of protomers are considered to be isoinhibitors.
Amino Acid Composition—A comparison of the amino acid compositions of inhibitor tetramers reconstituted from the individual protomers, A, B, C, and D with gel-filtered Inhibitor I, from which they were prepared, is shown in Table III. Significant differences among the four proteins are seen in several amino acids. It is clear that the amino acid composition is not constant among the tetramers despite the fact that they are all quite similar in their immunological reactivity. The protomers as isolated may be groups of variants that elute together as a result of similarities in their overall charge. All four of the protomer peaks were similar in exhibiting only an NH$_2$-terminal glutamic acid by the dansylation procedure. No other NH$_2$-terminal amino acids were detected in the four protomers or in gel-filtered inhibitor.

Immunological Cross-Reactivity—Fig. 9 shows that the cross-reactivity among the components is identical. The protomers therefore possess considerable structures that are immunologically very similar. The method, however, is only qualitative, and it is not known how much similarity these regions actually represent among the protomers.

The Effect of Acid pH and Pepsin on Inhibitor I Tetramers—Sephadex filtration studies demonstrated that gel-filtered inhibitor dissociates into dimers at pH 3. The decrease in $V/V_0$ of Inhibitor I when dissolved in acid corresponds to a molecular weight change from 39,000 to about 20,000 (Fig. 10).

Previous studies have shown crystallized Inhibitor I to be stable at pH 3 (2). However, it was susceptible to pepsin digestion at pH 3. Digestion with pepsin, at pH 3, of unfractionated dissociation-purified inhibitor, and of the four isolated variants A, B, C, and D, demonstrated that all of these species are not equally digested. Fig. 11 shows that the immunological reactivity of Species D was rapidly destroyed by pepsin, whereas the immunological reactivity of Species A was more stable than that of Species D, but less stable than those of Species B and C.

Immunoelectrophoresis of Inhibitor I and Hybridization of Its Protomers—Immunoelectrophoresis of gel-filtered inhibitor, the preparation from which these protomers were isolated, is shown at the bottom of Fig. 12. At the top of this figure is shown unhybridized tetrameric A, B, C, and D. The differences in electrophoretic mobilities among the four reassociated subunit components permitted the use of this technique to observe hybridization. In Fig. 12 is also shown Inhibitor I reconstituted to form hybridized mixtures of tetramers, prepared by dissolving equal quantities of the indicated protomers in $8\ M$ urea, $0.2\ M$ formic acid, pH 2.8, and diluting 10-fold to reassociate the tetramers. The subscripts represent the proportion of each protomer added.

Hybridization of a mixture of all four protomers was attempted, the quantities of each protomer added being approximately the amounts observed in the chromatographic separation of gel-filtered inhibitor preparations (labeled G75-P1) on sulfoethylcellulose. Immunoelectrophoresis of the resulting hybrids (Fig. 12, bottom) show similarities to the original gel-filtered inhibitor from which they were isolated, but the patterns are not identical.
TABLE III

Amino acid composition of Inhibitor I subunits and gel-filtered Inhibitor I

| Amino acid   | Amino acid residues per moleculea |
|--------------|----------------------------------|
|              | Subunits                         | Gel-filtered Inhibitor I |
|              | A      | B      | C      | D      | A      | B      | C      | D      |
| Lysine       | 5.2    | 5.0    | 5.9    | 5.8    | 5.2    |        |        |        |
| Histidine    | 0.4    | 0.4    | 0.7    | 1.0    | 0.6    |        |        |        |
| Arginine     | 3.9    | 3.6    | 4.6    | 3.9    | 3.6    |        |        |        |
| Aspartic acid| 11.6   | 10.4   | 10.3   | 8.4    | 9.1    |        |        |        |
| Threonine    | 3.6    | 2.8    | 3.1    | 3.3    | 2.9    |        |        |        |
| Serine       | 5.1    | 4.0    | 4.2    | 3.4    | 4.2    |        |        |        |
| Glutamic acid| 11.1   | 9.3    | 8.6    | 8.0    | 9.2    |        |        |        |
| Proline      | 6.6    | 7.1    | 5.4    | 4.3    | 5.4    |        |        |        |
| Glycine      | 6.9    | 5.5    | 7.1    | 8.1    | 6.3    |        |        |        |
| Alanine      | 6.5    | 2.3    | 2.4    | 2.5    | 4.7    |        |        |        |
| Half-cystine | 2.5    | 2.1    | 2.6    | 2.1    | 2.1    |        |        |        |
| Valine       | 9.6    | 8.0    | 8.7    | 6.1    | 7.6    |        |        |        |
| Methionine   | 1.1    | 0.6    | 0.6    | 1.4    | 0.8    |        |        |        |
| Isoleucine   | 7.7    | 6.3    | 7.7    | 5.6    | 5.9    |        |        |        |
| Leucine      | 8.0    | 9.8    | 10.4   | 6.9    | 7.7    |        |        |        |
| Tyrosine     | 2.3    | 0.6    | 1.0    | 1.3    | 1.3    |        |        |        |
| Phenylalanine| 3.1    | 3.2    | 2.3    | 1.9    | 2.4    |        |        |        |

a Calculated on the basis of a molecular weight of 9500 for all species of proteins.

FIG. 10. The estimation of the molecular weight of Inhibitor I on a Sephadex G-75 column (1.5 X 90 cm) with buffers of either 0.05 M ammonium bicarbonate, pH 7.7, or 0.1 M formic acid, pH 3.0. Standard proteins were (1) Kunitz trypsin inhibitor (mol wt. 6,600); (2) lima bean inhibitor (mol wt. 8,400); (3) ribonuclease (mol wt. 13,000); (4) chymotrypsin (mol wt. 25,000); and (5) serum albumin (mol wt. 67,000).

FIG. 11. The digestion by pepsin of dissociation-purified Inhibitor I and of Inhibitor I tetramers reassociated from its protomers. The protomers are from the peaks indicated in the upper right hand corner, isolated from sulfonylethylulose chromatography (Fig. 6). G75PI refers to gel-filtered inhibitor. Conditions, 1.5 mg of each tetrameric species was incubated with 15 ìg of pepsin in 0.01 M NaCl adjusted to pH 3.0 with hydrochloride. Two times crystallized swine pepsin (Worthington) was incubated at 25° with the inhibitor preparations. The remaining concentration of immunologically active tetramer in each incubation mixture at the times shown was determined by the radial diffusion method (4).

TABLE IV

Summary of molecular weight determinations of Inhibitor I and its complex with chymotrypsin

| Protein                  | Method                        | Molecular weight or equivalent weight |
|--------------------------|-------------------------------|---------------------------------------|
| Inhibitor I              | Ultracentrifuge, Archibald    | 38,000 ± 2,400a                       |
| Four times crystallized  | Ultracentrifuge, velocity     | 37,500 ± 2,800a                       |
| Dissociation-purified Inhibitor I | Ultracentrifuge, equilibrium | 39,900 ± 1,000                        |
| Monomers                 | Ultracentrifuge, equilibrium | 39,000 ± 1,100b                       |
|                         | Sephadex G-75, Tris buffer, pH 8.2 | 39,000 ± 1,100b   |
|                         | Septadex G-75, Tris buffer, pH 8.2 | 39,000 ± 1,100b |
|                         | Sephadex G-75, 4 M guanidine, pH 8.5 | 9,800 ± 200b  |
|                         | Sephadex G-75, 4 M urea, pH 2.8 | 10,000 ± 500  |
| Monomers                 | Sephadex G-75, 4 M guanidine, pH 8.5 | 9,300 ± 1,100b   |
|                         | Electrophoresis in 0.1% SDS     | 9,500 ± 500b      |
|                         | α-Chymotrypsin inhibition (synthetic substrate) | 9,500 ± 500b     |

a Average of data taken from Reference 1.

b Taken from Reference 3.

c SDS, sodium dodecyl sulfate.
Fig. 12. The demonstration of hybridization of Inhibitor I protomers by immunoelectrophoresis in 0.1 M sodium barbital, pH 8.6. Samples Aa, Bb, Cc, and Dd are protomers obtained from sulfoethylcellulose chromatography of gel-filtered Inhibitor I, dissolved in 8 M urea, and reassociated to the tetrameric species by dilution (1-10) with 10 volumes of water. In nonhybridizing conditions (Ad + Bc and Ba + Cc) the individual protomers were mixed in water only. In hybridization experiments (Aa + Bb; Cc + Dd) the individual protomers were dissolved together in 8 M urea, 0.2 M formic acid, pH 2.8, and after 30 min were diluted 10-fold with water to promote hybridization. The concentration of protein in each sample was adjusted so that each diffusion line represented 200 μg of inhibitor protein. Gel-filtered Inhibitor I is represented by G75PI.

This suggests that the hybridization of protomers at these concentrations does not produce exactly the same populations of hybrids originally present in the inhibitor, and, therefore, gel-filtered inhibitor preparations may not be composed of random hybrids.

Molecular Weights of Inhibitor I and of Its Complex with Chymotrypsin—In previous communications the molecular weight of the crystalline Inhibitor I was not clearly established. The earliest report (1), using the Archibald and sedimentation velocity techniques of ultracentrifugal analysis, gave values of 31,900 and 34,000, derived by extrapolating to zero concentration molecular weight determinations at three concentrations. A later study using a molecular sieving technique gave a molecular weight of 39,000 for nondissociated Inhibitor I and approximately 9,500 for the dissociated subunits (3). On examining the earlier data (1), we concluded that the extrapolation leading to the low values was probably not justified. The means of the experimental values from that study gave molecular weights of 38,000 ± 2,400 by the Archibald technique and 37,500 ± 2,800 by the sedimentation velocity technique. To clarify the discrepancy we have determined the molecular weight of dissociation-purified inhibitor utilizing the ultracentrifugal method of Edelstein and Schachman (16) and have also determined the molecular weight of four times crystallized Inhibitor I by the method of molecular sieving (9) with Sephadex G-75. The ultracentrifugal analysis of dissociation-purified inhibitor gave a partial specific volume of 0.743 cm³ per g and a weight average molecular weight of 39,900 ± 1,000. The molecular weight of crystallized Inhibitor I by the molecular sieve technique was 39,000 ± 1,000.

A Sephadex G-200 column was utilized to determine the molecular weight of the complex between Inhibitor I and chymotrypsin. Complex was formed by saturating a 1-mg sample of dissociation-purified inhibitor with an excess of chymotrypsin (3 mg) in 1 ml of 0.05 M ammonium bicarbonate buffer, pH 7.7. Fig. 13 shows the V/V₀ of the complex on Sephadex G-200 to be representative of a protein of 140,000 ± 4,600 molecular weight. This value is in agreement with a complex containing 4 molecules of chymotrypsin (mol wt 25,000) bound per tetramer of Inhibitor I (mol wt 39,000) and a protomer weight of 9,500 in which each subunit in the tetramer combines with 1 molecule of chymotrypsin.

A summary of our current information concerning the molecular weight of Inhibitor I and of the complex is shown in Table IV.

DISCUSSION

The large scale preparation of Inhibitor I was facilitated by its unusual stability to heat. This stability may be attributable to its subunit nature, and it is possible that the inhibitor dissociates at high temperature. In the dissociating agents, guanidine...
HCl and urea. Inhibitor I dissociates and can be reassociated to its tetrameric form by removal of the reagents. This latter property provided the means of chromatographic separation of monomers, in bulk, with Sephadex G-75 in 4 M guanidine HCl and the separation of individual monomers on sulfoethylcellulose in 8 M urea at pH 2.8. The separation of monomers provided a convenient method for obtaining a highly pure, heterogeneous mixture of Inhibitor I iso-inhibitors. Individual monomers, A, B, C, and D, obtained from sulfoethylcellulose chromatography in urea, provided purified iso-inhibitors composed primarily of the individual protomers.

The four protomeric types that comprise Inhibitor I tetramers appear to be homologous polypeptides. They all are of similar size, all have NH₂-terminal glutamic acid, and the tetramers reassociated from them cross-react immunologically. Differences among the tetramers, derived from individual protomers, were observed in amino acid composition, inhibitory activities toward trypsin and chymotrypsin, and electrophoretic mobilities.

The amino acid compositions of the four protomers clearly differed, indicating that several genes are coding for the monomers. It is also possible that each protomer type may contain more than one variant. Yet, despite these differences the immunological reactivity in the Ouchterlony double diffusion assay exhibits no differences among the protomers.

Differences existing among the protomers were evident in the activities of their reconstituted tetramers toward proteolytic enzymes. The inhibitory capacities toward chymotrypsin and trypsin vary among all four tetrameric species (Table II). Knowledge of the sequences at the active sites of the inhibitors should yield valuable information toward the understanding of the mechanism of inhibition and of the sequences that determine the inhibitory activities, both in specificity and potency.

The ability of the protomers to hybridize with each other to form stable tetrameric iso-inhibitors suggests that native Inhibitor I is a heterogeneous mixture of subunits whose properties reflect those of the individual proteins that comprise it. The immuno-electrophoretic data suggest that partially purified Inhibitor I does exist as a mixture of heterogeneous tetrameric species. It is possible that in nature interchange among protomers might occur depending on the turnover of the individual components in the tetramer. We observed that pepsin degrades tetrameric A and D much more rapidly than tetrameric C and D. Tetrameric A and B are in equally low concentration in Inhibitor I, and this might indicate their instability in vivo. This, as well as the other differences among Inhibitor I protomers, must be considered when looking into the larger problems of its function and regulation in plants.

The molecular weight of either crystallized Inhibitor I or dissociation-purified inhibitor, determined by ultracentrifugation or gel filtration, was near 39,000. The molecular weight of the complex formed between inhibitor and excess chymotrypsin was about 140,000 and suggests that 4 molecules of chymotrypsin are complexed per molecule of inhibitor. The equivalent binding weight of Inhibitor I with chymotrypsin is 9,250 which agrees well with the molecular weight of 9,500 reported for the subunits (3). Each subunit in each tetramer apparently possesses a maximum of one chymotrypsin binding site.

The tetrameric nature of Inhibitor I appears to be unusual among proteinase inhibitors, although, in general, inhibitors having the size of the Inhibitor I protomers are common. For example, lima bean inhibitor exists as a monomer of about 9,000 molecular weight (17), and a small (8,000 mol wt) trypsin and chymotrypsin inhibitor has been reported from soybeans (18) and another from groundnuts (19). Some other plant inhibitors apparently exist as polymers. Millar et al. (20) reported that the Bowman-Birk inhibitor from soybeans is a dimer, and Hochstrasser et al. (21) have reported that several plant proteinase inhibitors may have subunit components, mostly dimers. However, not all plant proteinase inhibitors with molecular weights over 10,000 possess subunit structures. Soybean trypsin inhibitor, the most studied example, does not have subunit structures (22).

No in vivo functions of the plant inhibitors are known for certain. The function of potato Inhibitor I has been studied recently, and it behaves as a specialized interin or storage protein during specific developmental stages of the potato plant (4) and accumulates in leaves upon insect attack, suggesting that it may serve as a protective agent against invading microorganisms or pests (23).

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