Ficins (EC 3.4.22.3). Purification and Characterization of the Enzymatic Components of the Latex of *Ficus glabrata*†

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ABSTRACT: The crude fig latex (from *Ficus glabrata*) has been shown to contain at least six ficin components. Three ficins were separated and purified by salt-fractionation, chromatography on CM-cellulose, and gel filtration on Sephadex G-75 at pH 4.5. Two ficins (II and III) were obtained crystalline and shown to be homogeneous by several criteria. The amino acid compositions of ficins II and III were determined, and differ notably in their histidine content (ficin II, three residues per mol; ficin III, one residue per mol). The equivalent weight of ficin III by titration with 5,5′-dithiobis(2-nitrobenzoic acid) is 26,000. The stoichiometry of inhibition of ficin by $HgCl_2$ was studied. When $[HgCl_2]/[ficin]$ was greater than 0.5, no free sulfhydryl groups were detected, indicating the formation of a dimer. Stability studies on the ficins indicate that autodigestion is not a serious problem during their purification and storage.

The latex of the fig tree (*Ficus*) yields a complex of proteolytic enzymes which has been given the name ficin (EC 3.4.22.3). Like other well-known plant proteinases, in particular papain and bromelain, ficin has an essential cysteine residue at the active site. The sequence of amino acids around this residue has been determined (Wong and Liener, 1964; Husain and Lowe, 1970a). It bears a high degree of homology with the corresponding sequences in papain (Light *et al.*, 1964; Drenth *et al.*, 1968) and stem bromelain (T. Murachi (1973), personal communication; Husain and Lowe, 1970b). Liener and Friedenson (1970) have shown that all of the ficins present in salt-fractionated *Ficus glabrata* latex contain identical active-site sequences.

Husain and Lowe (1970a) reported the cross-linking of the active cysteinyl residue of ficin to an adjacent histidine residue with the bifunctional reagent 1,3-dibromoacetone. The sequence of amino acids around the labeled histidine residue was determined. The corresponding histidine sequences in papain (Husain and Lowe, 1968) and stem bromelain (Husain and Lowe, 1970b) are very similar to the ficin sequence.

In 1938, Walti crystallized ficin from clarified fig latex. Partial purification by salt fractionation was achieved by Hammond and Gutfreund (1959). Liener (1961) reported that salt-fractionated ficin behaved as a homogeneous protein on CM-cellulose at pH 4.4. However, Metrione *et al.* (1967) found evidence for three bands in salt-fractionated ficin submitted to starch–gel electrophoresis at pH 4.5. Sgarbieri *et al.* (1964) reported the separation of nine proteolytic components from salt fractionated *F. glabrata* latex, by CM-cellulose chromatography at pH 7.0. Using a similar procedure, Williams and Whitaker (1969) isolated six of these components and examined their amino acid compositions. The components were not shown to be pure by any reliable criteria. In 1968, Englund *et al.* purified one of the *F. glabrata* enzymes by salt fractionation and CM-cellulose chromatography in the presence of sodium tetrathionate.

Jones and Glazer (1970) used Englund's method of purification to obtain five electrophoretically pure proteolytic components of *F. glabrata*. All successful purifications of *F. glabrata* enzymes have used sodium tetrathionate to inhibit the enzymes during purification. Several workers (Williams and Whitaker, 1969; Jones and Glazer, 1970) report, however, that autolysis of ficin is not observed experimentally.

In this, the first paper of a series, we report the purification and some properties of two enzyme components of *F. glabrata*. During the purification, the enzymes are maintained in the active (fully reduced) form by 5 mM cysteine. Four other enzyme components have been encountered in this work, and one of them partially purified. The second paper describes the specificities of ficins II and III toward amino acid ester substrates, and the pH dependencies of ficin-catalyzed hydrolyses of several of these substrates. The specificity of ficin toward peptide and protein substrates is also described and the results are compared with those obtained for the enzyme bromelain.

Experimental Section

Materials. Crude fig latex (from *Ficus glabrata*) was obtained from Koch-Light Laboratoris, England. *p*-Nitrophenyl hippurate was synthesized by the dicyclohexylcarbodiimide method (Bodanszky and du Vigneaud, 1962). The ester was recrystallized from chloroform–hexane, mp 170–171°, lit. (McDonald and Balls, 1957) mp 170–171°. The ester was 99.5% pure, based on the release of *p*-nitrophenol. L-Cysteine hydrochloride and EDTA were obtained from British Drug Houses Ltd., England. Acrylamide, *N*,*N*′-methylenebisacrylamide, and acetonitrile (Spectro Grade) were supplied by Eastman Organic Chemicals. Nbs_2[1] and cysteine hydrochloride were supplied by Sigma Chemical Co. Ammonium sulfate was obtained from Schwarz/Mann, New York, N. Y. All other chemicals were analytical grade reagents. CM-Cellulose (Type 20, 0.71 mequiv/g) was purchased from Carl Schleicher and Schuell Co., Keene, N. H. Sephadex G-75 was supplied by Pharmacia, Sweden.

Methods. Protein was estimated spectrophotometrically

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[1] Abbreviation used is: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

by measuring the absorbance at 280 nm (Warburg and Christian, 1941). Activity was measured using *p*-nitrophenyl hippurate as the assay substrate. In a typical assay, 3 ml of 0.1 M phosphate buffer (pH 6.0) was equilibrated at 25.0 ± 0.1° in a cuvet in the sample compartment of a Cary 14 spectrophotometer. A 75-μl aliquot of a stock solution (5.2×10^{-3} M) of *p*-nitrophenyl hippurate in acetonitrile was added, and the initial absorbance of the ester was measured. A suitable aliquot of enzyme (50 μl of a solution containing 0.1–0.5 mg/ml) was added to initiate the reaction. The hydrolysis of *p*-nitrophenyl hippurate was measured by observing the release of *p*-nitrophenol at 317 nm ($\Delta\epsilon$ on hydrolysis, 7400). The spontaneous hydrolysis of *p*-nitrophenyl hippurate was determined in a separate run with 50 μl of the enzyme buffer. One katal (kat) is defined as the amount of enzyme which catalyzes the transformation of 1 mol of *p*-nitrophenyl hippurate/sec under the defined conditions (International Union of Biochemistry, 1973). The terms microkatal (μkat) and nanokatal (nkat) follow the usual convention. The specific activity is defined as (μkat/l.)/A_{280}.

CM-Cellulose was cleared of fines by settling and decantation, and washed several times with 0.05 N sodium hydroxide and repeatedly with distilled water until the pH was ~6.5. The material was thoroughly washed with 0.01 M acetate buffer (pH 4.5), 1 mM in EDTA. The packed columns were equilibrated with buffer A until the pH and A_{280} of the feed and effluent were the same. CM-Cellulose chromatography was performed at a flow rate of 0.5 ml/min. Sephadex G-75 chromatography was performed at a flow rate of 0.3 ml/min.

Polyacrylamide gels were prepared at pH 4.5 by a modification of the method of Jordan and Raymond (1969). The following stock solutions were prepared: A, 20% w/v acrylamide monomer and 1% w/v bisacrylamide in water; B, 3% w/v H_2O_2 in water; and C, 0.24 M Tris, 0.24 M citric acid, adjusted to pH 4.5 with 2 M NaOH. The gel mixture, prepared at 4°, contained 40.8 ml of H_2O, 15 ml of A, 0.4 ml of B, 3.8 ml of C, 1 mg of $FeSO_4$ and 40 mg of ascorbic acid. Gels were set in polythene tubes (7 mm × 140 mm) at 4°. Electrophoresis buffer was a 1 in 20 dilution, in water, of solution C. Gels were preelectrophoresed for 1 hr under the conditions used for electrophoresis of the ficins (250 V, 2.5 mA/gel). Protein samples applied to the gels contained 40% sucrose. Gels were stained for protein with 0.3% Amido Black 10B (G. T. Gurr, London) in methanol–acetic acid–water (4:1:5, by vol) (Giri, 1956, 1957).

Ficins II and III were crystallized from buffer A (0.01 M acetate buffer (pH 4.5), 5 mM in cysteine and 1 mM in EDTA) in the following way. Solid ammonium sulfate was added to solutions (mg/ml range) of the proteins until the first turbidity appeared. This was removed by addition of a minimum amount of buffer and the solution was allowed to evaporate slowly at 4°.

Solutions of the two purified ficins (II and III) were dialyzed exhaustively against boiled distilled water under nitrogen, and then centrifuged. Aliquots were submitted to spectrophotometric measurement and to drying under high vacuum (10^{-5} mm) at 120–140° for 2 hr, to set up a weight standard for spectral measurements. Three 1-mg samples of each protein were similarly dialyzed, and lyophilized for amino acid analysis. The dried samples were hydrolyzed in constant boiling hydrochloric acid in sealed, evacuated tubes at 110° for 20, 40, and 70 hr.

Cysteic acid was estimated in the performic acid oxidized proteins by the method of Moore (1963). Analyses were performed in duplicate on a Technicon TSM amino acid analyzer. Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). A computer program was used to calculate the number of residues of each amino acid in a molecular weight of 25,000 from the experimental data (Scott *et al.*, 1974).

Sedimentation coefficients were determined in buffer A, in a Beckman Spinco Model E analytical ultracentrifuge at 20°. The temperature was controlled by a Spinco RTIC control unit. The concentration of ficin II was 2.5–5.0 mg/ml, and of ficin III was 2.5–5.1 mg/ml.

One of the purified ficins was titrated by the method of Ellman (1959). To 3 ml of 0.1 M phosphate buffer, pH 6.0, equilibrated at 25°, was added a suitable aliquot of activator-free ficin III (50–200 μl) prepared by dialysis of the enzyme against oxygen-free 0.01 M acetate buffer (pH 4.5). The final enzyme concentration was approximately 0.1 mg/ml. An aliquot of Nbs_2 (25 μl of a 10^{-2} M solution) was added, and the release of 2-nitro-5-mercaptobenzoic acid was followed at 412 nm using the 0–0.1 slide wire of a Cary 14 spectrophotometer. The molar absorption coefficient of 2-nitro-5-mercaptobenzoic acid at 412 nm was found to be 13,710, by reaction of a known concentration of Nbs_2 with an excess of cysteine in 0.1 M phosphate buffer (pH 6.0). The equivalent weight of ficin III (specific activity 107) was determined from the concentration of reduced sulfhydryl groups (obtained by titration with Nbs_2) and the corresponding protein concentration calculated from the A_{280} and the experimentally determined dry weight of ficin III. The equivalent weight was calculated from the expression: equiv wt = concn of protein (g/l.)/concn of reduced sulfhydryl groups (M).

The stoichiometry of the inhibition of ficin III by $HgCl_2$ was examined at pH 6.0. Aliquots of ficin (100 μl, final concentration 3.25×10^{-6} N) were added to a series of spectrophotometer cuvets containing 3 ml of 0.1 M phosphate buffer (pH 6.0). Increasing concentrations of $HgCl_2$ were added to each cuvet. Residual activity was determined on a 50-μl aliquot in the assay described above, and the concentration of free sulfhydryl groups remaining at each concentration of $HgCl_2$ was then determined by titration of each reaction mixture with Nbs_2.

The activation and stability of ficin at 4° in five "thiol-activating" systems were determined (Table IV). Activator-free ficin was prepared by dialysis of the enzyme against 0.01 M acetate buffer (pH 4.5); 50% of the enzyme activity was lost on dialysis. Reactivation of the enzyme in the same buffer with added "activator" was followed by assaying against *p*-nitrophenyl hippurate as described previously. Oxygen was not excluded from the enzyme during these experiments.

The stability of ficin II at 4° over the pH range 4.0–10.5 in the presence of 1 mM EDTA and of 1 mM EDTA and 5 mM cysteine was also investigated.

Results

The crude fig latex was extracted by a modified procedure of Hammond and Gutfreund (1959). Crude latex (25 g) was suspended in 450 ml of 0.1 M acetate buffer (pH 4.0), and the mixture stirred for 2 hr at 4°. The resulting suspension was dialyzed against 5 l. of 0.01 M acetate buffer (pH 4.5), 1 mM in EDTA, at 4° for 24 hr. Insoluble material was removed by centrifugation. The extract was salt fractionated by a modification of the procedure of Hammond and Gutfreund (1959) (Table I). After steps 1–3 (Table I), the precipitate was collected by centrifugation and resuspended in a minimum volume of 0.01 M acetate buffer (pH 4.5), 1 mM in EDTA, and

TABLE I: Salt Fractionation of Crude Fig Latex.

Step	Total Activity (nkat)	Specific Activity ($\frac{\mu kat/l.}{A_{280}}$)	A_{280}/A_{260}	% Yield[a]
1 Extraction	824	27.3	1.7	100
2 0–50% satd $(NH_4)_2SO_4$	636	46.8	1.82	77
3 0–30% satd $(NH_4)_2SO_4$	378	56.5	1.87	46
4 0–50% satd NaCl	378	66.1	1.90	46

[a] Yield calculated on total activity recovered in each step.

dialyzed against this buffer. After step 4, the precipitate was taken up in buffer A (0.01 M acetate (pH 4.5), 5 mM in cysteine and 1 mM in EDTA), and dialyzed against this buffer. The salt-fractionated ficin (step 4) was purified 2.4-fold and contained 46% of the total activity. The remainder of the activity could be precipitated from the supernatant in step 3 by 60% saturated $(NH_4)_2SO_4$.

The salt-fractionated ficin (after step 4) was applied to a CM-cellulose column (4 cm × 30 cm) and no protein passed through the column on washing with buffer A. A linear salt gradient from 0 to 0.6 M sodium chloride in 2.4 l. of the starting buffer was applied, followed by 0.6 M sodium chloride in the same buffer, resulting in the elution of two peaks of absorbance and activity (Figure 1). These two fractions (fractions 1 and 2) were concentrated by precipitation with ammonium sulfate at 60% saturation and dialyzed against buffer A. The enzyme activity in fraction 1 (ficins I and II) was rechromatographed on a CM-cellulose column (3 cm × 30 cm) equilibrated with buffer A. A linear salt gradient from 0 to 0.5 M sodium chloride in 2 l. of buffer A was applied, resulting in the elution of two peaks of absorbance and activity, centered at 0.24 and 0.32 M sodium chloride, respectively (Figure 2). The enzyme activity (ficin III) in fraction 2 (Figure 1) was rechromatographed on a CM-cellulose column (3 cm × 30 cm) equilibrated with buffer B (0.1 M acetate (pH 4.5), 5 mM in cysteine and 1 mM in EDTA). A linear salt gradient from 0 to 0.5 M sodium chloride in 2 l. of buffer B was applied, resulting in the elution of a peak of absorbance and activity centered at 0.28 M sodium chloride (Figure 3).

The specific activity was constant asross each single protein peak eluted from CM-cellulose, and elctrophoresis of the three ficins usually showed that complete separation of all three had been obtained. In some cases, however, rechromatography was necessary. The enzyme components obtained from CM-cellulose chromatography were applied to a Sephadex G-75 column (3 × 70 cm) equilibrated with buffer A. Sephadex G-75 filtration increased the maximum specific activities of all three enzymes. Maximum specific activities were recorded on the trailing side of each elution profile (Figure 4). These were 73.8, 101.8, and 107.5 for ficins I, II, and III, respectively.[2] Ficins II and III appear homogeneous electrophoretically at this stage (Figure 5). However, ficin I contains three components and requires further purification. Yields of 70 mg of ficin III and 30 mg of ficin II were obtained from 25 g of fig latex.

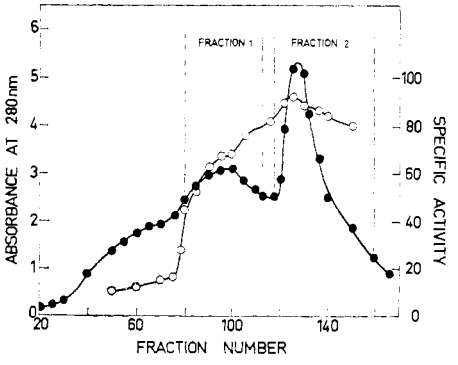

FIGURE 1: Chromatography of salt-fractionated ficin on CM-cellulose at pH 4.5; load, ~2490 mg; fraction size, ~14 ml; gradient complete at fraction 114; subsequent fractions eluted with 0.6 M NaCl: (●) A_{280}; (○) specific activity.

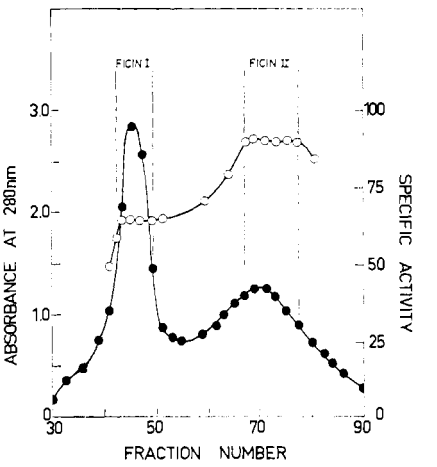

FIGURE 2: Rechromatography of fraction 1 on CM-cellulose at pH 4.5; load, ~390 mg; fraction size, 12 ml: (●) A_{280}; (○) specific activity.

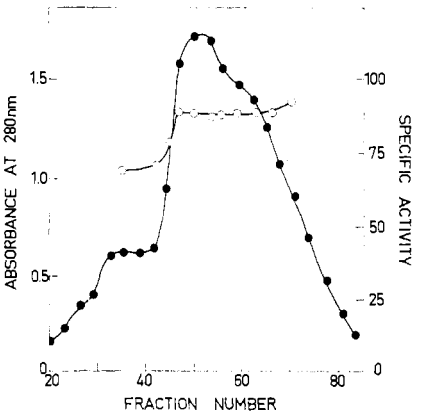

FIGURE 3: Rechromatography of fraction 2 on CM-cellulose at pH 4.5; load, ~400 mg; fraction size, 15 ml: (●) A_{280}; (○) specific activity.

Ficins II and III crystallize as fragile, thin, hexagonal plates (Figure 6). Spectral data for the two proteins are shown in Table II. The results of amino acid analysis of ficins II and III are shown in Table III. There are significant differences between the two proteins, notably in histidine content (ficin II, three residues per mol; ficin III, one residue per mol). In the ultracentrifuge, ficins II and III sedimented with a symmetrical boundary characteristic of a homogeneous protein. The sedimentation coefficients determined in buffer A were 2.65 ± 0.1

[2] The initial specific activity of salt-fractionated ficin obtained from some early batches of fig latex was low, and the specific activities of the isolated ficins II and III were correspondingly low. Maximum specific activities for ficins II and III were achieved only when the salt-fractionated latex had a specific activity of 60–70.

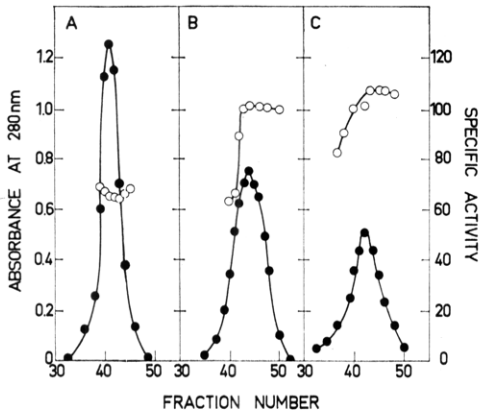

FIGURE 4: Gel filtration of the ficins on Sephadex G-75: (A) ficin I; (B) ficin II; (C) ficin III; (●) A_{280}; (○) specific activity. Ficin III load is one-fifth of the yield from the previous step.

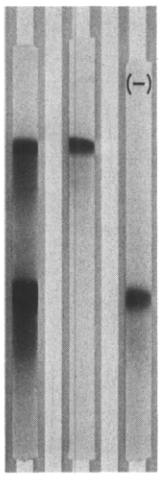

FIGURE 5: Polyacrylamide gel electrophoresis of ficins at pH 4.5; left to right: ficins II and III, ficin II, ficin III.

S for the two ficins, indicating that they are of the same molecular size.

The equivalent weight of ficin III of specific activity 107, determined by titration with Nbs_2, was 26,000 ± 500. A sample of this enzyme was stored for 2 months in buffer A at 4°. Its specific activity decreased to 70.1 and the equivalent weight increased proportionately to 41,500 ± 800.

The inhibition of ficin III by $HgCl_2$ is shown in Figure 7. One g-atom of Hg^{2+} per mol of active ficin was required for complete inhibition of enzyme activity. However, no free sulfhydryl groups were detected when the ratio $[HgCl_2]$/[ficin] was greater than 0.5.

The activation and stability of ficin II in the presence of three thiol-reducing agents, cysteine, dithiothreitol, and mercaptoethanol, with and without EDTA, were studied and the results are shown in Table IV. Complete reactivation was obtained only in the presence of a thiol and the chelating agent. Ficin showed good stability on storage at 4°. Even after 4.5 months, ~50% of the activity remained. The activation

TABLE II: Spectral Data for Ficins II and III.

Enzyme	λ_{max} (nm)	A_{280}/A_{260}	$A^{1\%}_{1\,cm}$
Ficin II	280	1.98 ± 0.02	21.50
Ficin III	279	1.98 ± 0.02	24.04

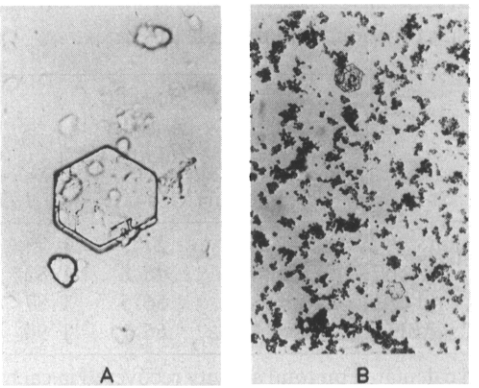

FIGURE 6: Crystalline ficins: (A) ficin II (× 205); (B) ficin III (× 82).

and stability of ficin III were found to be the same as those observed for ficin II. The stability of ficin II at 4° over the pH range 4.5–8.5 in the presence of 1 mM EDTA and of 1 mM EDTA and 5 mM cysteine was studied. Although the rate of inactivation of the enzyme increased with increasing pH, and was faster in the presence of cysteine, the inactivation was largely reversible. For example, after 15 days at pH 8.0, in the presence of cysteine and EDTA, the activity of the enzyme had decreased to 11% of its original value; dialysis of the enzyme against buffer A resulted in the reactivation of the enzyme to 80% of its original value.

Discussion

The latex of *F. glabrata* has been found to contain several enzymatically active components. Enzyme prepared by the salt-fractionation procedure of Hammond and Gutfreund (1959) contained three major components (ficins I, II, and III) which were separated and purified on CM-cellulose and Sephadex G-75. The homogeneity of the purified ficins was examined in the ultracentrifuge and by polyacrylamide gel electrophoresis. Ficins II and III behaved as homogeneous proteins. Their relative mobilities at pH 4.5 suggest that ficin II is a more basic protein than ficin III. Three protein bands were detected when ficin I was electrophoresed at pH 4.5. It is not known whether all bands have enzymatic activity. Ficin I was not purified further.

Ficins II and III were obtained in crystalline form. The crystals of both enzymes appear as thin hexagonal plates, and were of the same form as the crystals obtained by Walti (1938) from fresh clarified fig latex of *F. glabrata*. These crystals are markedly different from the long, thin needles obtained for ficin D (from *Ficus carica var. Kadota*) by Kramer and Whitaker (1964).

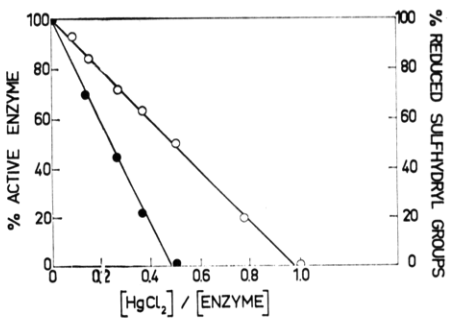

FIGURE 7: Inhibition of ficin III by $HgCl_2$ at pH 6.0: (○) residual activity determined using *p*-nitrophenyl hippurate as assay substrate; (●) percentage of reduced sulfhydryl groups determined by titration with Nbs_2.

TABLE III: Amino Acid Compositions of Ficins II and III.

	Ficin II[a]	Ficin II[c]	Ficin II[d]	Ficin H[e]	Ficin III[b]	Ficin III[f]	Ficin III[g]	Ficin[h]
Lysine	8.30	8	8	8	5.46	6	6	5
Histidine	2.93	3	3	3	0.96	1	1	1
Arginine	12.87	13	13	13	10.47	11	11	10
Aspartic acid	19.90	20	20	21	18.58	19	19	17
Threonine	8.75	9	9	8	9.16	9	10	8
Serine	15.18	16	15	15	14.12	15	15	14
Glutamic acid	24.36	25	25	25	25.58	27	27	25
Proline	9.43	10	10	10	9.86	10	10	11
Glycine	29.47	30	30	31	30.53	32	32	28
Alanine	21.16	22	21	21	20.84	22	22	20
Half-cystine	8.30	8	8	9	7.52	8	8	8
Valine	18.37	19	19	19	19.58	20	20	18
Methionine	3.15	3	3	3	4.93	5	5	5
Isoleucine	7.18	7	7	7	7.54	8	8	7
Leucine	16.21	17	16	16	15.46	16	16	15
Tyrosine	10.69	11	11	12	15.60	16	16	16
Phenylalanine	5.42	6	5	5	5.31	6	6	5
Tryptophan	7.94	8	8	7	7.69	8	8	6
Mol wt[i]	25,000	25,554	25,135	25,151	25,000	26,058	26,159	

[a, b] Experimentally determined numbers of moles of each amino acid per 25,000 g of ficin. [c] Data in *a* multiplied by 3.00/2.93. Histidine becomes an integral value (= 3.00). All other results are given as the nearest integral values. [d] Data in *a* multiplied by 8.00/7.94. Tryptophan becomes an integral value (= 8.00). All other results given as nearest integral values. Edelhoch (1967) demonstrated that tryptophan could be estimated within 3% of the correct value for a series of standard proteins. His method has been employed in this laboratory to estimate tryptophan in the proteins α-chymotrypsin, trypsinogen, and papain (Inkerman, 1973). The results obtained were within 2% of the correct values in all cases. Therefore the use of tryptophan as a standard is justified. [e] Data of Jones and Glazer (1970). [f, g] Histidine normalized to 1.00 in *f*; tryptophan normalized to 8.00 in *g* (*cf.* footnotes *c* and *d*). [h] Data of Englund *et al.* (1968). [i] Molecular weight = Σ (number of residues × residue weight).

Three additional ficin components were separated and partially purified from the 30–60% saturated $(NH_4)_2SO_4$ fraction (ficins IV, V and VI). Two of these components (V and VI) were electrophoretically indistinguishable from ficin II

TABLE IV: Activation and Stability of Ficin II at pH 4.5 and 4°.[a]

	Activity[b]				
Time (days)	i	ii	iii	iv	v
0[c]	73.6	100	97.3	100	100
2	70.9	100	97.0	100	100
4	62.7	95.0	96.0	96.0	100
8		93.6	96.0	96.0	96.0
11	60.9	90.0	96.0	96.0	96.0
15	59.1	88.2	96.0	96.0	96.0
22	47.3	80.9		87.0	85.0
29	45.5[d]	78.2			85.0
57		66.8	74.6	79.8	83.3
135		48.9	48.4	56.0	52.0

[a] Ficin (0.35 mg/ml) in (i) 1 mM EDTA; (ii) 1 mM EDTA–5 mM cysteine; (iii) 1 mM EDTA–10 mM mercaptoethanol; (iv) 2 mM dithiothreitol; (v) 2 mM dithiothreitol–1 mM EDTA. [b] Expressed as % of original activity measured before removal of cysteine–EDTA by dialysis. [c] Activities were measured immediately after dilution of activator-free ficin into each of the "activating" systems. [d] Complete recovery of activity was obtained by performing assays in the presence of 4×10^{-4} M cysteine.

Further purification is necessary to identify these two ficins and their relationship to ficin II.

Jones and Glazer (1970) report the separation of six ficins from *F. glabrata* latex after 0–50% saturated sodium chloride fractionation. Of the five components purified, three (ficins F_1, F_2, and H) were relatively more basic than the other two (ficins B and E). A comparison of the electrophoretic mobilities, amino acid compositions, and tryptic peptide maps of F_1, F_2, and H reveals that they are almost identical proteins. It is likely that ficins II, V, and VI, prepared in the present work, correspond to ficins H, F_1, and F_2; a comparison of the amino acid compositions of ficin II and ficin H (Table III) supports this conclusion. Ficin III is almost certainly the same protein as was isolated by Englund *et al.* (1968) (Table III) and Jones and Glazer (1970) (ficin E).

The dry weight determinations of ficins II and III showed that enzyme solutions containing 1 mg/ml have absorbances (280 nm) of 2.150 and 2.404, respectively. Englund *et al.* (1968) reported 2.10 for their purified ficin (ficin III, this paper) and Hollaway (1967) obtained a value of 1.84 for ficin purified by the method of Sgarbieri *et al.* (1964).

The equivalent weight of purified ficin III was 26,000 ± 500, in good agreement with reported molecular weights of 25,000–26,000 (Williams and Whitaker, 1969; Englund *et al.*, 1968; Jones and Glazer, 1970). In addition, the equivalent weight was found to be inversely proportional to the specific activity of the enzyme; this is good evidence that the hydrolysis of *p*-nitrophenyl hippurate and the titration with Nbs_2 occur at one and the same site on the enzyme.

Englund *et al.* (1968) measured 0.92 equiv of reduced sulfhydryl group/25,000 g of ficin by amperometric titration.

The value is increased to 1.02 equiv/25,000 g of ficin by using the dry weight reported for ficin III in this paper instead of the value obtained by Englund *et al.* (1968).

The stoichiometry of the inhibition of ficin III with $HgCl_2$ showed the reaction of 1 g-atom of Hg^{2+}/mol of ficin when residual activity was measured. When the concentration of free sulfhydryl groups was determined in the presence of increasing concentrations of $HgCl_2$, no free sulfhydryl groups were detected above 0.5 g-atom of Hg^{2+}/mol of ficin. The reaction of ficin with $HgCl_2$ may be written as

$$\text{E-CH}_2\text{SH} + \text{HgCl}_2 \rightleftharpoons \text{E-CH}_2\text{S-Hg}^+ + \text{H}^+ + 2\text{Cl}^- \quad (1)$$

$$\text{E-CH}_2\text{S-Hg}^+ + \text{E-CH}_2\text{SH} \rightleftharpoons \text{E-CH}_2\text{S-Hg-SCH}_2\text{-E} + \text{H}^+ \quad (2)$$

Reaction 1 may be reversed by the addition of a suitable concentration of thiol. Reaction 2 is reversible on dilution. Thus when [$HgCl_2$]/[ficin] is 0.5 or less, the inhibition is described by reaction 2; dilution of this inhibited enzyme for assay results in dissociation of the dimer E-CH_2S-Hg-SCH_2-E, and the stoichiometry of the inhibition, measured in this way, is that of reaction 1. Liener (1961) performed a similar experiment in which residual activity of ficin was measured by hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester, and residual sulfhydryl groups were titrated with *N*-ethylmaleimide. He reported that the stoichiometry of the inhibition was 0.5 g-atom of Hg^{2+}/mol of ficin, by determination of either residual activity or residual sulfhydryl groups. The assay used to determine residual activity probably necessitated the use of a higher concentration of enzyme than was employed in the present work. Dissociation of the dimer (eq 2) may not have been complete under such conditions. The data presented on the graph showing percentage loss of activity *vs.* $HgCl_2$ concentration (Figure 6 of Liener (1961)) are, however, more consistent with a stoichiometry higher than 0.5 g-atom of Hg^{2+}/mol of ficin, indicating partial dissociation of the dimer in the assay system.

A thiol and a chelating agent were necessary for complete reactivation of ficin, after removal of cysteine and EDTA by dialysis. In the presence of 5 mM cysteine–1 mM EDTA, the enzyme showed good stability, even at pH 8.0. These results clearly demonstrate that autolysis is not a serious problem in the preparation and storage of ficin, in contrast to the suggestion of Englund *et al.* (1968).

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