Proteinase Inhibitor II from Potatoes: Isolation and Characterization of Its Protomer Components†

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ABSTRACT: Proteinase inhibitor II, an inhibitor of chymotrypsin and trypsin, is a heat-stable protein with a dimeric molecular weight of 21 000 that is a component of Russet Burbank potato tubers. Four monomeric isoinhibitor species of molecular weight 10 500 comprise inhibitor II and were isolated by chromatography on phosphocellulose in 8 M urea. Upon removal of the urea, each monomeric species dimerized to yield homogeneous dimers. The three major protomer species, called B, C, and D, and their homogeneous dimers were further characterized. They have similar molecular weights and amino acid compositions, and each has an N-terminal alanine residue. Dimers of purified protomers B, C, and D exhibited full cross-reactivities with each other in immunological double-diffusion assays. Reconstituted dimers possess two binding sites for bovine α-chymotrypsin, indicating that each monomer possesses one binding site for this enzyme. Significant differences were noted among the reconstituted dimers in their isoelectric points, immunoelectrophoretic mobilities, ion-exchange properties, and their inhibitory reactivities against trypsin. The properties of the inhibitor II dimeric species are similar but not identical to inhibitors IIa and IIb reported from Japanese potatoes (variety "Danshaku-Imo"), indicating the existence of intervarietal, as well as intravarietal, differences among potato tuber inhibitor II isoinhibitors.

Potato tubers are a rich source of a complex group of proteins and polypeptides that potently inhibit several proteolytic enzymes usually found in animals and microorganisms (Solyom et al., 1964; Hochstrasser and Werle, 1969; Iwasaki et al., 1972; Kaiser and Belitz, 1972; Melville and Ryan, 1972; Porath, 1972; Santarius and Belitz, 1972; Hojima et al., 1973b; Kiyohara et al., 1973; Ryan, 1973; Belitz et al., 1974; Ryan et al., 1974; Worowski, 1974; Rouleau and Lamy, 1975). The molecular weights of the potato inhibitors vary from as small as 4100 (Ryan et al., 1974) to as large as 39 000 (Melville et al., 1972; Kiyohara et al., 1973). Potato tuber proteinase inhibitors can be differentiated into two groups, based on their stability to a temperature of 80 °C for 10 min (Kaiser and Belitz, 1972). The heat-stable group of inhibitors has been under study in a number of laboratories, including our own, for several years, and the most thoroughly characterized of these inhibitors has been inhibitor I, mol wt 39 000 (Melville et al., 1972), carboxypeptidase inhibitor (CPI), mol wt 4100 (Ryan et al., 1974), inhibitors IIa and IIb, mol wt 20 700 (Iwasaki et al., 1972), and inhibitor A5, mol wt 26 000 (Santarius and Belitz, 1972). The proteinase inhibitors with molecular weights of 20 000 and above have been found to be composed of protomers, and the protomers can be heterogeneous mixtures of "isoinhibitors" that have similar amino acid compositions, but exhibit different physical and chemical properties (Iwasaki et al., 1972; Melville et al., 1972; Santarius and Belitz, 1972).

With Russet Burbank potatoes, we have found that the heat-stable, salt-free, soluble proteins are comprised almost entirely of proteinase inhibitors that can be separated by gel filtration into three major peaks, having molecular weights of 39 000, 21 000, and 4100–6000 (Melville et al., 1972; and Ryan et al., 1974). The 39 000 molecular weight protein peak is comprised almost entirely of inhibitor I, a tetrameric protein composed of four hybridized isoinhibitor protomer species (Melville et al., 1972). The 4000–6000 molecular weight peak is a doublet that contains at least three inhibitors. One, a carboxypeptidase inhibitor, has been characterized and sequenced (Ryan et al., 1974; and Hass et al., 1975), while the other two inhibitors are presently under study (Hass et al., in press).

In this communication, we report that the 21 000 molecular weight protein peak from the heat stable proteins of Russet Burbank potato tubers contains almost exclusively a dimeric inhibitor that is composed of four isoinhibitor protomer species. The protomers were purified under dissociating conditions and their properties, and those of their reconstituted dimers, are presented. Some of the properties of the reconstituted isoinhibitor protomers are very similar to those of inhibitors IIa and IIb, previously reported by Iwasaki et al. (1972), and inhibitor A5, reported by Santarius and Belitz (1972). We have adopted the designation of inhibitor II, used by Iwasaki (1973), for all of the dimers reconstituted from the isoinhibitor protomers.

Experimental Procedure

Bovine α-chymotrypsin (3× crystallized) was purchased from Worthington Biochemical Corp. BzTyrOEt, TosArgOMe,[1] and trypsin (2× crystallized) were from the Nutritional Biochemical Corp. Sephadex G-25 and G-75 were from Pharmacia. Russet Burbank potatoes were obtained through the Horticulture Department of Washington State University from experimental plots in Central Washington. Potato inhibitor I was prepared by the method of Melville and Ryan (1972). Pancreatic trypsin inhibitor, chymotrypsinogen A, and LBI were from Worthington Biochemical Co. Cyto-

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[1] Abbreviations used are: BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; TosArgOMe, tosyl-L-arginine methyl ester; EDTA, (ethylenedinitrilo)-tetraacetic acid.

chrome *c* and ribonuclease A were from Sigma Chemical Co.

Inhibitory activity against chymotrypsin and trypsin was determined as described previously (Melville and Ryan, 1972). Trypsin and chymotrypsin esterase activities were measured by the method of Hummel (1959). Protein concentrations were determined by the method of Lowry (1951). Concentrations of inhibitor I and II were estimated by immunological techniques according to the method of Ryan (1967) in agar plates containing 1% antiserum. Standards were pure samples of the two inhibitor proteins. The concentration of antigen was determined by plotting 1/concentration of antigen vs. the square of the diffusion ring diameter (Trautman et al., 1971).

Anti-inhibitor II rabbit serum was prepared from rabbits injected subcutaneously over a period of 3 weeks (2 mg twice weekly injection) with twice chromatographed inhibitor II, protomer B, suspended in incomplete Freund's adjuvant. Booster shots were given at 6-month intervals to insure a high titer of antiserum. Two weeks after the termination of injections, 10–20 ml of serum was collected every 2–3 days by bleeding through an ear vein. The blood was stored in a refrigerator at 2 °C and was allowed to clot overnight. The clear serum was removed and stored after centrifugation at 10 000*g* at 0 °C. Control serum was collected in a similar manner before the inoculation series was begun. All antiserum was stored at −20 °C.

Ouchterlony double-diffusion assays (Ouchterlony, 1949) were carried out in 2% Noble agar plates made up of 0.10 M sodium barbital, pH 8.2, in 0.9% sodium chloride and 0.0001% Thimerosol. Immunoelectrophoresis was performed as described previously (Melville and Ryan, 1972) on glass slides (2.5 × 7.5 cm) with 1.5% Noble agar in 0.10 M sodium barbital buffer, pH 8.6. Electrophoresis was for 3.5 h at 3 mA/slide.

Amino acid analyses of purified inhibitor II protomers were carried out according to the method of Moore and Stein (1963). Two-milligram samples were hydrolyzed under nitrogen gas in sealed ampules at 110 °C for 24 and 48 h. A standard norleucine aliquot was added to each sample before hydrolysis. The hydrolysates were dried under vacuum and the residues were dissolved in 0.2 M sodium citrate, pH 2.2, for analysis on a Beckman Model 120C automatic amino acid analyzer. Separate 1.0-mg samples were performic acid oxidized, hydrolyzed, and analyzed separately to determine half-cystine content (Spackman et al., 1958). Corrections for losses of threonine and serine during hydrolysis were made by extrapolation to zero-hydrolysis time, from which the final amino acid content was calculated. Values for other amino acids were determined from the averages of the analyses at 24 and 48 h. Free sulfhydryl groups were determined by titrating the inhibitor (2×10^{-4} M) with 5,5′-dithiobis(2-nitrobenzoate) in 0.003 M EDTA, 0.02 M sodium phosphate buffer, pH 7.4, containing 6 M guanidine hydrochloride (Ellman, 1959). N-terminal amino acid analyses were performed by the method of Edman and Begg (1967) utilizing a JEOL sequence analyzer JAS-47X.

Isoelectric focusing was carried out in polyacrylamide gels by the method of Wrigley (1971) with 1.9% ampholine, pH 3–10. The proteins, 100 μg, were polymerized in the gels for 90 min under fluorescent light and the gels were focused at 35 V/gel for 4 h. After focusing, the proteins were fixed with 10% Cl_3CCOOH for 24 h and stained with Amido black.

Ultracentrifuge analyses were made with a Spinco Model E analytical ultracentrifuge and a standard six-cell Yphantis centerpiece having sapphire windows. An Epon-Alum analytical An-D rotor with a 12-mm, 2.5° double-sector interference cell was utilized at a speed of 40 000 rpm at 20 °C. A computer program was employed for the analysis. The partial specific volumes were calculated from the amino acid analyses (Cohn and Edsall, 1943).

The molecular weight estimations by gel filtration (Whitaker, 1963) were determined with a column of Sephadex G-75 (90 × 0.8 cm) equilibrated with a nondissociating buffer of 0.05 M $(NH_4)HCO_3$, pH 8.2, or a dissociating buffer of 4.4% formic acid, respectively. The column was calibrated with cytochrome *c*, ribonuclease, LBI, chymotrypsinogen, and potato inhibitor I.

Results

Isolation of Proteinase Inhibitor II

Preparation of Crude Inhibitor. Inhibitor II is a component of "crude inhibitor" that contains all of the heat stable proteins of Russet Burbank potato tubers, prepared by methods previously described in the isolation of "crude inhibitor I" (Melville and Ryan, 1972). Soluble proteins were extracted from potato tubers by homogenization in 0.7% sodium dithionite. This extract was filtered to remove excess cellular debris, adjusted to pH 3.0 in 6 N HCl, and centrifuged to precipitate acid-insoluble proteins. Soluble proteins were precipitated by saturation in 70% ammonium sulfate, resuspended in distilled water, and heated at 80 °C for 10 min. The heated mixture was filtered and the clear filtrate was dialyzed for 48 h against distilled water. After dialysis, the solution was filtered to remove precipitated proteins and lyophilized. This preparation was the crude inhibitor and could be stored for months at 2 °C with no appreciable loss of activity.

Gel Filtration of Crude Inhibitor. Three grams of lyophilized crude inhibitor was dissolved in 60 ml of 0.05 M $(NH_4)HCO_3$ buffer, pH 8.2, clarified by centrifugation at 15 000 rpm, and applied to a 100 × 10 cm column of Sephadex G-75 equilibrated with the same buffer. Fifty-milliliter fractions were collected. Fractionation was achieved by upward flow with a rate of approximately 450 ml/h, monitored at 280 nm for protein. Each fraction was assayed for chymotrypsin and trypsin inhibitory activity and later, when antibodies were available, for inhibitor II by immunological assay. A typical Sephadex G-75 elution profile at 280 nm is shown in Figure 1.

The first peak to emerge does not contain appreciable inhibitory activity toward trypsin or chymotrypsin. Its size is variable, from not being present at all, to as large as shown in Figure 1. The peak size diminishes with longer heating times, but this also sacrifices some inhibitors so its presence is tolerated. The second peak (fractions 15–25) was identified as inhibitor I on the basis of (a) its V_e/V_0 ratio from Sephadex G-75, (b) the presence of chymotrypsin and trypsin inhibitory activity, and (c) immunological reactivity with inhibitor I antiserum. The third peak to elute (fractions 30–45), containing inhibitor II, was lyophilized and stored as a dry powder for further purification. This peak was designated as *gel-filtered inhibitor II.* The peak is composed primarily of inhibitor II, as demonstrated by quantitative immunological reactivity with rabbit anti-inhibitor II serum. Figure 1 shows the quantitation of inhibitor I in the fractions, assayed immunologically.

Preliminary Indications of the Protomer Nature of Inhibitor II. Gel-filtered inhibitor II, after dissolution in 8 M urea and subsequent removal of the urea by dialysis, fully recovered its inhibitory activity against both chymotrypsin and trypsin. When a solution of gel-filtered inhibitor II in 8 M urea was

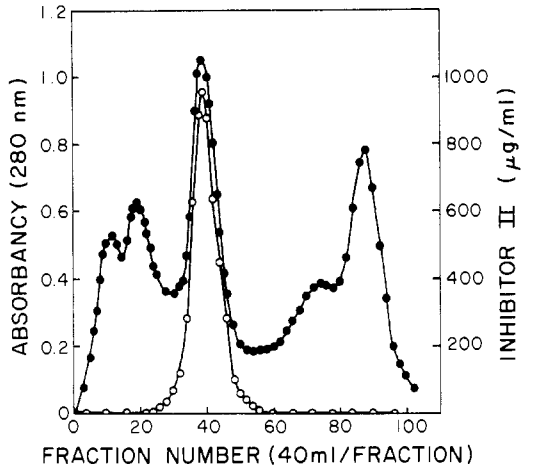

FIGURE 1: Chromatography of crude inhibitor on Sephadex G-75. The column (100 × 10 cm) was equilibrated in 0.05 M $(NH_4)HCO_3$ buffer, pH 8.2. Three grams of crude inhibitor was dissolved in 40 ml of buffer and centrifuged at 15 000*g* for 10 min. The supernatant was applied to the column and 40-ml fractions were collected at a flow rate of 450 ml/h. The absorbancy of each tube was measured (-●-●-) and inhibitor II was determined immunologically (-O-O-) by the radial diffusion assay (Ryan, 1967) after diluting 1:5 with water.

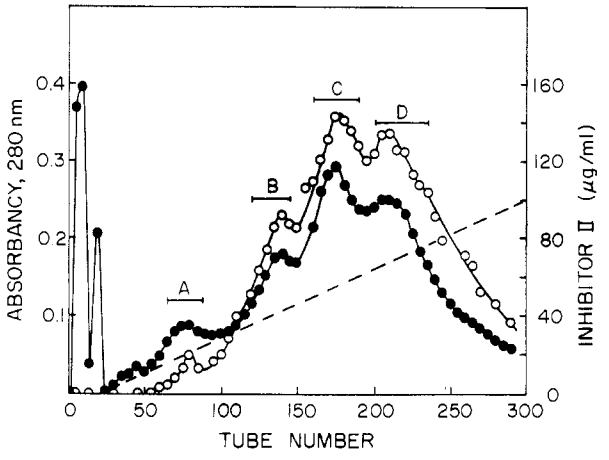

FIGURE 2: Ion-exchange chromatography of inhibitor II protomers on phosphocellulose. Salt-free, gel filtered inhibitor II (120 mg) was dissolved in 3 ml of 0.01 M citrate buffer containing 8 M urea, pH 4.3, and applied to the column as described in the text. Absorbancy of collected fractions (2.0 ml/tube) was determined spectrophotometrically at 280 nm (-●-●-) and inhibitor II concentration was determined immunologically by radial diffusion (-O-O-). Inhibitors in the peaks were collected as indicated, the buffer removed by dialysis, and recovered as salt-free proteins after lyophilization.

chromatographed on a Sephadex G-25 column, equilibrated with 8 M urea, the majority of proteins eluted with a V/V_0 of a protein with half the size of the original inhibitor II. When the urea was removed by dialysis, the inhibitor chromatographed again with its original V/V_0. This data indicated that the inhibitor was composed of protomers. Therefore, the further purification of inhibitor II was performed in dissociating buffers to facilitate recovery of the individual protomers.

Chromatography of Gel-Filtered Inhibitor II Protomers on Phosphocellulose in the Presence of 8 M Urea. Phosphocellulose was prepared by sequential washing with acid, base, and water, followed by equilibration with 0.01 M citrate, pH 4.3, in 8 M urea. A 2.2 × 21 cm column of phosphocellulose, equilibrated with the above buffer, was prepared having a flow rate of 0.75 ml/min. One hundred and twenty milligrams of G-75 inhibitor II in 3 ml of buffer was applied to the column,

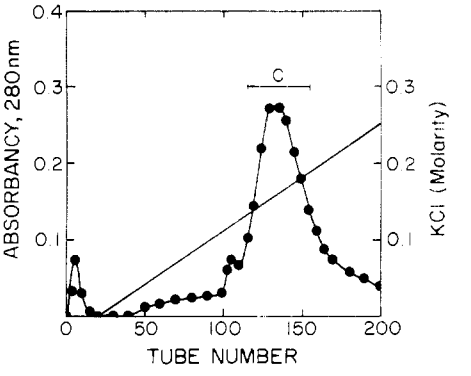

FIGURE 3: Rechromatography of 25 mg of salt-free protomer C. Conditions were as described in Figure 2.

TABLE I: Purification Summary for Inhibitor II.

	Total[a] Protein (mg)	Total[b] Inhibitor (mg)	Fold Purification	Recovery (%)
Original juice	630	10.2	0	58
Original juice, filtered at pH 3.0	452	14.9	2.0	86
0–70% ammonium sulfate precipitate	290	17.2	3.7	100
Heat, 80 °C 10 min, filter	81	11.9	9.1	69
48-h dialysis	43	11.5	16.6	66
Sephadex G-75	11	10	57	58
Chromatography in 8 M urea. Sum of individual protomers	7	7	62	40

[a] Determined by the method of Lowry (1951). [b] Radial diffusion assay (Ryan, 1967).

and eluted with the same buffer. Two-milliliter fractions were collected. After the initial peak was eluted from the column, a linear salt gradient of 0–0.35 M KCl in the citrate–urea buffer was begun. Inhibitor II protomers eluted as shown in Figure 2. Four inhibitor II protomer fractions, labeled A, B, C, and D, were collected and pooled, as shown in Figure 2. Each pooled fraction was dialyzed against distilled water until salt free and lyophilized.

The individually purified protomers were rechromatographed under the conditions described for Sephadex G-75 inhibitor II. As an example, Figure 3 shows the rechromatography of peak C, one of the two major protomer types. The rechromatographed protomers were pooled, desalted, and lyophilized. Inhibitor II, reconstituted from the pure rechromatographed protomers B, C, and D, was utilized for the remainder of the studies in this report. Protomer A was difficult to purify in quantities suitable for study, and its properties are not reported herein.

A summary of the purification of inhibitor II protomers from potato tuber juice is presented in Table I. The products represent a 40% recovery from the starting material with a 68-fold purification. The recovered protomers represented nearly 70% of the proteins in the Sephadex G-75 inhibitor II peak. An increase in immunologically reactive inhibitor was noted during the first three purification steps. The reasons for the increase are not known, but the inhibitor is apparently masked in some way. Calculations of yield were based upon the maximum activity achieved in step 3.

Properties of Inhibitor II

Immunological Characterization of the Purified Protomers.

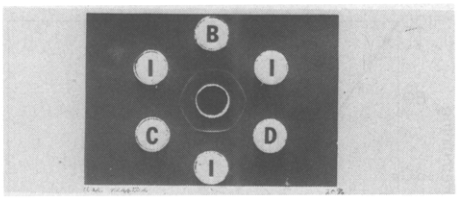

FIGURE 4: Ouchterlony double-diffusion assays of homogeneous reconstituted dimers of inhibitor II protomers B, C, and D compared with the gel-filtered inhibitor II (I) from which they were purified. Details of the analysis are in the text.

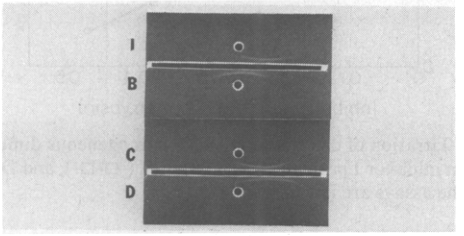

FIGURE 5: Immunoelectrophoresis of homogeneous reconstituted dimers of inhibitor II protomers B, C, and D compared with gel-filtered inhibitor II (I). The negative pole is to the right. Details of the analysis are in the text.

In order to confirm the isoinhibitor relationship of the purified protomers, rabbit antibodies against inhibitor II reconstituted from protomer B were prepared. The antiserum was utilized to identify cross-reactivity with the other protomers and with the gel-filtered inhibitor II by both double-diffusion and immunoelectrophoresis techniques. In double-diffusion assays, the proteins were challenged with the anti-protomer B serum. Figure 4 demonstrates that all four protomers fully cross-reacted with each other and with the gel-filtered inhibitor. This identity was further confirmed with immunoelectrophoresis (Figure 5). Inhibitor II reconstituted from each protomer migrated at different rates, confirming that they possess different ionic properties. In separate experiments, it was demonstrated with crude potato juice that only one cross-reacting protein is present, migrating at the same position as the gel-filtered inhibitor II.

Isoelectric Focusing. Isoelectric focusing of inhibitor II, reconstituted individually from the three major protomer types, is shown in Figure 6. Protomer B exhibited an isoelectric point of about 5.7, protomer C, about pH 7.2, and protomer D about 8.2. In several preparations of protomers B and C, bands appeared as doublets or with minor bands very close by, but no attempts were made to separate them. It is probable that within each protomer type, some further heterogeneity exists. However, protomer D always gave a sharp, single band with an isoelectric point near 8.2.

Amino Acid Analysis. Table II summarizes the results of the amino acid analysis of inhibitor II. The inhibitor II protomers are quite similar, and in general all have a high half-cystine content characteristic of several other naturally occurring proteinase inhibitors (Laskowski and Sealock, 1971). The protomers are also rich in glycine, lysine, aspartic acid, and glutamic acid.

The N-terminal amino acids of protomers B, C, and D were alanine. Protomers C and D also exhibited the presence of small quantities of lysine, indicating the possible presence of heterogeneity within these protomer preparations.

Molecular Weight Determinations. Elution volumes of pure protein samples of known molecular weight were determined with a Sephadex G-75 column (90 × 0.8 cm) equilibrated in nondissociating and dissociating buffers as described under

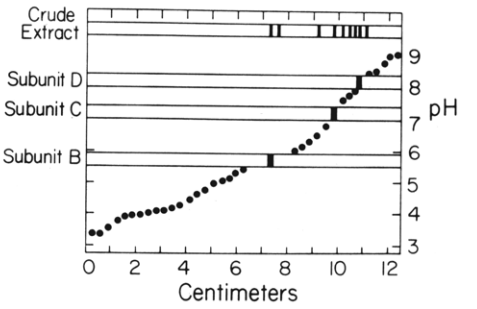

FIGURE 6: Isoelectric focusing of the homogeneous reconstituted dimers of inhibitor II protomers B, C, and D with crude inhibitor II. Details of the analysis are given in the text.

TABLE II: Amino Acid Analyses of Inhibitor II Protomers B, C, and D.

	Protomers					
	B		C		D	
Amino Acids	Moles[a]	Nearest Integer	Moles[a]	Nearest Integer	Moles[a]	Nearest Integer
Aspartic acid	10.04	10	8.82	9	11.09	11
Threonine	6.12	6	6.78	7	6.72	7
Serine	7.67	8	7.54	7–8	7.70	8
Glutamic acid	8.78	9	8.18	8	9.12	9
Proline	6.95	7	7.01	7	6.79	7
Glycine	15.07	15	12.64	13	15.34	15
Alanine	5.92	6	6.54	6–7	6.45	6–7
½-Cystine[b]	12.33	12	12.01	12	11.95	12
Valine	1.55	1–2	1.55	1–2	2.18	2
Methionine	0	0	0	0	0	0
Isoleucine	4.00	4	4.00	4	4.00	4
Leucine	3.38	3	3.70	4	3.40	3
Tyrosine	2.33	2	2.19	2	2.28	2
Phenylalanine	2.99	3	2.95	3	3.14	3
Lysine	8.93	9	9.27	9	8.23	8
Histidine	0.97	1	1.16	1	0.93	1
Arginine	2.39	2	1.91	2	2.98	3
Tryptophan		1		1		1
Totals		99–100		96–99		102–103
Mol wt		10 450		10 280		10 770

[a] Based on 4.00 isoleucine residues. [b] Performic acid oxidation, determined as cysteic acid.

Experimental Procedures. The void volume was determined with blue dextran. Semilog plots of V/V_0 against the corresponding molecular weights of the standard protein samples were linear. The molecular weight of inhibitor II in both dissociating and nondissociating buffers was calculated from its V/V_0 value. The estimate for the molecular weight of inhibitor II containing a chromatographically purified mixture of the four protomers in 0.05 M NH_4HCO_3, pH 8.2, was estimated to be 21 000. In the presence of 4.4% formic acid the molecular weight was 10 000 ± 1000.

Table III summarizes the molecular weight estimations of inhibitor II protomers and its dimers. On the average, inhibitor II in solution appears to have a molecular weight of 21 000 and is composed of two protomers with molecular weights of 10 500.

Binding Stoichiometry with Chymotrypsin and Trypsin. The inhibitory activities of inhibitor II, individually reconstituted from purified protomers B, C, and D, are shown in Figures 7 and 8. All three protomers, as dimers, inhibit 2 mol of

TABLE III: Molecular Weight Estimations of Inhibitor II and Its Protomers.

	Mol Wt
Gel filtration–Sephadex G-75	
0.05 M NH_4HCO_3	21 900 ± 2000
4.4% Formic acid	10 000 ± 1000
Sedimentation equilibrium	
Protomer B	19 250 ± 200
Protomer C	22 300 ± 200
Protomer D	21 300 ± 650
Amino acid analysis	
Protomer B	10 450
Protomer C	10 280
Protomer D	10 770
Average	
Dimer	21 200
Monomer	10 460

chymotrypsin/1 mol of inhibitor, when extrapolated to 100% inhibition, indicating each protomer probably has one binding site for chymotrypsin. Trypsin inhibitor activity is strongest with protomers B and C, but the stoichiometry was not one to one per protomer, but one to one per dimer. Protomer D is a very poor inhibitor of trypsin.

Because of the difficulty in interpreting kinetic data with large proteinaceous inhibitors that bind stoichiometrically (Laskowski and Sealock, 1971), K_I's of the reconstituted inhibitor protomers were not individually determined. However, a K_I for the inhibition of chymotrypsin was estimated from Lineweaver–Burk kinetics of inhibitor II containing a mixture of all of the protomers. These analyses exhibited competitive kinetics that yielded an estimate of the K_I as 2×10^{-8} M.

Stability. Inhibitor II was tested for its stability at room temperature in the following buffers: 0.05 M glycine–HCl, pH 2.2; 0.05 M Tris-HCl, pH 7.4; 0.05 M glycine–NaOH, pH 10.6; 0.05 M Na_2PO_4–NaOH, pH 11.6; and 0.05 M KCl–NaOH, pH 12.0. Aliquots of each solution were assayed immunologically as a function of time to determine the stability of inhibitor II over a wide pH range. At room temperature the inhibitor was found to be stable between pH 2.2 and 10.6, but was rapidly denatured at pH 11.6 and above. When heated for 30 min at 70 °C at pH 2.2 or 7.8, less than 6% of inhibitor II activity was lost. Inhibitor II was stable for several months in solution at room temperature and indefinitely as a lyophilized, salt-free powder.

Discussion

Inhibitor II is a major protein component of the heat-stable proteins of Russet Burbank potato tuber juice. It has a molecular weight of about 21 000 and is composed of dimers of four individually purified protomers of molecular weight of 10 500.

The dimers are dissociated by 8 M urea, and are reassociated when the urea is removed by dialysis. Thus, in 8 M urea, the individual protomers could be isolated on phosphocellulose, eluted with a linear salt gradient, and subsequently recovered in a salt-free, dimeric form after dialysis and lyophilization.

The four protomers that comprise inhibitor II are very similar in their amino acid contents, molecular weights, immunological cross-reactivities, and inhibitory capacities toward chymotrypsin and trypsin, although small individual differences are apparent among them. The most striking differences

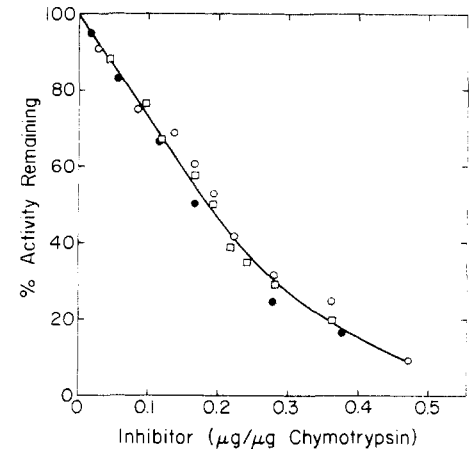

FIGURE 7: Titration of chymotrypsin with homogeneous dimers reconstituted from inhibitor I protomers B (-O-O-), C (-□-□-), and D (-●-●-). Details of the assays are in the text.

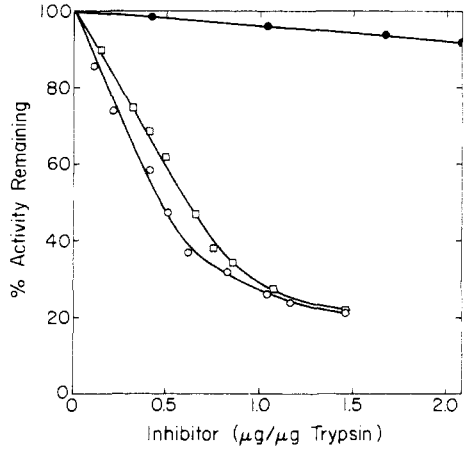

FIGURE 8: Titration of trypsin with homogeneous dimers reconstituted from inhibitor I protomers B (-O-O-), C (-□-□-), and D (-●-●-). Details of the assays are in the text.

among the protomers B, C, and D are (1) their ionic properties that are responsible for their different mobilities in immunoelectrophoresis (Figure 5), (2) their different isoelectric points (Figure 6), and (3) their different elution position during chromatography on phosphocellulose (Figure 2). The different ionic properties of the protomers are not predictable by their amino acid contents, and it is probable that the degree of amidation of glutamic and aspartic acids may play a major role in this regard. A full knowledge of both the amino acid sequences of the inhibitor protomers and the amidation of its acid groups should be helpful in discerning the nature of the differences in the physical and inhibitory properties reported here.

The protomers are all potent inhibitors of chymotrypsin. Each dimer inhibits two molecules of chymotrypsin, and it is probable that each monomer possesses a binding site for this proteinase. On the other hand, the inhibition of trypsin is not constant among the protomers. Reconstituted protomers B and C inhibit trypsin fairly well, but reconstituted D is a very poor trypsin inhibitor. It is not known if the inhibitory active sites for chymotrypsin are responsible for the trypsin inhibition or if separate sites for chymotrypsin and trypsin inhibition are present on each protomer, such as are found with some proteinase inhibitors from beans (Tan and Stevens, 1971; and Odani and Ikenaka, 1972).

The isolation procedure for purifying inhibitor II protomers is very similar to that used to purify the protomers of inhibitor

I, a heat-stable chymotrypsin and trypsin inhibitor previously isolated from potato tuber juice (Melville and Ryan, 1972). Both inhibitor I and II have four protomer species of molecular weights near 10 000, and both are potent inhibitors of chymotrypsin, and weaker trypsin inhibitors. However, from the data available, the two inhibitors do not appear to be homologous. Neither inhibitor type cross-reacts with antiserum prepared against the other (Ryan, unpublished data). Inhibitor I protomers each possess only two half-cystines (Melville and Ryan, 1972), whereas inhibitor II protomers each have 12. However, both inhibitors are rich in lysine, glutamic and aspartic acids, and glycine. The sequences of all four of the protomers of inhibitor I have been determined (Richardson, 1974; and Richardson and Cossins, 1974, 1975). It would be of interest if the sequence of inhibitor II protomers could be achieved and compared with the sequence of inhibitor I. This comparison is of even greater interest in that both inhibitor I and II are induced to accumulate in both potato and tomato leaves in response to wounding (Ryan, 1974). This response is mediated by a wound hormone called PIIF (Proteinase Inhibitor Inducing Factor) (Green and Ryan, 1972), and the inhibitors appear to be the primary gene products of this response (Ryan, 1974b).

Inhibitor II must be considered cumulatively as a family of dimeric inhibitors comprised of a heterogeneous mixture of the four protomer species isolated from phosphocellulose in the presence of urea. We have attempted to demonstrate hybridization by mixing protomers in urea and then diluting with water or neutral buffers, followed by isoelectric focusing or immunoelectrophoresis. No evidence of hybridization could be found. However, little is known of the strength or kinetics of association between monomers, and during the analyses the monomers may have segregated into homogeneous dimers as a result of their net charges.

Inhibitor II is similar in several respects with inhibitors IIa and IIb, isolated from Japanese potatoes ("Danshaku-Imo") by Iwasaki and associates (Kiyohara et al., 1973; and Iwasaki et al., 1972). Because of these similarities, we have maintained their nomenclature by calling all protomers and their dimers inhibitor II. The Japanese potatoes apparently contain only two inhibitor II species. These inhibitors are very similar to inhibitor II from Russet Burbank potatoes in the molecular weights of both monomers and dimers, and in having N-terminal alanines (Iwasaki et al., 1972). However, the Danshaku-Imo inhibitors IIa and IIb have different isoelectric points than any of the Russet inhibitor II dimers, and they also differ somewhat in amino acid contents (Iwasaki et al., 1972). It appears that considerable variability in the number of inhibitor II protomers and in their properties exists among potato varieties. It is not yet clear whether the variability results from genetic differences in the protomers or as a result of post-translational modifications, such as deamidation of aspartic or glutamic acids, or proteolysis.

Inhibitor II, or one of its protomers, may also be homologous with inhibitor A5, isolated from the German potato variety "Maritta" by Santarius and Belitz (1972). Although they report evidence that their inhibitor has a molecular weight of 26 000 and is a tetramer of molecular weight of 6000, the inhibitor is heat stable and has an amino acid analysis quite similar to that of inhibitor II.

Because of the protomer nature of many plant inhibitors, and the presence of isoinhibitors with considerable variability among them, we suggest that a nomenclature for plant proteinase inhibitors be established based on immunological cross-reactivities of the inhibitors with antiserum prepared against well characterized inhibitor proteins to identify classes of inhibitors. By utilizing such an identification system, it will be possible to greatly simplify the often confusing and sometimes conflicting data that appears in the literature. It is imperative that some form of nomenclature be established, preferably based on antigenic determinants rather than such diverse properties as electrophoretic, isoionic, or inhibitory activities.

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Protein Kinase Associated with Tubulin: Affinity Chromatography and Properties†

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ABSTRACT: Rat brain tubulin purified by colchicine–agarose affinity chromatography contains protein kinase activity. The kinase activity can be separated *completely* from tubulin by chromatography on casein columns and is not subsequently retained by colchicine affinity columns. Protein kinase activity associated with purified tubulin does not correlate with the total content of protein kinase activity in brain homogenates, since microtubules isolated from 48 000*g* fetal brain supernatants contain twice as much protein kinase activity than adult microtubules, although the total protein kinase activity is twice as high in the 48 000*g* adult supernatant. The protein kinase of tubulin preparations, while corresponding to a different molecule than tubulin, is probably not simply the result of contamination. These observations are interpreted in terms of specific associations between protein kinase and tubulin complexes. The protein kinase–tubulin association may be an important determinant in the regulation of tubulin function. Fetal tubulin polymerizes twice as well as adult tubulin in the absence of glycerol at the same tubulin concentration. The preferred substrate for the protein kinase either in vivo or in vitro (pH 7.4, 37 °C) is a specific high-molecular-weight protein, distinct from tubulin, which copurifies with tubulin through different kinds of isolation procedures (i.e., colchicine affinity chromatography and ammonium sulfate precipitation followed by diethylaminoethyl-cellulose chromatography). The tubulin-associated protein kinase is completely dependent on cyclic adenosine monophosphate ($K_m = 10^{-7}$ M), as demonstrated by the complete suppression of activity upon addition of the protein kinase modulator, a well-known specific inhibitor of cAMP-dependent protein kinases.

Microtubules are highly organized structures that are ubiquitously present in eukariotic cells and that result from the polymerization of the asymmetric dimeric protein, tubulin (Taylor, 1965; Renaud et al., 1968; Weisenberg, 1972). The formation and dissolution of microtubules are fast processes that occur in cells in response to changing physiological conditions. Mechanisms must therefore exist for regulating the tubulin–microtubule equilibrium. Recent experimental evidence suggests that proteins present in purified tubulin preparations may be fundamental to the processes that regulate microtubule formation (Kuriyama, 1975; Weingarten et al., 1975; Sandoval and Cuatrecasas, 1976). In addition, the multiplicity and diversity of microtubular functions suggest the existence of additional regulatory mechanisms controlling the functional role of the microtubule. Specialized microtubule functions can be the result of different types of tubulin emerging from diverse posttranslational modifications of the protein (Ratt et al., 1971). Alternatively, specialized organizational centers, whose activity may vary according to the physiological conditions of the cell, can functionally diversify an initially homogeneous population of tubulin (Gibbons et al., 1969; Inoue, 1964; Weisenberg et al., 1972; Witman, 1973).

Phosphorylation of tubulin has been proposed as a possible device for regulating tubulin function. Protein kinase activity is present constantly in tubulin prepared from different sources, e.g., *Tetrahymena axonemes* (Muro Fushi, 1973; Kaji, 1973), rat brain (Goodman et al., 1970), and by different procedures (Goodman et al., 1970; Soifer, 1975; Shigekawa et al., 1975). One of the subunits of the tubulin dimer appears to be in a phosphorylated state in vivo (Eipper, 1974a). cAMP,[1] a well-known effector in protein kinase reactions (Brostrom et al., 1970), is able to promote microtubule formation (Prasad et al., 1971) or dissolution (Bitensky et al., 1965) in the intact cell. Despite these findings, there is no conclusive evidence for the existence of a protein kinase specific for tubulin phosphorylation, and a variation in its state of phosphorylation has not been rigorously correlated with any change in function.

The present studies report that: (a) rat brain tubulin purified by affinity chromatography copurifies with a specific protein kinase that can subsequently be separated in an active state by chromatography on casein–agarose columns, (b) brain protein kinase content does not correlate with microtubule protein

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[1] Abbreviations used are: DAC, deacetylcolchicine; IDAC, isodeacetylcolchicine; (I)DAC, isodeacetylcolchicine–deacetylcolchicine; TMCA, trimethylcolchicinic acid; cAMP, adenosine 3′,5′-cyclic monophosphate; GTP, ATP, guanosine and adenosine 5′-triphosphates; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density; Mes, 4-morpholineethanesulfonic acid.