Multiple Molecular Forms of *Ficus glabrata* Ficin. Their Separation and Relative Physical, Chemical, and Enzymatic Properties[1,2]

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Abstract. Six of the proteolytic enzyme components of *Ficus glabrata* ficin have been isolated and shown to be chromatographically homogeneous. The molecular weights, the amino acid compositions, the electrophoretic and chromatographic behavior of the tryptic peptides, and the relative specificities of these 6 components have been determined. Within the experimental precision of the methods all 6 components are identical. They also have identical solubilities in sodium chloride and ammonium sulfate solutions. However, they are markedly different in their chromatographic properties. These multiple molecular forms of *Ficus glabrata* ficin may differ only in their conformational forms (conformers) or they may have minor differences in amino acid sequences which are sufficient to give different conformations and yet not be detected by the usual peptide mapping techniques. At the moment, we favor the latter possibility.

It has been shown that the latex of *Ficus glabrata* contains several proteolytic enzyme components which have markedly different chromatographic properties (2, 24). However, the solubility of these components are essentially identical as they cannot be separated by salt fractionation techniques (24). The realization that *Ficus glabrata* ficin is heterogeneous and the difficulty of isolating, in pure form, the enzyme components have slowed work on the enzyme. Limited data are available on the specificity (9, 13), the kinetic parameters (1, 7, 8) and the amino acid sequence around the essential sulfhydryl group of ficin (28). However, these data were obtained with preparations containing all the multiple molecular forms.

In our continuing work on this enzyme, it became essential to separate the multiple molecular forms of the enzyme and to study some of their physical, chemical and enzymatic properties. In this paper we examine the chromatographic properties, amino acid composition, tryptic peptides and relative specificities of 6 of the 8 proteolytic enzyme components of *Ficus glabrata* ficin. From the data presented here it appears that the properties (other than those based on charge) of the components are so similar that specificity and sequence studies performed on

the unseparated components will be essentially correct.

Materials and Methods

Materials. Two batches of *Ficus glabrata* latex were used. Liquid latex was obtained from Enzyme Development Corporation (Ref. AP/1). This material had been flown in from Peru especially for our use. The second batch was dried latex from Merck and Company (lot 63261).

Carboxymethyl (CM-) cellulose was prepared from Whatman coarse cellulose powder (subsequently sieved to pass 100 mesh screens) according to the method of Peterson and Sober (22). Before use, the CM-cellulose was washed with 0.01 M cysteine to remove any monochloroacetic acid left from the alkylation of the cellulose.

Cysteine hydrochloride was from Mann Research Laboratories, Incorporated. Sodium tetrathionate was from K and K Laboratories; Versene was from Eastman Kodak Company, and casein (Hammarsten quality) was from Nutritional Biochemical Corporation. Trichloroacetic acid was from Baker Chemical Company. Crystallized bovine serum albumin (lot V 69012) was from Armour Pharmaceutical Company. Bio-Gel P-30 (lot 50733, 100–200 mesh) was from Bio-Rad Laboratories. Sephadex G-100 (lot TO 8080, 40–120 μ) and Blue Dextran 2000 were from Pharmacia Fine Chemicals, Incorporated.

Pepsin (hog stomach, 2 × crystallized, lot 1827), trypsin (beef pancreas, 2 × crystallized, lot H 1090) and α-chymotrypsin (beef pancreas, 3 × crystallized, lot H 1252) were from Mann Research Laboratories, Incorporated. Cytochrome *c* (crystallized, horse heart, type III, lot 114B-7150) was obtained from Sigma Chemical Company.

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All chemicals used were of reagent grade and deionized water prepared by passage through a Barnstead Bantam Dimineralizer was used.

Methods. Purification of Proteolytic Enzyme Components of Ficus glabrata. The 2 batches of *Ficus glabrata* latex were purified independently by a modification of the salt fractionation procedure of Hammond and Gutfreund (9). The results are summarized in table I. The dried latex was suspended in 2 liters of 2.0×10^{-2} M cysteine to make a 20 % solution and stirred for 2 hr at 4°. Sodium tetrathionate (11.5 grams, 5×10^{-2} M) was added to inactivate the enzyme and the solution was centrifuged, then filtered through a layer of glass wool to remove insoluble material (Step 2, table I). The liquid latex was centrifuged at 0° and 50,000*g* for 1 hr to remove the gums. The supernatant liquid was filtered through glass wool and cysteine was added to give a final concentration of 1.0×10^{-2} M. After stirring for 3 hr at 4°, 14.4 grams sodium tetrathionate (5.0×10^{-2} M) was added. This represents the material in Step 2, table I. The remainder of the procedure was the same for both batches of latex.

The solution was dialyzed for 16 hr against 1.0×10^{-4} M sodium tetrathionate $- 1.0 \times 10^{-4}$ M Versene. Solid ammonium sulfate was added slowly with constant stirring, to 50 % saturation at 0°. After sitting for 3 hr with continuous stirring, the precipitated protein was removed by centrifugation. The precipitated protein was suspended in cold water and dialyzed for 16 hr against 1.0×10^{-4} M sodium tetrathionate $- 1.0 \times 10^{-4}$ M Versene (Step 3). Step 3 was repeated at 30 % saturated (0°) ammonium sulfate (Step 4). The dissolved and dialyzed precipitate fraction from Step 4 was made 50 % saturated (0°) with solid sodium chloride, stirred for 3 hr at 4°, centrifuged, the precipitate suspended in cold water and dialyzed against a solution of sodium tetrathionate $-$ Versene (Step 5). Step 5 was repeated at 30 % saturated sodium chloride (Step 6). The dialyzed solution was then chromatographed on CM-cellulose.

The equilibration of CM-cellulose and the preparation of columns were as described previously (24). The preparative columns were 2.1×45 cm. Aliquots of dialyzed solution from Step 6 (table I) were placed directly on top of the column and the protein eluted from the column with a step-wise elution scheme as shown in Fig. 1. Fractions of a single component, as represented by the cross-hatched bars in Fig. 1, were pooled and precipitated with ammonium sulfate (50 % saturated at 0°). The precipitate was suspended in a minimum volume of cold water and dialyzed against 1.0×10^{-4} M sodium tetrathionate $- 1.0 \times 10^{-4}$ M Versene for 16 hr. An aliquot of each component was then chromatographed on an analytical column of CM-cellulose (1.2×45 cm) under the conditions described in Fig. 2. Elution was by a linear gradient of increasing sodium chloride concentration designed to give conditions under which the component would be eluted at a volume of eluate at least 3 times that of the void volume of the column. If a component were found not to be homogeneous by this criterion it was again subjected to preparative chromatography followed by analytical chromatography to assess the degree of homogeneity. Components 2 and 8 were homogeneous after the first chromatography, components 3, 5, 6, and 7 after the third chromatography and components 4 and 9 were still heterogeneous after the fourth chromatography. For this reason, components 4 and 9 were not used for further studies.

Protein and Activity Determinations. The eluate fractions from the columns were read at 280 mμ in a Beckman DU spectrophotometer. The extinction coefficients of the various components were determined by the use of a modification of the biuret method (6) to determine the protein content of the solutions. In the modification the biuret reagent was prepared so as to be 4 times the normal concentration. In the determination 1.0 ml of biuret reagent and up to 4.0 ml of protein solution were used. This modification permitted the determination of the protein content of solutions more dilute than those normally needed for the biuret protein determination.

Activity determinations were performed on casein at pH 7.0 and 35.0° according to the procedure of Kunitz (18) as modified for the assay of ficin activity (24). A unit of enzyme activity is defined as that amount which gives an absorbance increase of 1.00 in 1 min under these conditions.

Amino Acid Analysis. Hydrolysis of the proteins was carried out according to the procedure of Moore and Stein (20) and the amino acids were separated and quantitatively determined on a Technicon Auto-Analyzer using an analysis time of 22 hr. Sufficient protein samples from each of the 6 homogeneous components (2, 3, 5, 6, 7, and 8) were dialyzed exhaustively against 0.1 M KCl, against deionized water and then lyophilized. Hydrolysis was carried out at 110 $\pm$ 1° for 20, 40, and 70 hr.

Cystine and cysteine were determined as cysteic acid according to the method of Hirs (10).

Areas on the chromatogram representing the individual amino acids were integrated and correlated with standard curves run with the same batch of reagents by use of a Technicon Integrator/Calculator Model AAG. These values, in micromoles of each amino acid, together with data on the volume of hydrolyzate applied to the column, total weight of protein, total volume of hydrolyzate, and molecular weight of each amino acid were entered into a computer. The output from the computer gave the average combining ratios, including standard deviation, of each amino acid in the protein, minimum molecular weight of the protein and the percent of the protein accounted for.

Molecular Weights by Gel Filtration. The

molecular weights of *Ficus glabrata* ficin components 2, 3, 5, 6, 7, and 8 were determined by gel filtration according to the method of Whitaker (26). Columns prepared from Bio-Gel P-30 (1.2 × 196 cm) and Sephadex G-100 (1.2 × 193 cm) were calibrated by the use of Blue Dextran 2000 (to determine the void volume), pepsin, trypsin, α-chymotrypsin and cytochrome *c*. The volumes of the fractions (approximately 1 ml) collected from the columns were determined by weighing.

Peptide Mapping of the Components. Fifty mg of each of the components was exhaustively dialyzed against 0.1 M KCl followed by exhaustive dialysis against deionized water. The material was then lyophilized. Twenty mg of each component was reduced and alkylated essentially as described by Hirs (11) with careful observation of the precautions he described.

After exhaustive dialysis of the alkylated protein against deionized water, it was subjected to tryptic hydrolysis. The alkylated component was placed in the reaction vessel of a Radiometer pH-Stat (Titrator 11, Titragraph SBR 2c, pH meter 25, biuret SBU1A) which was thermostated at 35.0° and continually flushed with N_2. After the pH of the solution was adjusted to 7.8, 0.1 ml of trypsin (1 mg/ml in 0.001 N HCl) was added and the reaction allowed to proceed for 6 hr. The pH was maintained at 7.8 by automatic addition of 0.04 N NaOH. At the end of 6 hr, another 0.1 ml aliquot of trypsin solution was added and the reaction continued for another 6 hr. At this time the rate of uptake of base was negligible. The digest was lyophilized and dissolved in 0.2 ml of 0.1 M NH_4HCO_3.

The digest was subjected to high voltage electrophoresis followed by paper chromatography. An aliquot of the digest containing 1 mg of material was applied at the center of a 2 × 22.5 inch strip of Whatman 3MM cellulose paper. The spot was dried, the strip was carefully wet with the buffer (pyridine: acetic acid:water, 25:1:225, v/v) and placed on the flat plate of a Savant High Voltage Electrophoresis apparatus. Electrophoresis was performed at 3000 volts for 35 min. (The current varied from 195 *ma* at the start to 225 *ma* at the finish.) The strip was removed, dried for 20 min at 80° and then sewn between a paper strip (3.5 × 22.5 inch) and a full-sized sheet (18.5 × 22.5 inch) of Whatman 3MM cellulose paper. Chromatography was performed at right angles to the direction of electrophoresis for 15.5 hr using the upper phase of a butanol:acetic acid:water (4:1:5, v/v) solution. The atmosphere was saturated with the lower phase of the solvent mixture. After drying at 80° for 30 min the chromatogram was sprayed with 0.2% ninhydrin in methanol and again dried at 80° for 20 min. The spots were outlined in pencil.

Relative Specificity of the Proteolytic Enzyme Components. The extent of hydrolysis of casein by each of the components was measured by following the uptake of NaOH in a pH-Stat at 35.0° and pH 8.0. The reaction mixture consisted of 1.5 ml of 2% casein, 0.1 ml of 0.25 M Versene, 0.1 ml of 0.25 M cysteine·HCl, 0.25 ml of 0.25 M NaOH and 1.05 ml of water. The pH was adjusted to pH 8.0 and 0.1 ml (0.5 mg/ml) of a solution of a component was added. The uptake of base was followed until the reaction reached completion. At this point 0.1 ml of a solution of another component was added to ascertain if further hydrolysis could occur. The average pK of the amino groups liberated by the hydrolysis was determined by titrating a hydrolyzed reaction mixture of casein from pH 6 to 10. Given the average pK of the liberated amino groups, the amount of base uptake, the weight of casein used and the molecular weight of casein, the total number of peptide bonds hydrolyzed per molecule casein can be calculated.

Results

Purification of the Proteolytic Enzyme Components of Ficus glabrata *Latex.* A summary of the purification of *Ficus glabrata* latex by ammonium sulfate and sodium chloride fractionation is given in table I. In this procedure, as well as all steps throughout the purification, the enzyme was kept inactivated with sodium tetrathionate which reacts with the essential sulfhydryl group of the enzyme. From an original total activity of 182,000 units in the crude liquid latex, 23,300 units (12.8% recovery) of activity were obtained with a 3.65 fold purification. In a similar purification starting with dried latex, 69,500 units of purified enzyme were obtained from a starting value of 342,000 units (20.3% recovery). In this case the fold purification was 7.43. This reflects the lower specific activity of the dried latex. In some other preparations we have started with dried latex with considerably lower specific activity. Regardless of the specific activity of the starting material one ends up with a specific activity of 2.2 to 2.5 for all preparations. The chromatographic patterns on CM-cellulose were qualitatively identical for all preparations.

An aliquot of the salt-purified enzyme was further fractionated on a CM-cellulose column as shown in Fig. 1. With the exception of the presence of 1.0 × 10⁻⁵ M sodium tetrathionate in all the buffers, the step-wise elution scheme was identical to that used previously for uninhibited enzyme (24). The chromatographic pattern (first chromatography, Fig. 1) with the inhibited enzyme was essentially identical to that with the uninhibited enzyme (24).

Table II gives a summary of the results obtained from the first chromatography of material from Step 6 of table I on CM-cellulose. From a total of 92,800 units of activity of salt-purified enzyme, 48,300 units were recovered. The difference between the 2 values is largely the result of saving only the center part of each component (fractions indicated

Table I. *Purification of* Ficus glabrata *Latex by Ammonium Sulfate and Sodium Chloride Fractionation*

Step	Condition	Total volume	Activity per ml[1]	Protein per ml[2]	Specific activity	Fold purification	Total activity	Activity recovery
		ml			Liquid latex			*%*
1	Original latex	944	191	317	0.603	1.0	182,000	100
2	Removal of gums[3]	940	155	258	0.601	0.997	146,000	80.3
3	50 % $(NH_4)_2SO_4$	890	127	159	0.799	1.33	113,000	62.1
4	30 % $(NH_4)_2SO_4$	905	103	76	1.35	2.24	93,300	51.3
5	50 % NaCl	910	78.8	46.5	1.69	2.80	71,800	39.4
6	30 % NaCl	180	129	58.8	2.20	3.65	23,300	12.8
					Dried latex			
1	Original powder[4]	2050	167	500	0.334	1.0	342,000	100
2	Centrifugation[5]	2065	136	103	1.33	3.98	281,000	82.2
3	50 % $(NH_4)_2SO_4$	1780	161	205	0.783	2.34	286,000	83.7
4	30 % $(NH_4)_2SO_4$	1570	134	118	1.14	3.43	210,000	61.4
5	50 % NaCl	1395	98.3	48.6	2.02	6.05	137,000	40.2
6	30 % NaCl	870	80.0	32.2	2.48	7.43	69,500	20.3

[1] Determined with casein as substrate (Δ absorbance/min.).
[2] Absorbance at 280 mμ.
[3] Crude latex after inactivation with sodium tetrathionate, dialysis and centrifugation to remove plant gumns and insoluble material.
[4] 400 g dried powder suspended in 2000 ml of 2.0 $\times$ 10^{-2} M cysteine.
[5] Suspended powder after inactivation with sodium tetrathionate, dialysis and centrifugation to remove insoluble material.

by cross-hatched bars). Based merely on the specific activities of the components as compared with the specific activity of the salt-purified enzyme there was very little further increase in fold purification. This indicates that the salt-purified enzyme is composed entirely of the various proteolytic enzyme components. When the fold purification is based on the fraction of each component present in the

salt-purified enzyme then the fold purification by CM-cellulose ranges from 21 for component 5 to 118 for component 7.

The material representing each component was checked for homogeneity on analytical CM-cellulose columns (Fig. 2). If the material was not homogeneous by this criterion it was rechromatographed again as shown in Fig. 1. The data for the second,

Table II. *Results of Preparative Carboxymethyl Cellulose Chromatography of* Ficus glabrata *Ficin After Ammonium Sulfate and Sodium Chloride Salt Fractionation*

Material	Total volume	Activity per ml[1]	OD $_{280}$	Specific activity[2]	Fold purification[3]	Total activity units	Activity recovery
	ml						*%*
Original latex[4]	2,990	175	443	0.396	1.0	524,000	100
Salt purified enzyme[4]	1,050	88.4	36.7	2.41	6.09	92,800	17.7
Component 1 Discarded because of limited amount of material.							
Component 2	49	16.4	7.29	2.25	5.69 (114)	806	0.154
Component 3	260	36.6	14.4	2.55	6.44 (30)	9,530	1.82
Component 4	95	31.5	12.6	2.50	6.34 (78)	2,990	0.571
Component 5	140	129	50.4	2.56	6.47 (21)	18,000	3.44
Component 6	198	20.2	9.58	2.11	5.33 (74)	4,000	0.764
Component 7	60	40.0	17.2	2.32	5.86 (118)	2,400	0.458
Component 8	175	42.3	15.5	2.73	6.89 (61)	7,400	1.41
Component 9	51	62.8	23.0	2.73	6.89 (61)	3,200	0.611

[1] Determined with casein as substrate (Δ absorbance/min.).
[2] Units activity/OD$_{280}$.
[3] The values in parentheses indicate the fold purification based on the approximate amount of each component present in the salt purified enzyme as shown by chromatography on CM-cellulose (Fig. 1), the values not in parenthesis represent the fold purification based only on comparison of specific activity with the original.
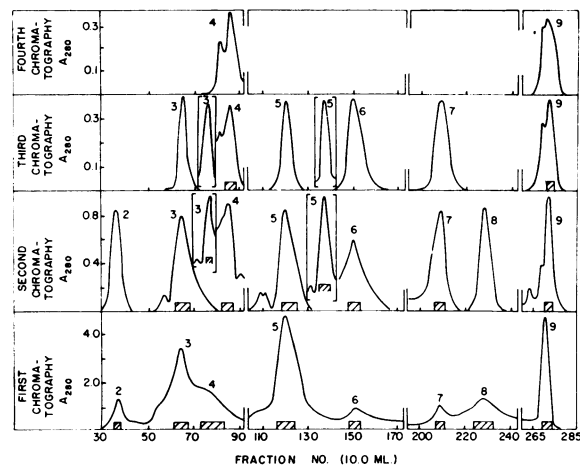[4] Combined preparations of table I.

FIG. 1. Chromatography of salt-purified *Ficus glabrata* ficin on CM-cellulose followed by rechromatography of the individual components on CM-cellulose. The cross-hatched bars indicate the fractions of each component pooled for rechromatography. Chromatography was performed at 2° on 2.1 × 45 cm columns initially equilibrated with 0.01 M phosphate, pH 7.0. The following step-wise changes in eluting solution (all prepared in 0.01 M phosphate at pH 7.0) were: fraction 36, 0.04 M NaCl; fraction 99, 0.1 M NaCl; fraction 130, 0.12 M NaCl; fraction 192, 0.20 M NaCl and fraction 252, 1.0 M NaCl. All eluting solutions contained 1.0 × 10⁻⁵ M sodium tetrathionate.
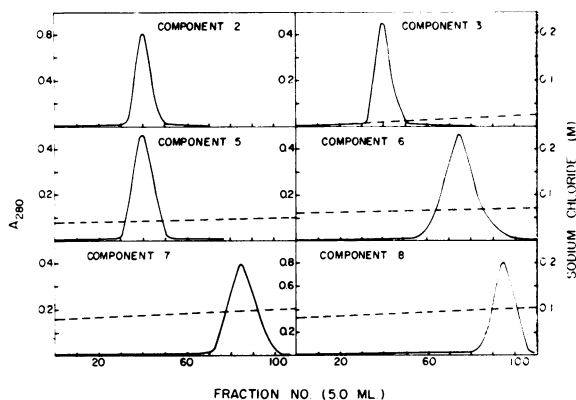

FIG. 2. Chromatography of purified proteolytic enzyme components on analytical columns (1.2 × 45 cm) of CM-cellulose at pH 7.0 and 2°. Component 2 was chromatographed under starting buffer conditions of 0.005 M acetate buffer, pH 5.0. The other components were chromatographed on columns initially equilibrated with 0.01 M phosphate, pH 7.0. Elution was with a linear gradient prepared as follows (400 ml in each container; mixing container concentration listed first; all solutions had 0.01 M phosphate; pH 7.0) : component 3, 0.00 M NaCl — 0.06 M NaCl; component 5, 0.04 M NaCl — 0.12 M NaCl; component 6, 0.06 M NaCl — 0.11 M NaCl; component 7, 0.08 M NaCl — 0.24 M NaCl; component 8, 0.08 M NaCl — 0.24 M NaCl. The dashed straight line indicates the NaCl concentration.

and subsequent, chromatographies represent a composite of chromatograms run for each component. As shown by the data of Fig. 1, each component rechromatographed in the identical position it was eluted initially. In the second chromatography of component 4 there was an appreciable amount of component 3. This material representing component 3 eluted in the position expected. This is indicated by the data being placed in brackets. More than half of the material assigned to component 6 obtained from the first chromatography was component 5 as shown by results of the second chromatography (Fig. 1, the material representing component 5 chromatographed in the position expected of component 5). As shown by the data for the third chromatography components 3 and 5 separated from components 4 and 6, respectively during the second chromatography were homogeneous. Even a fourth chromatography of components 4 and 9 failed to give chromatographically homogeneous material. Because of limitations on material no further attempts were made to purify these components and further studies were not performed with them.

The chromatographic homogeneity of components 2. 3, 5, 6, 7, and 8 is indicated by the data of Fig. 2 where the conditions were designed to give true chromatography of the components. Further data on these chromatographically homogeneous components are summarized in table III. It will be noted that the specific activities of $E^1 \%_{280}$ values for these components are nearly identical.

Amino Acid Analysis of the Proteolytic Enzyme Components. The amino acid composition, expressed in residues per 10,000 grams of protein, for components 2, 3, 5, 6, 7, and 8 are given in table IV. The most surprising results of these data is the great similarity in amino acid composition among the components. While in a few cases there appear to be approximately one residue difference in certain amino acids among the components it is hard to decide whether this difference is outside of experimental error. In the case of components 2, 3, 5, and 6 there appears to be more tyrosine than in components 7 and 8 while components 7 and 8 appear to have more histidine and lysine than the other components. The arginine content of components 2 and 3 appears to be statistically different from that of components 5 and 6 which are in turn statistically different from that of components 7 and 8. The ammonia content of components 3 and 6 are markedly different from that of the other components. However, determination of ammonia directly from the autoanalyzer data is semiquantitative at best. One must conclude that, if the components don't all have the same amino acid composition, they are extremely similar. The amino acid composition reported here for the several components is quite similar to that reported previously for *Ficus glabrata* ficin which had not been separated into its individual components (19, 25) and for one component (presumably component 5) isolated from

Table III. *Summary of Purification, to Chromatographic Homogeneity, of the Proteolytic Enzyme Components in* Ficus glabrata *Latex*

Component	Total volume	Activity per ml[1]	Protein	Specific activity[2]	Fold purification[3]	Total activity	Activity recovery	$E_{280}^{1\%}$
	ml		mg/ml				%	
2[5]	49	16.4	3.36	2.25	5.68 (114)	806	0.154	21.7
3[6]	88.5	62.3	11.0	2.55	6.44 (30)	5510	1.05	22.2
5[7]	86	112	20.3	2.55	6.44 (21)	9660	1.84	21.6
6	40	43.3	8.59	2.15	5.43 (75)	1730	0.330	23.4
7	22.5	54.2	9.85	2.69	6.79 (136)	1220	0.233	20.4
8[5]	175	42.3	7.55	2.73	6.89 (61)	7400	1.41	20.5

[1] Determined with casein as substrate (Δ absorbance/min).

[2] Units activity/OD_{280}.

[3] Based on the original crude latex. The values in parenthesis indicate the fold purification based on the approximate amount of each component present in the salt purified enzyme as shown by chromatography on CM-cellulose (Fig. 1), the values not in parenthesis represent the fold purification based only on comparison of specific activity with the original.

[4] Based on the original crude latex.

[5] Homogenous after first preparative chromatography.

[6] Another 200 units activity of component 3 was obtained upon further chromatography of component 4. This material is not included in the above summary.

[7] Another 809 units activity of component 5 was obtained upon further chromatography of component 6. This material is not included in the above summary.

the mixture (2). However, the amino acid composition is quite different from that of *Ficus carica* variety Kadota ficin components C and D (17).

Peptide Mapping of the Proteolytic Enzyme Components. As indicated above, the amino acid composition of components 2, 3, 5, 6, 7, and 8 were very similar if not identical. It does not follow from the identity of amino acid composition that the amino acid sequence of the components is similar or identical. Innumerable examples, starting with the

Table IV. *Amino Acid Analysis of the Proteolytic Enzyme Components in* Ficus glabrata *Latex[1]*

Amino acid	Component 2		Component 3		Component 5		Component 6		Component 7		Component 8	
Aspartic acid	7.61	± 0.25	7.38	± 0.11	6.93	± 0.43	7.64	± 0.29	7.64	± 0.13	8.11	± 0.31
Threonine[2]	3.57		3.70		3.19		3.49		3.55		3.41	
Serine[2]	5.70		5.62		5.40		5.64		5.67		5.76	
Glutamic acid	9.27	± 0.45	9.25	± 0.08	9.81	± 0.12	9.26	± 0.29	8.85	± 0.35	9.38	± 0.03
Proline	4.45	± 0.04	4.28	± 0.06	4.48	± 0.10	3.64	± 0.05	3.94	± 0.05	3.80	± 0.19
Glycine	12.3	± 0.12	12.3	± 0.10	12.3	± 0.10	11.6	± 0.42	11.7	± 0.64	11.9	± 0.07
Alanine	7.79	± 0.06	8.07	± 0.02	7.68	± 0.11	8.20	± 0.20	7.79	± 0.10	7.86	± 0.06
Valine	6.72	± 0.12	6.58	± 0.05	6.90	± 0.12	7.41	± 0.18	6.79	± 0.24	6.90	± 0.37
1/2-Cystine[3]	2.52	± 0.08	2.54	± 0.01	3.09	± 0.08	3.12	± 0.01	3.12	± 0.10	2.70	± 0.08
Methionine	1.43	± 0.06	1.45	± 0.04	1.87	± 0.01	1.65	± 0.06	1.20	± 0.04	1.29	± 0.04
Isoleucine	3.63	± 0.22	3.49	± 0.02	2.78	± 0.13	2.94	± 0.19	2.81	± 0.04	3.00	± 0.05
Leucine	6.61	± 0.21	6.33	± 0.11	5.98	± 0.19	5.66	± 0.12	6.42	± 0.37	6.75	± 0.06
Tyrosine	5.76	± 0.01	5.90	± 0.02	6.11	± 0.10	5.93	± 0.22	4.69	± 0.05	4.62	± 0.13
Phenylalanine	2.27	± 0.12	2.12	± 0.07	2.15	± 0.04	2.17	± 0.07	2.17	± 0.04	2.14	± 0.12
Lysine	3.11	± 0.16	2.69	± 0.02	2.47	± 0.07	2.70	± 0.09	3.33	± 0.12	3.18	± 0.30
Histidine	0.721	± 0.16	0.720	± 0.07	0.566	± 0.002	0.705	± 0.20	1.19	± 0.09	1.17	± 0.18
Arginine	3.49	± 0.16	3.40	± 0.01	4.32	± 0.05	4.37	± 0.27	5.16	± 0.06	5.15	± 0.37
Tryptophan[4]	4.36	± 0.21	4.99	± 0.10	4.60	± 0.15	4.74	± 0.09	4.46	± 0.12	4.19	± 0.13
Ammonia[5]	12.8	± 0.01	6.24	± 0.40	10.8	± 0.32	29.3	± 2.80	12.3	± 0.05	11.6	± 0.51

Amino acid residues per 10,000 grams protein

[1] Values listed are the average of the experimental values of 20, 40, and 70 hr hydrolysis (to 0.01 residue) and the standard deviation

[2] Corrected for decomposition during hydrolysis.

[3] Determined as cysteic acid

[4] Determined spectrophotometrically.

[5] Determined as a constituent of the protein hydrolyzate along with the amino acids. The values should be considered as approximate only.

classical work of Ingram on hemoglobin (12) have demonstrated the utility of peptide mapping for showing small differences among similar proteins. Initially, an attempt was made to prepare peptide maps of the components following alkaline denaturation. The components were treated with 0.01 M NaOH for 45 min at room temperature in order to denature them. They were then treated with trypsin as described in Methods Section. After digestion there was a large amount of insoluble material present. When the protein was reduced and alkylated before tryptic digestion the digest was completely soluble. The results are shown in Fig. 3. The results were so surprising that they were

repeated several times beginning with different preparations of reduced and alkylated proteins. The results were consistently the same. All 6 components gave 13 peptides. As with the amino acid composition, there appears to be little if any difference among the components with respect to their peptide maps. Peptide 4 of component 2 appears to behave a little differently from peptide 4 of the other components. Peptides 12 and 13 of components 6 and 8 appear to have slightly different properties than do these peptides of the other components.

Molecular Weights of the Proteolytic Enzyme Components of Ficus glabrata *Latex.* The molecular weights of components 2, 3, 5, 6, 7, and 8 were determined by 3 methods: gel filtration, amino acid composition, and peptide mapping. The last 2 are interrelated. The results are shown in table V. The molecular weights as determined by gel filtration on Sephadex G-100 are considerably lower than those determined by amino acid composition (with exception of components 7 and 8) and by peptide mapping. This anomolous behavior of ficin on Sephadex G-100 has been reported before (2). It might be suggested that this behavior is due to retardation caused by the aromatic residues of the protein as it is well-known that aromatic amino acids, peptides containing aromatic amino acids, and other aromatic compounds (5, 23) are retarded on Sephadex. To check this possibility, the molecular weights were determined on Bio-Gel P-30. The molecular weights determined on this polyacrylamide material were even lower than those obtained on Sephadex G-100. The explanation for the peculiar behavior of the ficin components on gel filtration is unknown. It does not appear that all ficins show this peculiar behavior since the molecular weights of *Ficus carica* variety Kadota ficin components C and D determined by gel filtration were similar to those determined by other methods (17).

The lower molecular weights of components 7 and 8 as calculated from the amino acid composition and by peptide mapping are largely the result of an apparently higher content of histidine and arginine in these 2 components.

Comparative Specificity of the Proteolytic Enzyme Components. The comparative specificity of proteolytic enzymes can be determined conveniently
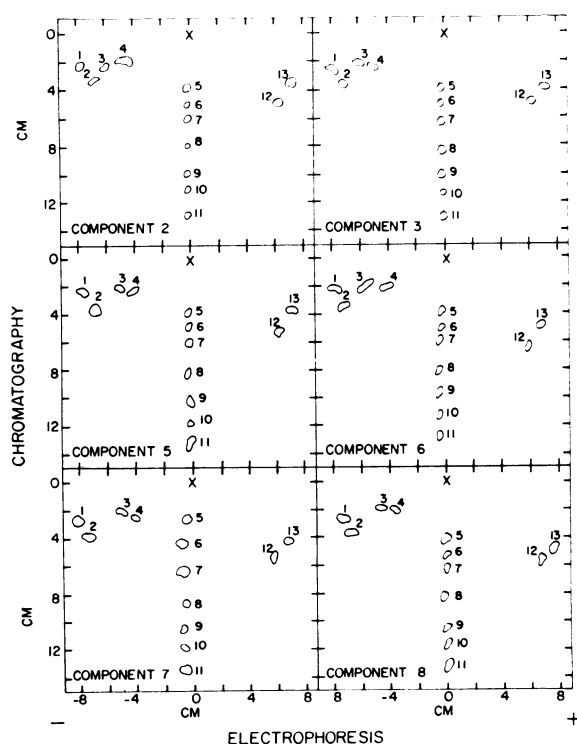

FIG. 3. Tryptic peptide maps of the proteolytic enzyme components of *Ficus glabrata* ficin. X indicates position at which the hydrolyzate was applied. See text for details of experiment.

Table V. *Molecular Weights of Proteolytic Enzyme Components of* Ficus glabrata *Latex*

Component	Method			
	Sephadex	Bio-Gel	Amino acid composition[1]	Peptide map[1]
2	12,025 ± 55	9,817	16,481 ± 506	18,200 ± 880
3	11,520 ± 70	8,590	16,125 ± 1,935	19,700 ± 100
5	10,420 ± 50	9,954	19,538 ± 775	17,700 ± 290
6	11,455 ± 25	10,425	17,062 ± 523	17,000 ± 860
7	11,900 ± 440	9,078	10,270 ± 342	14,200 ± 250
8	11,750 ± 220	8,790	9,861 ± 842	14,400 ± 1,200

[1] Minimum molecular weights.

and rapidly by adding a second enzyme to a digest after another enzyme has had an opportunity to hydrolyze all the peptide bonds in the protein for which it has specificity. The experiment is then repeated in a reverse fashion. If the 2 enzymes have identical specificity no further hydrolysis will occur on addition of the second enzyme to a digest prepared with the first enzyme. The results of such experiments performed with components 2, 3, 5, 6, 7, and 8 are shown in table VI. It appears that the specificity of all components is nearly identical. Components 2, 3, and 6 have identical specificity but components 5, 7, and 8 appear to be able to hydrolyze 1 more peptide bond in casein. The specificity of ficin, as determined on synthetic substrates, is directed largely toward the peptide bonds in which arginine, lysine and glycine contribute the carbonyl group. The number of glycine, arginine and lysine residues in casein [based on a molecular weight of 20,000 (4)] are 7, 5, and 11, respectively (15). Therefore, the total of these residues, 23, corresponds exactly to the maximum number of peptide bonds hydrolyzed by the components (22.9 for component 8).

Discussion

This work, as well as that reported previously (2, 24) indicates that Ficus glabrata latex contains a number of proteolytic enzyme components. In this work, the components were inactivated with sdoium tetrathionate in order to prevent any autolysis from occurring during the purification. The chromatographic behavior of the inhibited enzyme components is essentially identical to the behavior of the

Table VI. *Comparative Specificity Studies on the Proteolytic Enzyme Components in* Ficus glabrata *Latex*

Component	Bonds hydrolyzed/ molecule casein[1]	Added component[2]	Additional bonds hydrolyzed/ molecule casein
2	20.7 ± 0.8	3	None
3	19.8 ± 0.1	2	None
		5	0.51 ± 0.02
5	22.6 ± 0.1	3	None
		6	None
6	21.9 ± 0.2	5	0.24 ± 0.02
		7	0.27 ± 0.03
7	22.6 ± 0.5	6	None
		8	0.42 ± 0.08
8	22.9 ± 0.4	7	None

[1] Calculated for a molecular weight of casein of 20,000 (4).

[2] Reaction allowed to go to completion with original component and then second component added to check for additional hydrolysis.

uninhibited components (24). This is somewhat surprising in view of the marked effect sodium p-chloromercuribenzoate has on the chromatographic pattern of Ficus carica variety Kadota ficin (17). Presumably, each of these reagents would react with the essential sulfhydryl group of the enzyme to add 1 additional negative charge to the enzyme. As was concluded previously (17) it appears that sodium p-chloromercuribenzoate has additional effects on the chromatographic properties of the enzyme components.

The results presented here confirm those reported earlier (2, 24) in showing that salt-purified Ficus glabrata ficin still contains a number of chromatographically distinct components. These components all rechromatographed as unique components with no indication of randomization (interconversion) produced by the experimental conditions. Components 2, 3, 5, 6, 7, and 8 were isolated and shown to be chromatographically homogeneous.

The amino acid composition of components 2, 3, 5, 6, 7, and 8 were essentially identical despite their marked differences in chromatographic properties. In order to determine if this similarity in amino acid composition would be reflected in similarity in amino acid sequence each component, after reduction and alkylation, was subjected to tryptic hydrolysis and the peptides separated by electrophoresis-chromatography. The data indicate that all 6 components gave identical peptide maps (Fig. 3).

What do these results mean in terms of the reason for multiple molecular forms of ficin? Evidence against these multiple forms being the result of autolysis subsequent to collection from the plant is extensive (16). Studies on the enzymes from several species of Ficus and several varieties of Ficus carica have indicated that the multiple molecular forms are distinctive for the source of the enzyme (24, 27). This would imply that the multiple forms have their origin at the genetic level and therefore should differ in amino acid composition and/or amino acid sequence. Based on the very marked differences among the components in chromatographic behavior (see Fig. 1 for example) one would expect they would differ noticeably in amino acid content and/or sequence. The data reported here do not bear out these expectations, however.

Recently, evidence has indicated that multiple molecular forms of an enzyme may be the result of the same peptide chain being folded in different ways (different conformers) so that the exposed groupings are different among the differently folded species (3, 14). This could account for the different electrophoretic and chromatographic properties of the conformers. In order for the conformers to be separable, the activation energy needed to convert one conformer to another must be sufficiently large so that the rate of interconversion between the conformers is very slow. However, conditions should exist which would permit the ready interconversion

of conformers. We have not seen this with the ficin components with one possible exception (16, 17).

The conformer hypothesis could only explain the multiple molecular forms found within a ficin isolated from a single species or variety. It could not explain the variation among the components found in ficins from different species of *Ficus* and different varieties of *Ficus carica*. According to the conformer hypothesis the components of all ficins would be the same. The amino acid composition of *Ficus glabrata* and *Ficus carica* variety Kadota ficins are quite different (17) indicating that, at the species level, the ficins are the expression of different genes. The distinct possibility does exist that the biosynthesis of ficin is under the control of a single gene in each plant, that this gene is different for each species and variety, and that the multiple molecular forms within each ficin are determined by the folding of the peptide chain (conformer).

Another interpretation of the results is also possible. There could be homologous replacements of one amino acid for another in the amino acid sequence while the amino acid composition is constant (or essentially so). These changes might be minor so that they do not affect the electrophoretic and chromatographic properties of the peptides (where there is no maintained conformation) but they do lead to quite marked changes in the conformation of the protein. Short of determining the complete amino acid sequence or the X-ray crystallographic structures of the components, we have no way of testing this hypothesis. In support of this hypothesis is the recent report of Neurath *et al.* (21) which indicates that a valine-leucine replacement in 2 multiple molecular forms of carboxypeptidase A produces marked chromatographic and stability differences between the 2.

Acknowledgment

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