Studies on Ficin. I. Its Isolation and Characterization*

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ABSTRACT: The sulfhydryl proteinase ficin has been isolated from commercial preparations by salt fractionation and chromatography on carboxymethylcellulose. During the isolation, proteolytic activity was reversibly inhibited by sodium tetrathionate. Several active components were found, but only the major one was characterized. In its inactive form, the purified enzyme could be stored as a salt precipitate at 4° for at least 15 months without any loss of activity. The major component was homogeneous by several criteria. Its amino acid composition, molecular weight, amino-terminal residue, and optical rotatory dispersion were determined. Its enzymatic specificity was studied using the oxidized B chain of insulin as a substrate. It was found that a wide variety of peptide bonds were hydrolyzed, but peptide bonds following an aromatic residue appeared to be hydrolyzed more efficiently than the others.

Ficin, the proteolytic enzyme elaborated by the fig tree was first crystallized by Walti (1938). It is a powerful enzyme of rather poor stability which belongs to that class of proteinases known as "sulfhydryl enzymes." These enzymes contain a cysteine residue which plays an essential role in the enzymatic activity.

Another member of this class, papain, has received far more study. Apparently ficin shares many common properties with papain. The amino acid sequence neighboring the essential cysteinyl residue has been determined (Wong and Liener, 1964) and found to resemble closely the corresponding one in papain (Light *et al.*, 1964). It is therefore not surprising that the mechanism of action of both enzymes is also known to be similar (Whitaker and Bender, 1965), and one in which the catalytic intermediate is undoubtedly an acyl enzyme with the essential thiol group acylated (Lowe and Williams, 1965).

Despite the interesting and informative investigations already made, ficin has not been well characterized with regard to purity, molecular weight, or amino acid composition, perhaps due in part to the problem of autodigestion. There is, therefore, the need of a method of purification which circumvents in some way the inherent difficulty and will yield the enzyme in a stable inactive form which can be easily and quickly reconverted to the active form. In the previous work isolation attempts have been made on the active enzyme. It was isolated from the clarified latex of a fig tree by direct crystallization (Walti, 1938) or by salt fractionation (Hammond and Gutfreund, 1959). Both of these preparations were inhomogeneous when examined by chromatography on CM-cellulose (Sgarbieri *et al.*, 1964). The salt-fractionated material contained as many enzymatically active components as the crude extract, while the crystalline material contained mainly one component. Liener (1961) has reported the homogeneity of the salt fractionated material on CM-cellulose chromatography, but our experience indicates it to be a separation artifact because of the low buffering capacity of the eluting agent. Kramer and Whitaker (1964) also observed that there was band spreading on rechromatography of the purified enzyme. This behavior was confirmed by our own experiences and suggested that autodigestion of the enzyme was occurring during purification.

Therefore, in our work the enzyme was first inactivated by treatment with sodium tetrathionate which converts the essential thiol group to a sulfenyl thiosulfate $(SSSO_3)^-$. This inactivation can be reversed by reaction with an excess of thiol reagents (Sanner and Pihl, 1963). With this inactive enzyme, the problem of instability during isolation and storage was overcome, and reproducible preparations of the enzyme become available. We have carried out several characterization studies of it, as reported in the findings to be detailed below.

Experimental Section

Materials. Crude preparations of ficin were obtained from Nutritional Biochemicals Corp. (lots 2019 and 9477) and Calbiochem Corp. (lot 503239). Indistinguishable results were obtained with these samples. This crude material was from the latex of *Ficus glabrata* and had been prepared by clarifying the latex with diatomaceous earth, filtering, and drying (communication from Nutritional Biochemicals Corp.).

Sodium tetrathionate was prepared by the method of Gilman *et al.* (1946) and was stored at 4° in a desiccator. Benzoyl-DL-arginine *p*-nitroanilide hydrochloride

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(BAPA)[1] was prepared by the method of Erlanger *et al.* (1961). Dithioerythritol (Cleland, 1964) was purchased from Cyclo Chemical Corp.

Carboxypeptidases A and B, papain (two-times crystallized) and trypsin (two-times crystallized) were from Worthington Corp. They contained full enzymatic activities as checked with the appropriate synthetic substrates.

The following ion-exchange resins were obtained from Bio-Rad Corp.: Cellex-CM (0.6 mequiv/g), Bio-Rex 70 (10 mequiv/g), and Aminex-MS (Blend Q-15). Sephadex G-25 and G-75 were from A. B. Pharmacia.

Preparative dialyses were carried out with Visking $^{23}/_{32}$ casing which had been washed with 5% HOAc followed by 0.01 M Versene. Deionized-distilled water was used for all the experiments.

Isolation of Ficin. Crude ficin (20 g) was suspended in 20 ml of solution containing 0.001 M disodium Versene and 0.01 M sodium tetrathionate. The crude enzyme in the absence of any activating agent exhibited about 25% of its potential activity and this concentration of tetrathionate was sufficient to inactivate the enzyme completely.

The pH of the suspension was 4.8, but was brought to neutrality by the addition of 11.0 ml of 1.0 M sodium hydroxide. The suspension was stirred for 3 hr at room temperature and then was centrifuged to remove insoluble material. A salting-out step was carried out by addition of 17.7 g of sodium chloride/100 ml of extract. After several hours at 4°, the precipitate was collected by centrifugation and suspended in 100 ml of pH 7.1 buffer containing 0.005 M sodium phosphate, 0.001 M sodium tetrathionate, and 0.001 M Versene. The preparation was then dialyzed overnight at 4° against the pH 7.1 buffer. After dialysis, some insoluble material was removed by centrifugation and the extract was applied to a column of Cellex-CM (20 × 4 cm) equilibrated with the pH 7.1 buffer. The column was then washed with 300 ml of the pH 7.1 buffer and elution was carried out with sodium phosphate in a linear gradient from 0.005 (pH 7.1) to 0.185 M (pH 6.9). (The 0.185 M buffer was prepared by dissolving 39.75 g of $NaH_2PO_4 \cdot H_2O$ and 64.17 g of Na_2HPO_4 in 4 l. of solution; the 0.005 M buffer was prepared by a 27/1000 dilution of the 0.185 M solution.) The gradient was arranged so the sodium phosphate concentration had changed linearly from 0.005 to 0.185 M when 3000 ml had passed through the column. The eluting buffer also contained Versene and sodium tetrathionate, each in a concentration of 0.001 M. The column was run at room temperature, and fractions of about 25 ml were collected at a flow rate of 150 ml/hr. The phosphate concentration of the gradient was followed by conductance measurement. The fractions were analyzed by enzymatic assays to be described below, by absorbance at 280 mμ, and by ninhydrin analysis after alkaline hydrolysis (Crestfield *et al.*, 1963).

The tubes containing fraction III, as indicated in Figure 2, were pooled and salted out by the addition of 35 g of sodium chloride/100 ml of effluent solution. The precipitated ficin was centrifuged and stored at 4° as a suspension in a small volume of the concentrated salt solution. In several experiments the ficin was salted out by saturating with magnesium sulfate, and then stored in that salt solution. The stored ficin could be centrifuged and redissolved, or else it could be dissolved directly on removal of the salt by dialysis. The latter procedure could be rapidly and conveniently carried out by the thin-film dialysis method (Craig and King, 1962).

The ficin in fraction III has an $\epsilon^{1\%}_{280\,m\mu}$ of 2.1 in 0.05 M NH_4HCO_3. This value was determined by evaporation of a 1-ml aliquot of the enzyme solution of known absorbance in a tared platinum shell and then weighing the residue after drying at 100° and 1 mm for 15 min.

In several experiments it was necessary to prepare active ficin in the absence of any activating agent. The enzyme was first activated by dissolving the inactive precipitate (6 mg) in 2 ml of 0.1 M sodium phosphate–0.001 M Versene (pH 7.0) containing either a 5000-fold molar excess of mercaptoethanol or of a 300-fold molar excess of dithioerythritol. The solution was quickly passed through a column of Sephadex G-25 (50 × 0.9 cm). The column was equilibrated with a freshly degassed buffer of 1×10^{-3} M disodium Versene–1.5×10^{-4} M HCl (pH 4.1). The duration between the activation and the elution from the column was less than 15 min, and during that time the enzyme remained fully active.

Methods. Activity was measured using casein and BAPA as substrates. In both assay procedures, the measured activity was directly proportional to the quantity of enzyme in the concentration range studied.

The casein procedure is similar to that of Kunitz (1947). A total volume of 2.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0–8 μg of ficin, 0.007 M mercaptoethanol, 0.001 M Versene, and 0.5% Hammersten casein was incubated for 20 min at 37°. The reaction was terminated by the addition of 3.0 ml of 5% trichloroacetic acid, and after centrifugation the absorbance of the supernatant was measured at 280 mμ. One unit of activity is defined as the quantity of enzyme required to bring about an absorbance change of 1.0.

The BAPA procedure is similar to that of Erlanger *et al.* (1961). A total volume of 0.8 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0–100 μg of ficin, 0.02 M mercaptoethanol, and 0.001 M Versene was warmed to 37°. BAPA (3 ml) (0.001 M) (prepared by dissolving 43.5 mg of BAPA in 1.0 ml of dimethyl sulfoxide and diluting to 100 ml with 0.1 M sodium phosphate–0.001 M Versene pH 7.0), also warmed at 37°, was added to the solution containing the ficin. After 1 hr at 37°, the reaction was terminated by the addition of 0.2 ml of glacial acetic acid. The extent of hydrolysis was determined by measurement of

[1] Abbreviations used: BAPA, benzoyl-DL-arginine *p*-nitroanilide hydrochloride; FDNB, 1-fluoro-2,4-dinitrobenzene.

absorbance at 410 mμ (ε 8940 for *p*-nitroaniline). One unit of activity is defined as the quantity of ficin required to catalyze the hydrolysis of BAPA at the rate of 1.0 μmole/min. The BAPA concentration used in this assay is less than that required for V_{max} and, consequently, the rate of hydrolysis depends upon the actual substrate concentration. The 0.001 M BAPA solution is supersaturated and the substrate tends to crystallize slowly on standing. Therefore, it is necessary to use freshly prepared solutions for reproducible results.

Amino acid analyses of ficin were carried out on a Spinco amino acid analyzer (Moore and Stein, 1963). Ficin, which had been subjected to rechromatography on the system used for isolation, was desalted by dialysis against water followed by passage through a mixed-bed ion-exchange column (Dintzis, 1952). Lyophilized samples of 4–5 mg were hydrolyzed in 6 M hydrochloric acid in sealed-evacuated tubes at 110° for 24 and 72 hr. Moisture, ash, and Kjeldahl nitrogen determinations were carried out on samples from the same preparation. Tryptophan was measured spectrophotometrically (Goodwin and Morton, 1946). Half-cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963) and as carboxymethylcysteine after reduction and alkylation by a method modified from that of Anfinsen and Haber (1961). In order to bring about complete cleavage of the disulfide bonds, it was found necessary to carry out the reaction under more vigorous conditions. Ficin (12 mg) was dissolved in 10 ml of 5 M guanidine hydrochloride–0.0005 M Versene containing 0.484 g of Tris. Under nitrogen atmosphere, 50 μl of mercaptoethanol was added. After incubation overnight at 37°, another aliquot of mercaptoethanol (25 μl) was added followed by 1 ml of 5 M guanidine hydrochloride containing 298 mg of iodoacetic acid. After 15 min at 25°, the reaction was terminated by adding 1 ml of mercaptoethanol. On dialysis against water the protein precipitated and was collected by centrifugation.

The amino-terminal residue of ficin was determined by the FDNB method. Ficin (1.3 μmoles) was dissolved in 19 ml of 50% ethanol containing 33 mg of sodium bicarbonate and 125 mg of FDNB. After stirring at 25° for 2.5 hr the solution was rotary evaporated to near dryness. The excess FDNB was removed by extraction with peroxide-free ether, and the sample was dried. The DNP-ficin was hydrolyzed in 6 M hydrochloric acid *in vacuo* for 24 hr at 110°. After removal of the acid, the hydrolysate was fractionated by countercurrent distribution in a system of ethyl acetate–phosphate buffer (Hausmann *et al.*, 1955). The fractions obtained from the distribution were examined by paper chromatography (Levy, 1955).

Attempts to determine the carboxyl-terminal residue were made by the carboxypeptidase method similar to that of Guidotti (1960). Reactions were run at 25° in a 0.2 M sodium phosphate buffer (pH 7.6). Carboxypeptidases A and B were used separately and together. The mixture for each reaction was 6.0 ml containing 0.37 μmole of ficin and about 0.02 μmole of carboxypeptidase. Aliquots were taken at intervals from zero time to 140 min, and the reaction was terminated by the addition of 0.05 ml of 6 M hydrochloric acid. The precipitated ficin was centrifuged and washed, and the combined supernatant and washing was examined on the amino acid analyzer.

Attempts to measure hexose and pentose were made by the orcinol–sulfuric acid method (Tsugita and Akabori, 1959) and by the orcinol–ferric chloride method (Volkin and Cohn, 1954). Standards were run with glucose, fructose, arabinose, and glucuronic acid.

Sulfhydryl groups of ficin were determined by measuring its carboxymethylcysteine content following reaction with iodoacetic acid and hydrolysis. These groups were also determined by amperometric titration with silver ion (Benesch *et al.*, 1955). The disulfide content of ficin was measured by titration of the liberated thiol following Na_2SO_3 cleavage of the bonds in 8 M urea (Carter, 1959). The experimental details are given in Tables IV and V and Figure 8.

Thin film dialysis (Craig and King, 1962) of ficin was carried out at 40° in a Visking $^{20}/_{32}$ membrane which had been stretched linearly and circularly. The solvent was 0.015 M ammonium acetate, pH 6.0. The membrane was calibrated with chymotrypsinogen, which exhibited a half-escape time of 17 hr.

The molecular weight of ficin was determined by equilibrium ultracentrifugation in a Spinco Model E ultracentrifuge (Yphantis, 1964). The ficin, which had been stored as a suspension in saturated magnesium sulfate, was dissolved in a pH 6.6 buffer containing 0.1 M sodium chloride, 0.01 M disodium Versene, 0.008 M sodium hydroxide, and 0.001 M sodium tetrathionate. The magnesium sulfate was completely removed by gel filtration on a column of Sephadex G-25 (50 × 0.9 cm) equilibrated with the pH 6.6 buffer. Dilutions were prepared and solutions of approximately 0.6, 0.2, and 0.1 mg/ml were placed in a six-channel cell with opposite channels containing buffer. The sample was run at 39,640 rpm at 23.5°, and equilibrium was attained within 16 hr. The density of the solvent, as measured by a Westphal balance at 25°, was 1.0041 g/cc. The partial specific volume (0.725 cc/g) was calculated from the amino acid composition (Cohn and Edsall, 1943).

Optical rotatory dispersion of ficin was determined on a Rudolph Model 80 polarimeter with a mercury arc lamp as the light source. A 2-dm polarimeter cell was used. Solvent refractive indices were measured on an Abbe refractometer. For calculations according to the Moffit equation, a value of λ_0 212 mμ was used.

The enzymatic specificity of ficin was investigated by digesting the oxidized B chain of bovine insulin and identifying the peptides which were produced. Insulin was oxidized by performic acid and the B chain was isolated by countercurrent distribution (Craig *et al.*, 1961). The digestion was carried out in 7.25 ml of pH 7.8 buffer containing 0.05 M sodium phosphate, 0.01 M Versene, 0.42 mg of dithicerythritol,

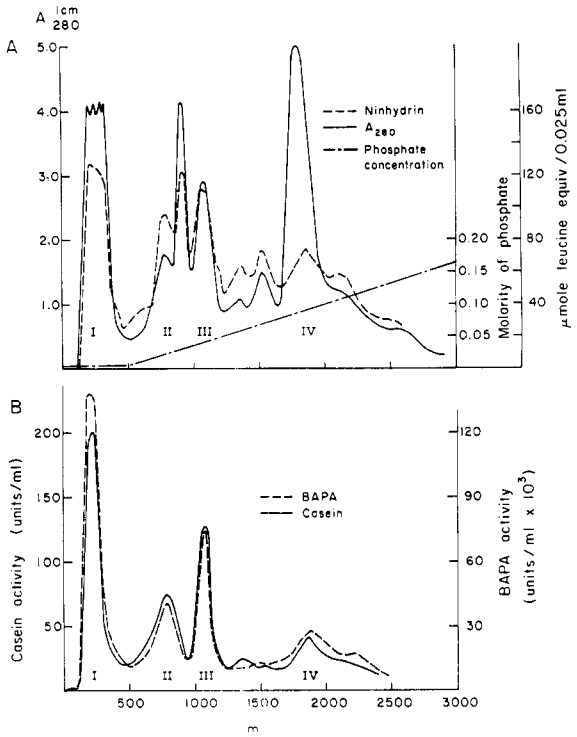

FIGURE 1: Chromatography on a 20 × 4 cm column of Cellex-CM (0.6 mequiv/g) of an extract of 10 g of crude ficin. There had been no preliminary salt fractionation of the extract. The column was equilibrated with 0.005 M sodium phosphate (pH 7.1) and the concentration increased linearly to 0.185 M sodium phosphate (pH 6.9). The buffers contained 0.001 M sodium tetrathionate and 0.001 M Versene. The column was run at room temperature, and 25-ml fractions were collected at a flow rate of 150 ml/hr. The BAPA activity was 25% lower than that in Figure 2 because of a slightly lowered substrate concentration (see Methods).

2.3 μmoles of B chain, and 0.0035 μmole of ficin. After 8 min at 37°, the reaction was terminated by the addition of 0.175 ml of 6 M hydrochloric acid. The acid-precipitated ficin was removed by centrifugation, and the insulin peptides were fractionated directly on a column of Aminex-MS blend Q-15 cation-exchange resin (0.9 × 15 cm). The pH 3.25 and 5.28 amino acid analyzer buffers were used, except that the BRIJ-35 and the thiodiglycol were omitted. A linear gradient was set up between the pH 3.25 and 5.28 buffers. The gradient was complete after 250 ml, and the column was then eluted with pH 5.28 buffer and finally with 0.2 M sodium hydroxide. The column was run at 34 ml/hr at 55°. Fractions of 1.5 ml were collected and the peaks were located by ninhydrin analysis of alkaline hydrolysates of 0.1-ml aliquots (Crestfield *et al.*, 1963). Tubes containing peptides were pooled and hydrolyzed in 6 M hydrochloric acid in the presence of the buffer salts. The peptides were identified from their amino acid compositions.

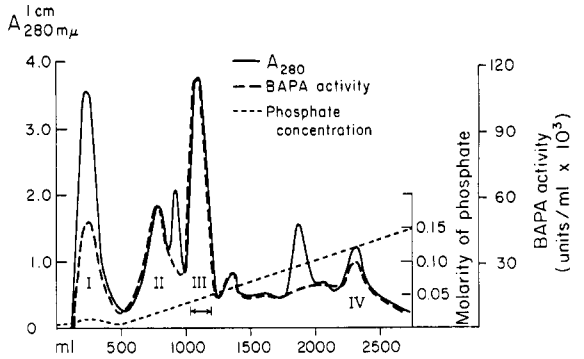

FIGURE 2: Chromatography on a 20 × 4 cm column of Cellex-CM (0.6 mequiv/g) of an extract of 20 g of crude ficin. There had been a preliminary sodium chloride fractionation as described in the text. The buffers and experimental conditions used for this column were identical with those described in the legend of Figure 1.

Results

Chromatograms of the extract of tetrathionate-inactivated crude ficin are illustrated in Figures 1 and 2. In Figure 1 the extract was chromatographed directly, but in Figure 2 the extract had undergone a preliminary sodium chloride precipitation. The same distribution of enzymatically active components is present in both chromatograms though they are present in slightly different ratios. These components have been designated as fractions I–IV. On rechromatographies of the fractions, only III appeared to behave as a single component. Fraction III from the experiment described in Figure 1 contained some nonenzymatic material as evidenced by an increase in its specific activity on rechromatography, but this impurity was not present in the sodium chloride fractionated extract of Figure 2. Therefore, the salt fractionation procedure was used routinely for the isolation of fraction III. As fraction III appeared to be homogeneous, it was selected for further studies. In the remainder of this paper, we shall often refer to this material simply as ficin.

The data in Table IA show the yields during the isolation of ficin without the sodium chloride fractionation, and the data in Table IB show the yields with the fractionation. In both cases the recovery of the activity from the chromatography column was quantitative. Although the data in Table IB reveals that only 35% of the activity was recovered during the sodium chloride precipitation step, it was preferable to sacrifice a high yield in favor of purer fraction III.

The tubes indicated by the arrows in Figure 2 were pooled and the protein was recovered by salting out the sodium chloride. During this precipitation, a small fraction always became irreversibly insoluble, but the amount of insoluble material apparently did not increase during storage. After 15 months of storage at 4°, about 5% of the ficin was insoluble but that

TABLE I: Yield of Ficin from Crude Preparation.

	BAPA Act. Units	A_{280} Units	Wt Recov (mg)
A.[a] Chromatography of Extract without Salt Fractionation			
Extract	81	7100	
Column effluent	80	6600	
Fraction 3	13	410	
B.[b] Chromatography of Extract after Salt Fractionation			
Extract	168		2250
Supernatant after salt fractionation	90		1370
Precipitate after salt fractionation	68	4000	1035
Column effluent	70	3850	1060
Fraction 3	14.8	475	225

[a] Gives the recovery of activity and absorbance in the experiment which is illustrated in Figure 1. The extract was prepared from 10 g of crude ficin. [b] Gives the recovery of activity and absorbance in an experiment similar to that illustrated in Figure 2. The extract, which was prepared from 20 g of crude ficin, had undergone a salt fractionation as described in the text. The weight recovery is based on the approximation that 1 BAPA activity unit is equal to 15.2 mg of enzyme, and the value includes all of the active components of ficin. Although there were about 225 mg of fraction III, a narrow cut was taken which contained about 150 mg. The isolation could be scaled up to 30 g of starting material without sacrificing resolution.

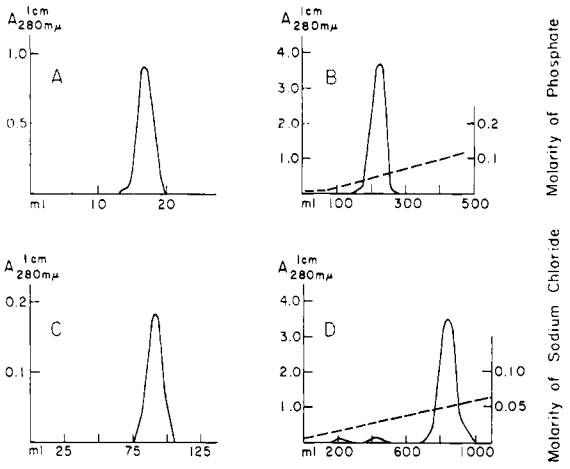

FIGURE 3: Chromatography studies. (A) Chromatography of 2.2 mg of ficin on a 0.9 × 20 cm column of Bio-Rex 70 (10 mequiv/g) equilibrated with pH 6.1 buffer composed of 0.1 M sodium phosphate and 0.001 M sodium tetrathionate. Fractions of 2.2 ml were collected at a rate of 13 ml/hr. (B) Chromatography of 80 mg of ficin on a 2 × 20 cm column of Cellex-CM (0.6 mequiv/g). The gradient was from 0.005 M sodium phosphate (pH 7.1) to 0.185 M sodium phosphate (pH 6.9). The buffer contained 0.001 M sodium tetrathionate. Fractions of 14 ml were collected at a rate of 70 ml/hr. (C) Chromatography of 1.1 mg of ficin on a 2 × 20 cm column of Bio-Rex 70 (10 mequiv/g) equilibrated with a pH 6.1 buffer composed of 0.03 M disodium Versene, 0.015 M sodium hydroxide, and 0.001 M sodium tetrathionate. Fractions of 2.3 ml were collected at a rate of 18 ml/hr. (D) Chromatography of 190 mg of ficin on a 4 × 20 cm column of Cellex-CM (0.6 mequiv/g). The column was equilibrated with a pH 6.3 buffer composed of 0.010 M disodium Versene, 0.0055 M sodium hydroxide, and 0.001 M sodium tetrathionate. Elution was carried out by a linear sodium chloride gradient from 0 to 0.13 M. Fractions of 20 ml were collected at a rate of 130 ml/hr.

which was soluble exhibited chromatographic and enzymatic properties identical with the freshly isolated enzyme.

When ficin is kept in solution it is desirable that sodium tetrathionate be kept in excess. When the tetrathionate was removed by gel filtration or dialysis, the ficin was found to exhibit up to 1.5% of its activity after incubation at 37° for 48 hr, and consequently became susceptible to autodigestion. Solutions were normally stored and chromatographed in 0.001 M sodium tetrathionate, as under these conditions there was no detectable enzymatic activity.

After lyophilization from water, dilute acetic acid, or ammonium bicarbonate solution, ficin solubility and activity decreased markedly, and consequently, this technique could not be used in preparing ficin for storage. When ficin was salted out with ammonium sulfate in the presence of sodium tetrathionate, it unexplainably sometimes became irreversibly insoluble, and therefore ammonium sulfate was not used for salting out. The insolubility may have been caused by an impurity in some batches of ammonium sulfate.

The homogeneity of fraction III was tested by gel filtration, thin-film dialysis, and chromatography on several ion-exchange systems using both carboxymethylcellulose and polymethacrylic acid resins. The results of the chromatography experiments are shown in Figure 3. In each case the ficin was eluted as a single peak, and there was quantitative recovery of enzymatic activity and of material absorbing at 280 mμ. Furthermore, the ratio of activity to A_{280} was constant across each peak. The results of gel filtration on Sephadex G-75 are shown in Figure 4. The pattern for fraction III is illustrated, but the other active fractions gave identical results. Ficin was eluted from the G-75 column slightly after ribonuclease, even though ribonuclease has a molecular weight about 55% of ficin. The unusual retention of ficin might be the consequence of an especially compact conformation, or it might

TABLE II: Amino Acid Composition of Ficin and Papain.[a]

Amino Acid	Ficin: Present Values: Measured Value[b]	Ficin: Present Values: Value to Nearest Integer	Ficin: Previously Reported Values: A[h,i]	Ficin: Previously Reported Values: B[h,j,k]	Ficin: Previously Reported Values: C[h,j]	Papain D[h]
Lysine	5.21 ± 0.05	5	7	9	9	9
Histidine	1.08 ± 0.04	1	2	2	2	2
Ammonia	25.3 ± 1.5[c]	25			52	19
Arginine	10.1 ± 0.4	10	8	7	10	10
Aspartic acid	17.1 ± 0.6	17	20	21	21	17
Threonine	8.4 ± 0.3[d]	8	10	8	10	7
Serine	14.4 ± 0.6[d]	14	13	10	16	11
Glutamic acid	25.2 ± 1.0	25	22	23	25	17
Proline	10.9 ± 0.4	11	11	12	13	9
Glycine	28.2 ± 1.1	28	28	30	32	23
Alanine	20.2 ± 0.8	20	19	21	21	13
Cystine 1/2	7.9 ± 0.3[e]	8	7	4	9	7[l]
Valine	17.5 ± 0.8	18	15	15	19	15
Methionine	5.12 ± 0.27	5	3	3	4	0
Isoleucine	7.25 ± 0.25[f]	7	7	10	10	10
Leucine	14.9 ± 0.6[f]	15	14	17	17	10
Tyrosine	15.5 ± 0.6[d]	15	11	14	15	17
Phenylalanine	5.12 ± 0.20	5	6	5	6	4
Tryptophan	5.7[g]	6	3		9	5

[a] Number of residues per mole of protein. [b] Average of three determinations values are per 25,000 g of protein. [c] Corrected for decomposition of serine, threonine, and cysteine. [d] Extrapolated to zero hours of hydrolysis. [e] Determined as cysteic acid and as carboxymethylcysteine. [f] Determined after 72-hr hydrolysis. [g] Spectrophotometric determination. The tyrosine value found by this procedure was 15.3. [h] References are (A) Marini-Bettolo *et al.* (1963), (B) Metrione (1963), (C) Gould (1964), and (D) Smith and Kimmel (1960). [i] This preparation was from *Fiscu anthelmintica;* others in the table were from *Ficus galbrata.* [j] Ficin purified by salt fractionation. [k] Uncorrected for decomposition of serine, threonine, cystine (1/2), and tyrosine. [l] From Glazer and Smith (1965).

be due to the high content of aromatic residues in ficin. It is known that aromatic compounds are strongly adsorbed to Sephadex (Porath, 1962). In the thin film dialysis experiment illustrated in Figure 5, ficin behaved as a single component as its escape followed a straight-line logarithmic plot, and the specific activity of the diffusate was constant throughout the experiment.

The molecular weight of ficin, as determined by equilibrium ultracentrifugation, was 25,500 ± 750. Within experimental error there was no evidence for contamination with either larger or smaller material, although there was some evidence for a concentration-dependent association in which monomer was in equilibrium with a small concentration of trimer. The molecular weight calculated from the amino acid composition (Table II) is 23,800 ± 700 which is about 5% less than the value obtained from the ultracentrifuge. In other experiments of this paper, an average value of 25,000 was used for the molecular weight of ficin. Previously reported values for the molecular weight of ficin have been 30,000 (Rothen, 1942), 26,000 (Bernhard and Gutfreund, 1956), and 26,500 (Marini Betto o *et al.*, 1963).

The various analytical data of ficin are collected in Table III. The values of the optical rotatory dispersion parameters given in the table were obtained from the plots of the dispersion data shown in Figure 6. Within experimental error, the active ficin and the tetrathionate-inactivated ficin possess identical dispersion parameters. A value for λ_c of 261 mμ has been reported for ficin (Jirgensons, 1959) which is in fair agreement with the present value.

The amino acid composition (Table II) is compared with values obtained by other investigators and is also compared with the composition reported for papain. Because of the central role which has been postulated for histidine residues in active sites of a number of enzymes, it is an interesting finding that ficin contains only one such residue. However, there was a complication in the measurement of the histidine content of ficin in that different values were obtained depending upon whether crushed resin or the newer high-speed

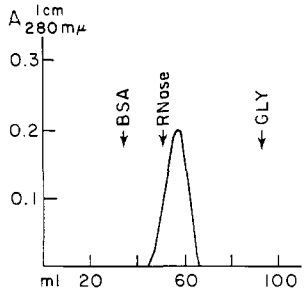

FIGURE 4: Gel filtration of 1.1 mg of ficin on a 150 × 0.9 cm column of Sephadex G-75. The solvent was a pH 7.6 buffer composed of 0.05 M Tris, 0.04 M hydrochloric acid, 0.001 M Versene, and 0.001 M sodium tetrathionate. The column was calibrated with bovine serum albumin, ribonuclease, and glycine and was run at room temperature. Fractions of 2.0 ml were collected at a rate of 10 ml/hr.

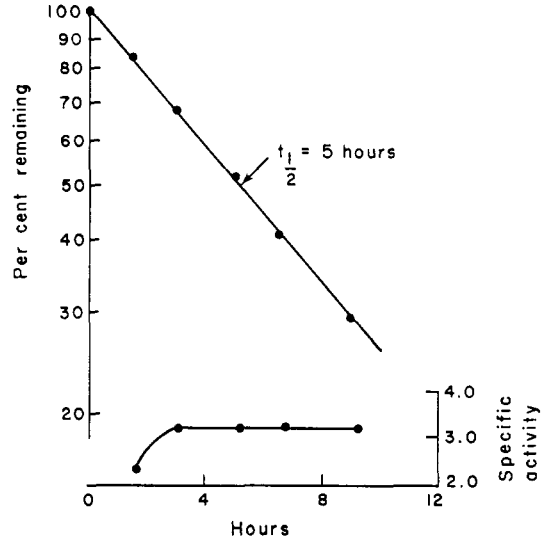

FIGURE 5: Thin-film dialysis of ficin at 40° in a Visking $^{20}/_{32}$ membrane stretched linearly and circularly. The solvent was 0.15 M ammonium acetate (pH 6.0). Assays were performed with BAPA as substrate. The specific activity is defined as the change in A_{410}/hr produced by 1.0 ml of diffusate of A_{280} 1.0. The point at 1.5 hr has an apparent low specific activity because rapidly dialyzed tetrathionate contributed significantly to the A_{280}.

bead resin was used for amino acid analysis. Using the 15-cm column of the analyzer (Aminex-MS resin, Blend Q-15), the histidine value was always found to be one residue, whereas if an aliquot of the same hydrolysate was run on the 5-cm column (Beckmann custom research resin, type AA-27), a double peak was observed in the histidine region whose total area often corresponded to approximately two residues. If the temperature of the column jacket fell to slightly below 55°, the two peaks would merge into one. The second peak did not appear on the 15-cm column because it was eluted from this resin in the position of ammonia; it could be detected, however, if the ammonia was first removed by elevating the pH of the hydrolysate to 12 and distilling the ammonia into sulfuric acid. It was easiest to measure the unknown by running the 5-cm column at 65°, when histidine, ammonia, and the unknown were almost completely separated from one another. The unknown material was found in amounts of 0.5–1.0 residue in crystalline ficin (Walti, 1938) and in ficin obtained from Nutritional Biochemicals Corp. and Calbiochem Corp. It was not destroyed if the hydrolysis was carried out at 142° instead of 110°, but it was partially destroyed by performic acid oxidation or hydrolysis in the presence of oxygen. Chromatography of authentic samples of 1-methylhistidine, 3-methylhistidine, and ethanolamine ruled out the possibility that the unknown was one of these substances. However, some preparations of ficin were consistently found to lack the unknown although they were isolated by the same procedure involving multiple dialysis and chromatography steps. For this reason, it seems likely that this is an impurity which is not covalently bonded to the protein. Since it is absent in some preparations, it is apparently not essential for any enzymatic function.

For the analysis of an amino-terminal residue, the hydrolysate of the dinitrophenylated protein was fractionated by countercurrent distribution. The distribution pattern is illustrated in Figure 7. The peaks with K = 0, ∞, and 1.1 are primarily ε-DNP-lysine, 2,4-dinitroaniline, and 2,4-dinitrophenol, respectively. The peak with K = 4.6 is in the position where DNP-leucine or DNP-isoleucine was expected to appear.

TABLE III: Summary of Analytical Data.

Weight recovered as amino acids (%)	97
Nitrogen, calculated from amino acid composition (%)	16.86
Nitrogen Kjeldahl analysis (%)	16.77
Ash (%)	0.66
Molecular weight, determined by ultracentrifugation	25,500 ± 750
Molecular weight, from amino acid composition	23,800 ± 700
Partial specific volume, calculated from amino acid composition (cc/g)	0.725
$A_{280}^{1\,cm}$ for c = 1 mg/ml	2.1
A_{280}/A_{260}	1.95 ± 0.05
$[\alpha]_{546}$ (deg)	−44.1
λ_c (mμ)	267
a_0 (deg)	−200
b_0 (deg)	−166

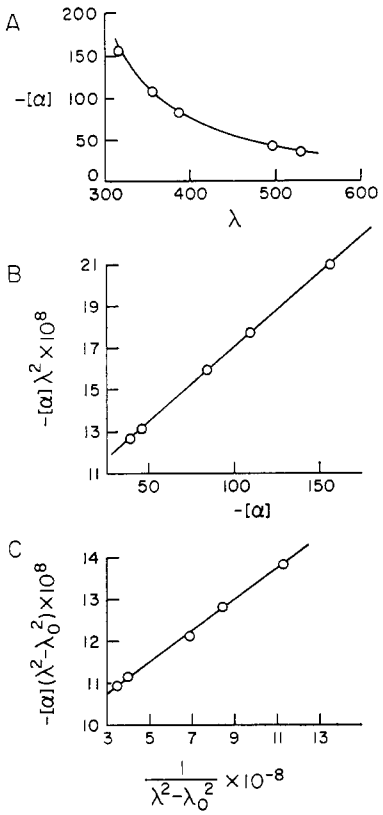

FIGURE 6: Optical rotatory dispersion of tetrathionate inactivated ficin at 23° in 0.1 M sodium phosphate–0.001 M Versene (pH 7.0). (A) The specific rotation is plotted *vs.* wavelength. (B) The data in A are plotted according to the method of Yang and Doty (1957). (C) The data in A are plotted according to the Moffit equation (Moffit and Yang, 1956).

Each of the countercurrent fractions was subjected to two-dimensional paper chromatography, and no α-DNP-amino acids were detected except for the K = 4.6 fraction which chromatographed identically with DNP-leucine or DNP-isoleucine. The identity of the amino-terminal residue was confirmed to be leucine by amino acid analysis of the K = 4.6 fraction after removal of the DNP group by hydrolysis at 110° in saturated barium hydroxide (Mills, 1950). The yield of DNP-leucine, as determined by integration of the countercurrent peak, was 86%. Previous reports of the amino-terminal residue of ficin were made by Metrione (1963) and Gould (1964), and both found that leucine was the predominant end group. Both reported the presence of contaminating DNP-amino acids, but the contamination is probably a consequence of a heterogeneous sample of ficin.

Attempts to detect a carboxyl-terminal residue were unsuccessful. Neither carboxypeptidase A nor B would liberate significant quantities of any amino acid from native ficin. In contrast to these results, Gould (1964) reported that 0.9 residue of alanine, followed by smaller quantities of valine and leucine, were liberated by carboxypeptidase A from a mercury derivative of ficin. His reaction conditions did not differ significantly from those described in this communication, yet differing results were obtained.

TABLE IV: Reactive Sulfhydryl Groups of Ficin as Measured by Carboxymethylation.

	Carboxymethylcysteine[a]
Inactive ficin[b]	0
Active ficin[c]	1.10
Denatured ficin[d]	2.06

[a] Calculated on the basis that the content of phenylalanine is five residues per mole. [b] The carboxymethylation of inactive ficin was carried out in 9 ml of Tris-HCl buffer (pH 8) containing ficin (3.5 mg) and Tris-iodoacetate (10 μmoles). After 15 min at 25°, excess reagents were removed by passage through Sephadex G-25 (50 × 2 cm) using 0.005 M NH_4HCO_3 as buffer, and then the protein lyophilized. [c] The carboxymethylation of active ficin was carried out in a like manner; the ficin (4.4 mg, 0.16 μmole) was activated *in situ* with diethioerythritol (13 mg, 80 μmoles) and an excess of Tris-iodoacetate (190 μmoles) was used. [d] A sample of carboxymethylated ficin (2.9 mg) (prepared as in footnote 2) was subjected to a second treatment with iodoacetatic acid (30 mg) in 5 M guanidine hydrochloride buffered at pH 7.3 with 0.3 M phosphate. After 2 hr at 37°, the product was recovered after dialysis. Amino acid analysis also revealed the presence of 0.5 equiv of *S*-carboxymethylhomocysteine, indicating some methionine alkylation.

Carbohydrate analysis suggested that ficin is not a glycoprotein. The presence of pentose was ruled out by the orcinol–ferric chloride test, and the presence of hexose was shown to be unlikely by the orcinol–sulfuric acid test. Although in the latter test the color yield at 530 mμ was sufficient to account for the four to five molecules of glucose per molecule of ficin, the spectrum of the reaction product was completely different from that which was produced by any of the sugars used as standards.

The dependence of ficin's enzymatic activity on a sulfhydryl group makes it of interest to make a careful analysis of the number of these groups. Ficin was alkylated with iodoacetic acid under several experimental conditions, and carboxymethylcysteine was measured by amino acid analysis. The results of these experiments are given in Table IV. There is no readily detectable sulfhydryl group in tetrathionate-inactivated ficin, there is one in active ficin upon which the activity depends, and there is a second one which is detectable only in denatured ficin. The results of the amperometric titration experiments are illustrated in Figure 8. The titration revealed the presence of 0.92 equiv of sulfhydryl group/25000 g of ficin and the absence of a

TABLE V: Determination of Disulfide Bonds of Tetrathionate Inactivated Ficin by Sulfite Cleavage.

Expt	Reaction Condition[a]	Moles of Thiol Liberated	Comments
1	Incubated in 8 M urea at 37°, then Na_2SO_3 added. Titrated at 37°.	0.03	The disulfide bonds and the $SSSO_3^-$ derivative of the essential thiol group were not cleaved. Also the buried thiol group was not liberated at 37°.
2	Incubated in 8 M urea at 50° for 20 min, then Na_2SO_3 added. Titrated at 50°.	2.2	The result suggested incomplete reduction.
3	Incubated in 8 M urea at 58° for 90 min. Temperature lowered to 37° and Na_2SO_3 added. Titrated at 37°.	3.4	The expected thiol content is 4 moles from the reduction of three disulfide bonds and the liberation of a buried thiol group. The lower value might be due to oxidation or carbamylation (Stark, 1964).

[a] Ficin (0.1 μmole) was dissolved in 1×10^{-3} M disodium Versene and 1.5×10^{-4} M HCl, and solid urea was added to a final concentration of 8 M. The final volume was 15 ml. After incubation, 50 μl of freshly prepared solution of saturated Na_2SO_3 was added. The liberated thiol was determined immediately by amperometric titration.

sulfhydryl group in ficin which had been inactivated by tetrathionate. It also confirmed the fact that enzymatic activity depends upon this single sulfhydryl group.

The presence of eight half-cystine residues in ficin, and the detection of two of these residues as cysteine, suggests that the remaining six are paired in three disulfide bonds. Evidence supporting this value was obtained by titration of a denatured sample in which the disulfide bonds had been cleaved by sodium sulfite (Carter, 1959). This reagent reacts quantitatively with disulfide bonds to yield an SSO_3^- group and a sulfhydryl group. The sulfhydryl group is then titrated with silver ion amperometrically. The results of these experiments on inactive ficin are given in Table V. Besides providing evidence for the existence of three disulfide bonds in ficin, these experiments also demonstrate the resistance of ficin to urea denaturation. This resistance to urea denaturation was also found in the attempts to reduce and carboxymethylate ficin in 8.0 M urea.

These findings on sulfhydryl groups and disulfide bonds of ficin are consistent with those previously reported in the literature. A number of workers using various reagents have reported the existence of one reactive sulfhydryl group in ficin upon which the

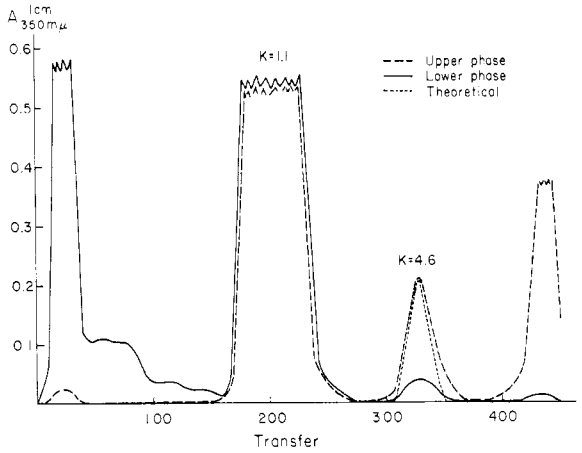

FIGURE 7: Countercurrent distribution of the hydrolysate of DNP-ficin (1.25 μmoles) after 451 transfers. The solvent system was prepared from equal volumes of ethyl acetate and 0.13 M NaH_2PO_4–1.20 M K_2HPO_4. The upper and lower phases had volumes of 2.3 and 3.0 ml, respectively.

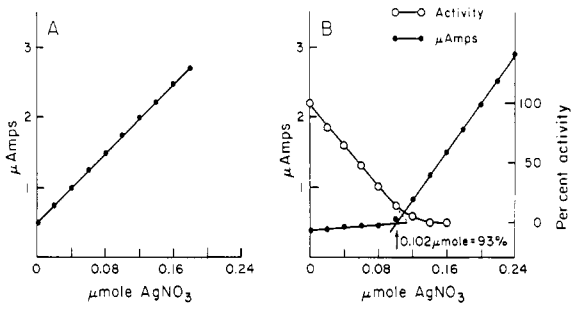

FIGURE 8: Amperometric titration of ficin in 6.0 ml of pH 7.4 buffer containing 0.25 M Tris-nitrate, 0.019 M potassium chloride, and 0.00075 M Versene. The titrant was 0.002 M silver nitrate. Aliquots of 0.20 ml were removed for assay against BAPA (mercaptoethanol was omitted in these assays), and correction was made for the quantity of ficin removed. (A) Titration of 0.096 μmole of tetrathionate-inactivated ficin. (B) Titration of 0.110 μmole of freshly activated ficin.

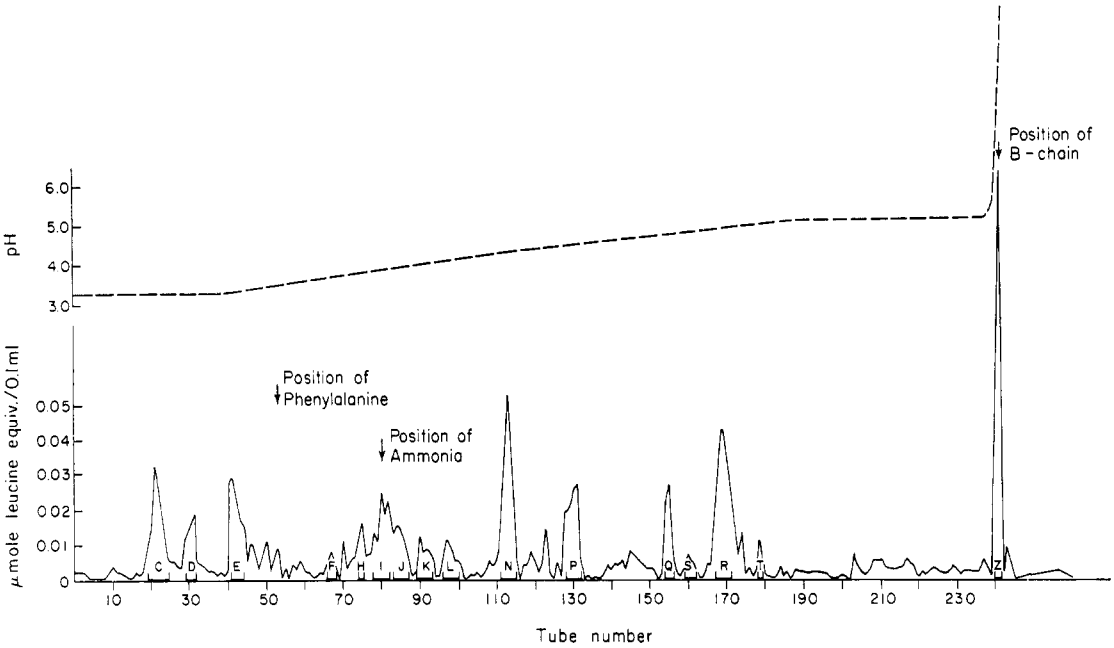

FIGURE 9: Separation of insulin B chain peptides on a 15 × 0.9 cm column of Aminex MS blend Q-15 resin. Elution was by gradient from 0.2 M sodium citrate (pH 3.25) to 0.35 M sodium citrate (pH 5.28) followed by 0.2 M sodium hydroxide. The column was run at 34 ml/hr at 55° and 1.5-ml fractions were collected. Peptides were detected by alkaline hydrolysis of 0.1-ml aliquots followed by ninhydrin analysis. Elution positions of phenylalanine, ammonia, and B chain are indicated.

activity depends (Liener, 1961; Chang and Liener, 1964; Hollaway *et al.*, 1964), and Liener (1961) has reported the existence of a buried sulfhydryl group. Liener could detect only one disulfide bond, but the reason for the low value is probably the resistance of ficin to denaturation.

In Table VI the enzymatic activity of ficin, using BAPA and casein as substrates, is compared with the activity of crystalline papain and trypsin. Whereas ficin has the greatest activity with casein, it shows a relatively low level of activity with BAPA.

TABLE VI: Activity of Ficin, Papain, and Trypsin against BAPA and Casein.[a]

	Ficin	Papain	Trypsin
BAPA (mole/mole min)	1.6	2.7	25
Casein (activity units/μmole)	2310	1580	1035

[a] Ficin and papain assays were performed as described in Methods. Trypsin was assayed under conditions identical with those described for ficin except that versene and mercaptoethanol were omitted; the level of casein activity at pH 7 is about 10% less than the activity at the optimum pH (Northrop and Kunitz, 1932).

An investigation of ficin's specificity was made by determining the points of cleavage catalyzed by ficin on the B chain of oxidized insulin. The peptides were separated by chromatography as is shown in Figure 9, and their composition was determined by amino acid analysis. The composition of some of the peaks are shown in Figure 10. Peaks P, K, L, H, and R are homogeneous peptides as indicated by their composition, but peaks C, D, I, and J are contaminated mixtures. The likely position of these peptides in the B chain is given in the figure, and the cleavages shown are only the major ones. If larger amounts of ficin were used or if the hydrolysis was carried out for a longer duration, the reaction mixture became too complex to make any interpretations. There is no obvious specificity pattern which can be readily detected although, because cleavages occurred after four of the five aromatic residues, it is likely that ficin has some preference for aromatic substrates. Surprisingly, no splits were detected after the basic residues lysine and arginine, as it has been previously thought that arginyl bonds were among the most favorable for ficin-catalyzed hydrolysis (*e.g.*, Smith and Kimmel, 1960). Unfortunately, there are no methionine residues in the oxidized insulin B chain, as studies with synthetic substrates by Fruton and coworkers have suggested that methionine derivatives are excellent substrates for ficin (Dekker *et al.*, 1949).

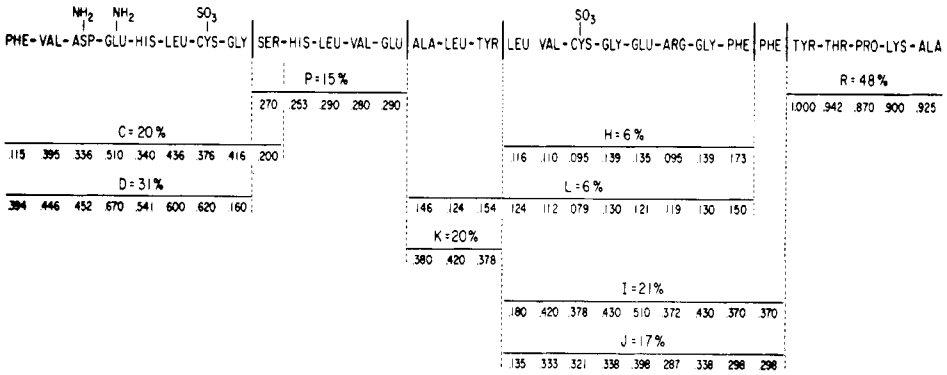

FIGURE 10: Ficin-catalyzed hydrolysis of the B chain of insulin. The horizontal lines delineate the peptides which were isolated from the digest, and are identified by letters referring to peaks in the chromatogram in Figure 9. Yields of each peptide are indicated, and the figures under the horizontal lines refer to μmoles of each amino acid/1.92 μmoles of B chain.

Discussion

Crude ficin latex was found to contain several enzymatically active fractions (Figure 1). Sgarbieri *et al.* (1964) have reported such an observation. The four main fractions in Figure 1 have the same ratio of casein to BAPA activity, but a significant difference among the four fractions is their solubility in sodium chloride solution. Comparison of the chromatograms in Figures 1 and 2 shows that fraction I was not salted out as effectively as fractions II and III during the preliminary sodium chloride precipitation of the extract.

The essential cysteine residue in the major portion of crude ficin is blocked in some manner, as crude extracts contain only 25% of the potential activity when assayed in the absence of activators. This heterogeneity, however, is not the cause of the chromatographic dispersity of ficin. The same chromatographic pattern was obtained with an extract which had been activated initially with cysteine and then deactivated immediately with tetrathionate, as with an extract inactivated directly with tetrathionate.

The reason for crude ficin exhibiting only 25% of its potential activity is not known. This inactivity is reversed readily by reaction with thiol reagents, and there is no change in the molecular size or conformation accompanying the activation, as detectable by sedimentation, chromatography on Sephadex G-75 and optical rotatory dispersion. It is conceivable that the blocked thiol group in inactive ficin is in the form of an intra- or intermolecular disulfide bond. The presence of an intramolecular disulfide bond involving the buried cysteine residue can be ruled out, as the sulfite reduction experiment of inactive ficin indicates that the buried cysteine residue is in the oxidation state of a thiol. An intermolecular disulfide bond comprising two molecules of ficin is unlikely, since the inactive and the active ficins have the same molecular size as determined by gel filtration. On the other hand, there is an indication of mixed disulfide bonds with low molecular weight sulfhydryl compounds; when the purest available ficin preparations were activated by cysteine, a mercaptan-like odor was detected. A volatile mercaptan of unknown structure has been found in streptococcal proteinase linked by a mixed disulfide bond to its essential cysteine residue (Ferdinand *et al.*, 1965). Small quantities of cysteine (up to 0.28 mole) had been found bound to papain in a mixed disulfide bond (Glazer and Smith, 1965), but in this case, the result could have been an artifact due to the high concentration of cysteine used during the extraction of papain from the latex (Kimmel and Smith, 1958).

It has been found on the basis of amperometric titration and analysis of carboxymethylcysteine that ficin contains a single reactive sulfhydryl group and that enzymatic activity depends upon this group. When various preparations were assayed after activation with mercaptoethanol a characteristic and reproducible specific activity was obtained. Although no active-site titrations or all-or-none assays have been carried out on ficin, these results are consistent with each molecule of enzyme being active. However, it has been reported with papain that different preparations of enzyme, even after treatment with excess activating agents, have variable sulfhydryl contents (often as low as 0.5 sulfhydryl group per mole) and consequently they have variable specific activities. As expected, the specific activity of a papain sample is directly proportional to its sulfhydryl content (Finkle and Smith, 1958). The reason for the variable sulfhydryl content in papain is unknown, although it must involve only the reactive cysteine residue or its immediate environment because crystalline papain fulfills many criteria of homogeneity.

The present studies on ficin have provided several additional points of similarity between ficin and papain. Although papain (molecular weight 21,000) is slightly smaller than ficin, both proteins are probably single-chain polypeptides. Both contain three disulfide bonds, and both have a single cysteine residue upon which activity depends. Papain, however, has no unreactive

sulfhydryl group (Glazer and Smith, 1965). Papain has an isoelectric point of 8.75 (Smith and Kimmel, 1960), and, although this value was not determined for the ficin preparation isolated in this paper, its basicity is suggested by its binding to carboxymethylcellulose at pH 7. An isoelectric point of pH 9 was reported for a preparation of crystalline ficin (Cohen, 1958). Both ficin and papain are irreversibly denatured by acid. Ficin is denatured in solutions as weakly acidic as 0.01 M acetic acid. Neither enzyme is completely unfolded by 8 M urea or by elevated temperature. Ficin was found to be inactive in 8 M urea or 5 M guanidine hydrochloride, but papain is active in 8 M urea in the presence of Versene (Gundlach and Turba, 1965). The amino acid compositions of both enzymes are similar, although notable differences are the absence of methionine residues in papain and the presence of two histidine residues in papain, whereas there is only one histidine residue in ficin.

Both enzymes are noted for their broad specificity and their effectiveness at digesting protein, but the preferred amino acid residues are different. The best synthetic substrates for papain are derivatives of benzoylarginine or -lysine and carbobenzoxyglutamine (Smith and Kimmel, 1960), whereas for ficin the best known synthetic substrate is carbobenzoxymethionine amide (Dekker *et al.*, 1949). The broad specificity of ficin is demonstrated by the large number of peptides obtained from its digestion of oxidized B chain of insulin (Figures 9 and 10). Attention may be drawn to its efficient cleavage of a Phe-Tyr bond in the B chain. A similar efficient cleavage of a Tyr-Phe bond in a hexapeptide by papain has been reported (Konigsberg and Hill, 1962). When the data on the ficin-catalyzed hydrolysis of the B chain is compared with the papain-susceptible peptide bonds (reviewed by Hill, 1965), the specificity differences of these two enzymes is no longer so readily discernible.

These marked similarities in the properties of ficin and papain make them an excellent pair of complementary proteins in the study of sulfhydryl proteinases. Information gained on one of them may likely apply to the other.

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An Infrared Study of Bound Carbon Monoxide in the Human Red Blood Cell, Isolated Hemoglobin, and Heme Carbonyls*

James O. Alben† and Winslow S. Caughey‡

ABSTRACT: The binding of carbon monoxide to hemes has been studied by high-resolution infrared spectroscopy. Inductive and resonance effects of substituents both on the porphyrin ring (*cis* effects) and on pyridine that is coordinated to the iron(II) (*trans* effects), as well as solvent effects in simple synthetic heme derivatives are reported and compared with the infrared difference spectrum of carbonyl–hemoglobin (HbCO − HbO_2) to yield a picture of intramolecular interactions that is consistent with hemoglobin structure. Assignment of the infrared absorption bands (ν_{CO}) of HbCO and heme–CO has been confirmed by isotopic substitution with [^{13}C]- or [^{18}O]carbon monoxide. The isotopic data for HbCO appear to be more consistent with Hb–Fe–O≡C (oxygen coordinated to the iron) than with the more conventional structure, Hb–Fe–C≡O (carbon coordinated to the iron), while a structure in which CO is bound parallel to the heme appears unlikely.

The reactions of carbon monoxide with hemoglobins, cytochrome *c* oxidase, and other hemoproteins, as well as with simple iron(II) porphyrins (hemes), have long been of both biochemical and chemical interest. Because structural and chemical interpretations of bonding in transition metal carbonyls have been based on studies of carbon–oxygen stretching frequencies (*cf.* Cotton and Wilkinson, 1966), the availability of infrared instrumentation of high sensitivity and resolution suggested the possibility that CO stretching frequencies (ν_{CO}) could also be observed and interpreted for carbon monoxide bound to the intact red blood cell or to isolated hemoglobins. Here we report and discuss such ν_{CO} values obtained with the normal human red blood cell and isolated hemoglobin and with variously substituted deuterohemes. A portion of this work has appeared in preliminary communications (Alben and Caughey, 1962, 1966; Caughey *et al.*, 1965, 1967).

Experimental Section

Materials. The hemes (Alben *et al.*, 1968) and metal-free porphyrins (Caughey *et al.*, 1966a) were prepared and characterized as reported earlier. Normal human hemoglobin A that was used with $^{12}C^{16}O$, kindly donated by Professor Keith Richardson, Department of Physiological Chemistry, Ohio State University, was prepared from washed red blood cells by lysis through freezing and thawing, removal of the stroma, and passage through a column of diethylaminoethylcellulose in 0.005 Tris-phosphate buffer (pH 8). Normal human hemoglobin A that was used with $^{13}C^{16}O$ or $^{12}C^{18}O$ was obtained in concentrated solution as a biproduct from the purification of genetically abnormal hemoglobin by Professor Samuel Charache, Department of Medicine, The Johns Hopkins School of Medicine. The carbon monoxide complex

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