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Preparation of Fully Active Ficin from *Ficus glabrata* by Covalent Chromatography and Characterization of its Active Centre by using 2,2′-Dipyridyl Disulphide as a Reactivity Probe

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1. Fully active ficin (EC 3.4.22.3) containing 1 mol of thiol with high reactivity towards 2,2′-dipyridyl disulphide (2-Py–S–S–2-Py) at pH4.5 per mol of protein was prepared from the dried latex of *Ficus glabrata* by covalent chromatography on a Sepharose-glutathione–2-pyridyl disulphide gel. 2. Ficin thus prepared is a mixture of ficins I–IV and ficin G, in which ficins II and III predominate. The various ficins exhibit similar reactivity characteristics towards 2-Py–S–S–2-Py. 3. Use of 2-Py–S–S–2-Py as a reactivity probe demonstrates (*a*) that in ficin, as in papain (EC 3.4.22.2), the active-centre thiol and imidazole groups interact to provide a nucleophilic state at pH values of approx. 6 additional to the uncomplicated thiolate ion that predominates at pH values over 9, and (*b*) a structural difference between ficin and papain that leads to a much higher rate of reaction of 2-Py–S–S–2-Py with ficin than with papain at pH values 3–4. This difference is suggested to include a lack in ficin of a carboxyl group conformationally equivalent to that of aspartic acid-158 in papain. 4. The high electrophilicity of the 2-Py–S–S–2-PyH$^+$ monocation allows directly the detection of the exposure of the buried thiol group of ficin at pH values below 4.

Most of the work on the *Ficus* proteinases, known collectively as ficin (EC 3.4.22.3), has been carried out on enzyme prepared from commercially available dried latex of *Ficus glabrata* (see Glazer & Smith, 1971).

The early kinetic work on ficin revealed a striking similarity in the catalytic properties of this enzyme and those of the much better characterized thiol proteinase papain (EC 3.4.22.2) (see Smith & Kimmel, 1960). This similarity in many respects has been confirmed by subsequent kinetic and structural work (see Glazer & Smith, 1971). The essential thiol group of ficin (Bernhard & Gutfreund, 1956; Hollaway *et al.*, 1964; Wong & Liener, 1964) is part of its catalytic site and is transiently acylated during catalysis (Lowe & Williams, 1964, 1965). The sequence around the essential cysteine residue bears a close resemblance to that around the essential (and only) cysteine residue of papain (Wong & Liener, 1964) and, as in papain, the essential thiol group of ficin is close to the imidazole group of a histidine residue (Husain & Lowe, 1968; Gleisner & Liener, 1973).

Although papain and ficin are closely similar in many respects, some differences in properties have been demonstrated. For example, some differences in kinetic behaviour suggest that various rate constants of the kinetic mechanism may contribute differently to the kinetic parameters that characterize the hydrolysis of the same substrates by papain and by ficin (Whitaker, 1969). Also, some differences are seen in the patterns of cleavage of the oxidized insulin B-chain by ficin (Englund *et al.*, 1968) and by papain (Johansen & Ottesen, 1968). For a review of the thiol proteinases, see Lowe (1976).

We have pointed to the particular value of 2,2′-dipyridyl disulphide (2-Py–S–S–2-Py) as a reactivity probe for the detection of interaction between thiol groups and other acid/base groups (Brocklehurst, 1974) and have used this probe to characterize the papain active site (see Shipton *et al.*, 1975, and references therein). The present paper reports that, as in papain, the essential thiol group of ficin interacts with another ionizing group (most probably the neighbouring imidazole group analogous to that of histidine-159 of papain) to provide a nucleophilic state additional to that at high pH, where all the active-centre groups are proton-deficient. In contrast with papain, however, ficin does not appear to possess a carboxyl group functionally equivalent to that of aspartic acid-158 of papain.

The high electrophilicity of the 2-Py–S–S–2-PyH$^+$ monocation permits direct detection at pH values below 4 of the buried thiol group of ficin, which has no analogue in papain.

Use of an insoluble derivative of 2-Py–S–S–2-Py provides a convenient method of preparation (covalent chromatography) and storage of fully active ficin.

Materials and Methods

Acetonitrile ('Special-for-Spectroscopy' grade) and dimethyl sulphoxide were purchased from BDH (Poole, Dorset, U.K.). 2-Mercaptoethanol, α-*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride, α-*N*-benzoyl-L-arginine ethyl ester hydrochloride, L-cysteine free base and dithiothreitol were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. *p*-Nitrophenylhippurate was a gift from Dr. C. W. Wharton, Department of Biochemistry, University of Birmingham, Birmingham, U.K. 2,2'-Dipyridyl disulphide and the buffers used in kinetic studies have been described previously (Stuchbury *et al.*, 1975).

Ficin (*EC* 3.4.22.3)

Ficin was extracted from the dried latex of *Ficus glabrata* (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) by using (i) the method of Englund *et al.* (1968) or (ii) the method described below.

Preparation of fully active ficin containing 1 mol of thiol with high reactivity towards 2-Py–S–S–2-Py at pH4.5/mol of protein

Dried latex (40g) was dissolved in 400ml of 10mM-L-cysteine free base containing 1mM-EDTA at 22°C. The pH of the solution (initially pH4) was adjusted to 7.0 with 1M-NaOH and stirring was continued for 30min, during which time no precipitate formed. In trial experiments no precipitate formed when the mixture was stirred for 1h. NaCl (A.R. grade, 17.7g/100ml of solution) was then added at 22°C, and after the mixture had stood for 1h at 4°C the precipitate was isolated by centrifugation (14000*g* for 30min at 4°C). The precipitate was washed with 50ml of 0.1M-sodium acetate buffer, pH4.5 (0.036M-sodium acetate and 0.064M-acetic acid) containing 0.3M-KCl and 1mM-EDTA and was then dissolved in 400–500ml of the same buffer at 22°C. This solution was mixed with 150ml of Sepharose–glutathione–2-pyridyl disulphide gel (Brocklehurst *et al.*, 1973, 1974) that had been allowed to equilibrate in the sodium acetate buffer, and the mixture was stirred gently for 20min at 22°C. The gel was isolated on a sintered-glass filter and washed rapidly with the acetate buffer (2 litres). The gel was then washed with 0.5M-NaCl containing 1mM-EDTA (approx. 3 litres) until the values of E_{280} and E_{343} of the filtrate had each fallen below 0.03. The gel was then allowed to equilibrate with 3 litres of 0.1M-Tris/HCl buffer, pH8.0 (0.045M-Tris and 0.055M-Tris/HCl), containing 0.3M-NaCl and 1mM-EDTA, and the covalently bound ficin was released by treatment with L-cysteine. A solution of L-cysteine (250ml, 20mM) in the Tris/HCl buffer was added to the gel, and the mixture was stirred gently for 1h and then subjected to vacuum filtration. The gel was washed on the filter with a further 250ml of the Tris/HCl buffer containing 20mM-L-cysteine, and these washings were added to the L-cysteine filtrate. To the combined filtrate and washings was added $(NH_4)_2SO_4$ (25g/100ml) and the mixture was left at 4°C for 1–2h. The precipitate was isolated by centrifugation (14000*g* for 30min at 4°C) and washed with 50ml of the sodium acetate buffer, pH4.5, containing 12.5g of $(NH_4)_2SO_4$ and 1mM-EDTA. The solid material was stored under a small volume of the washing buffer at 4°C until required.

Fully active ficin could then be obtained from this suspension by incubation for 20min at 22°C with the Tris/HCl buffer, pH8.0, containing 20mM-L-cysteine, 5mM-dithiothreitol and 1mM-EDTA, and separation from low-molecular-weight material by chromatography on Sephadex G-25 (see Brocklehurst *et al.*, 1973, 1974).

Ion-exchange chromatography on CM-cellulose (CM32) of ficin prepared by covalent chromatography

Sodium phosphate buffer (pH6.9, 0.185M) was prepared by dissolving 44.94g of $NaH_2PO_4,2H_2O$ and 64.17g of Na_2HPO_4 in 4 litres of 1mM-EDTA (see Englund *et al.*, 1968). Some of this solution (27ml) was diluted with 1mM-EDTA to give a final volume of 1 litre which provided 5mM-sodium phosphate buffer, pH7.1. Ficin prepared by covalent chromatography (approx. 250mg) was dissolved in 50ml of the 5mM-sodium phosphate buffer and this solution was dialysed against 2×5 litres at 4°C during 24h. The ficin in the resulting solution was mainly (at least 90%) in the reversibly blocked state. The ficin solution was divided into two portions (10ml and 40ml) and each was applied at 22°C to a separate column (15cm×1.5cm) of CM-cellulose (CM32) which had been precycled and allowed to equilibrate with the 5mM-sodium phosphate buffer. The column was washed with 40ml of the equilibrating buffer and the protein was eluted with sodium phosphate buffer in a linear gradient from 5mM (pH7.1) to 0.185M (pH6.9), essentially as described by Englund *et al.* (1968). Similar elution patterns were obtained from both columns. A similar elution pattern was obtained also when fully active ficin reversibly blocked by reaction with sodium tetrathionate was subjected to chromatography on CM-cellulose. Ficin III prepared by the method of Englund *et al.* (1968) was shown to be homogeneous by chromatography on a second column of CM 32.

Eluate analysis

The fractions were analysed by conductance measurement, E_{280} measurement and measurement of catalytic activity towards α-*N*-benzoyl-DL-arginine

p-nitroanilide essentially as described by Englund *et al.* (1968).

Michaelis parameters for ficin-catalysed hydrolyses

(*i*) *α-N-Benzoyl-L-arginine ethyl ester.* Initial rates were determined at 25.0°C and pH 6.6 with a Radiometer titrator as a pH-stat and 0.1 M-NaOH as titrant.

The assay mixture (5ml) contained substrate (5–100mM), NaCl (0.1 M) and L-cysteine free base (1 mM). The reaction was usually started by addition of the enzyme (2–5 μM). Linear progress curves were obtained. Parameter evaluation was carried out as described previously (Brocklehurst *et al.*, 1973).

(*ii*) *p-Nitrophenylhippurate.* Michaelis parameters were evaluated from complete progress curves by using the integrated form of the Michaelis–Menten equation as described by Hollaway *et al.* (1969). The reactions were carried out at 25.0°C in a Cary 16K spectrophotometer. The assay mixture (3ml) contained sodium phosphate buffer, pH 5.9, $I = 0.1$ mol/l, containing 8.2% (v/v) acetonitrile, and enzyme (0.2–0.5 μM). The reaction was started by the addition of *p*-nitrophenylhippurate (approx. 0.1 mM) in acetonitrile (50 μl). The concentration of the substrate was determined as a routine by complete hydrolysis in 1 M-NaOH, by using $\Delta\varepsilon_{400} = 18900$ litre·mol^{-1}·cm^{-1} (Goren & Fridkin, 1974).

Initial rates of the catalysis by ficin of the hydrolysis of α-N-benzoyl-DL-arginine p-nitroanilide

The procedure used was based on the sampling assay described by Arnon (1970) for the papain-catalysed hydrolysis, modified to provide a continuous assay.

The substrate in the form of its hydrochloride (43.5mg) was dissolved in 1ml of dimethyl sulphoxide, and 99ml of 0.05M-Tris/HCl buffer, pH 7.5, containing 2mM-EDTA was added. The reaction was started by addition of 0.1ml of enzyme solution to 2.9ml of the substrate solution at 37°C and monitored by recording the increase in E_{410}. In addition to runs carried out in the absence of added thiol, two other types of experiment were performed. Sometimes an enzyme solution was preincubated for 15–30min with 5–20mM-L-cysteine at pH 6–7 before 0.1ml was withdrawn and used to start the reaction. In other runs, 5–20mM-L-cysteine was incorporated into the stock solution of the substrate. The latter type of experiment revealed the presence of reversibly blocked ficin in the crude latex. The unblocking of the ficin was seen as an accelerating phase in the progress curve. Initial rates (in *E* units/s) were determined from the linear part of the progress curves. For *p*-nitroaniline (pH 5–10.5) $\lambda_{max.} = 380$nm, $\varepsilon_{max.} = 13500$ litre·mol^{-1}·cm^{-1}; $\varepsilon_{410} = \Delta\varepsilon_{410}$ for production of *p*-nitroaniline = 8800 litre·mol^{-1}·cm^{-1} (Erlanger *et al.*, 1961).

Kinetics of the reaction of ficin with 2-Py–S–S–2-Py

Complete progress curves were recorded on an oscilloscope by using a Durrum stopped-flow spectrophotometer or an Aminco–Morrow DW2 stopped-flow spectrophotometer. The reactions were studied at 25.0°C and $I = 0.1$ mol/l. The two syringes (1) and (2) contained (1) ficin (approx. 20 μM) in 0.1 M-KCl containing 1mM-EDTA, and (2) 2-Py–S–S–2-Py (400–500 μM) and buffer, $I = 0.2$ mol/l. Data pairs (E_{343}, time) were taken from photographs of the progress curves and pseudo-first-order rate constants computed from conventional first-order plots. These rate constants were divided by [2-Py–S–S–2-Py] to provide second-order rate constants (k). [2-Py–S–S–2-Py] was determined using $\varepsilon_{281} = 10200$ litre·mol^{-1}·cm^{-1} [T. Stuchbury & K. Brocklehurst, unpublished work; Grassetti & Murray (1967) give $\varepsilon_{281} = 9730$ litre·mol^{-1}·cm^{-1}]. The characterizing parameters of the pH–k profile (Fig. 4) were determined as follows. The data in the pH range 1.3–4.8 were used to evaluate $\tilde{k}_1$, pK_I and pK_{II} using the computer optimization procedure given in the following paper (Shipton *et al.*, 1976). These parameters were then used to calculate the contribution from this bell-shaped component to the value of k at pH 7. Subtraction of this contribution therefore yielded a provisional estimate of $\tilde{k}_2$. This and other (lower) estimates of $\tilde{k}_2$ were subtracted from the values of k obtained in the pH range 7.0–10.1, and values of $\tilde{k}_3$ and pK_{III} were determined by computer optimization. The values of $\tilde{k}_3$ and pK_{III} with the lowest standard errors were selected and these, together with the corresponding value of $\tilde{k}_2$ and the values of $\tilde{k}_1$, pK_I and pK_{II}, were used to compute the theoretical line in Fig. 4.

Protein concentrations

These were determined from measurements of E_{280}, by using $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1} (Hollaway, 1967).

Thiol concentrations

These were determined by using 2-Py–S–S–2-Py (Brocklehurst & Little, 1973) and $\varepsilon_{343} = 8080$ litre·mol^{-1}·cm^{-1} (Stuchbury *et al.*, 1975).

Spectroscopic study of the exposure of the buried thiol group of ficin

Some progress curves for the reaction of ficin with 2-Py–S–S–2-Py at pH values ≤ 4 were recorded by using a Cary 16K spectrophotometer. This was a convenient way to record the progress of the slow phase of the reaction that follows the essentially instantaneous phase, which was investigated separately by using the stopped-flow technique.

Results and Discussion

Preparation of fully active ficin by covalent chromatography

The ficin molecule contains two L-cysteine thiol groups not engaged in disulphide bonds, but one of these is buried in the native protein and its reactions with electrophilic reagents have been observed only in denaturing media, such as sodium dodecyl sulphate (Liener, 1961) and guanidinium chloride (Englund *et al.*, 1968). The exposed thiol group of ficin is part of its catalytic site (see the introduction) and thus a fully active ficin preparation must possess 1 mol of titratable thiol group per mol of protein.

Most methods of preparation of ficin (e.g. Hammond & Gutfreund, 1959; Englund *et al.*, 1968) fail to separate active ficin from protein which is devoid of thiol and is therefore presumably catalytically inactive. In the few instances in which thiol contents of ficin have been reported (see, e.g., Husain & Lowe, 1968, 1970; Wong & Liener, 1964) the preparations contain 0.6–0.7 mol of thiol/mol of protein and can therefore be only 60–70% active at most. In the present work, ficin prepared by the method of Englund *et al.* (1968) was found to contain 0.65 mol of thiol/mol of protein. Anderson & Hall (1974) have subjected ficin prepared by the method of Englund *et al.* (1968) to chromatography on an agarose-mercurial column, but thiol contents and Michaelis parameters were not reported.

The present paper reports a simple method involving covalent chromatography by thiol-disulphide interchange (Brocklehurst *et al.*, 1973, 1974) for the preparation of ficin containing 1.0 mol of thiol/mol of protein. All of this thiol has much higher reactivity (approx. 50 times) towards 2-Py–S–S–2-Py at pH approx. 4 than at pH approx. 8. This type of reactivity, not found with simple low-molecular-weight thiols, is characteristic of intact catalytic-site geometry in papain (Brocklehurst & Little, 1973).

The amidase activities and thiol contents of the various fractions collected during the preparation of ficin by the present method are presented in Table 1.

In the routine preparation of fully active ficin (see the Materials and Methods section), the *Ficus* latex was dissolved in an aqueous solution of L-cysteine and EDTA. The fraction that precipitated when NaCl (17.7%, w/v) was added to the extract was dissolved

Table 1. *Purification of ficin: thiol contents and amidase activities of the fractions*

Values given are the means of three determinations; standard errors were less than ±5% of the mean for a given preparation. Specific activities were determined by using the continuous assay towards α-*N*-benzoyl-DL-arginine *p*-nitroanilide described in the text. After activation with 20mM-L-cysteine and 5mM-dithiothreitol, no significant further increase in activity was obtained by preincubation with 5mM-L-cysteine for 30min. The activity of each fraction was abolished by addition of 2-Py–S–S–2-Py. Thiol contents were determined by titration with 2-Py–S–S–2-Py at pH4.5. Protein content or apparent protein content in the case of impure fractions was estimated by using $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1}.

Fraction no.	Fraction	$10^5 \times$ Specific activity (E_{410}/s per mol of 'protein' per litre): 'Protein' estimated by using $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1}	'Protein' estimated from thiol content	Thiol content (mol/mol of protein)
1	Solution of dried latex in 1mM-EDTA	36	—	0.09
2	Fraction 1 activated by treatment with L-cysteine and dithiothreitol, and separated from low-molecular-weight material on Sephadex G-25	226	309	0.77
3	The part of Fraction 1 insoluble in 17.7% (w/v) NaCl, after activation and Sephadex G-25 chromatography	217	418	0.52
4	The part of Fraction 1 soluble in 17.7% (w/v) NaCl, after activation and Sephadex G-25 chromatography	191	300	0.67
5	Ficin prepared by covalent chromatography after activation and Sephadex G-25 chromatography	440	440	1.0

in the sodium acetate buffer, pH4.5, and applied to a Sepharose–glutathione–2-pyridyl disulphide gel. Non-covalently bound material was removed by washing. The enzyme can be stored attached to the gel and released when required. The enzyme was released from the gel in which it is bound as a mixed disulphide by treatment with L-cysteine at pH 8.2, and can be precipitated by using $(NH_4)_2SO_4$ to prepare a more concentrated solution. Over a shorter period, the enzyme can be stored as the $(NH_4)_2SO_4$ suspension in dilute L-cysteine solution. Under these conditions, the enzyme becomes reversibly blocked presumably as a ficin–L-cysteine mixed disulphide, analogous to the well-known papain–L-cysteine mixed disulphide prepared by the method of Kimmel & Smith (1954) (see Brocklehurst & Kierstan, 1973). Ficin can be fully activated from this reversibly oxidized state as described in the Materials and Methods section and separated from low-molecular-weight material on Sephadex G-25.

Table 1 provides data also for a step additional to those used as a routine. When the *Ficus* latex is dissolved in EDTA solution in the absence of added low-molecular-weight thiol, the solution contains less than 10% of the thiol content and esterolytic activity (per E_{280} unit) of purified ficin. Treatment of the extract with low-molecular-weight thiol results in large increases in both specific activity and thiol content. No significant increase in specific activity appears to be achieved by the NaCl-precipitation step, but a twofold increase is obtained when the salt precipitate is subjected to covalent chromatography.

Only protein molecules containing a thiol group that is reactive towards the mixed-disulphide groups of the gel at pH4.5 will be collected on the gel and subsequently released from it. The enzyme released from the covalent-chromatography column and subsequently separated from low-molecular-weight material should therefore possess 1.0 mol of thiol/mol of protein. If this thiol content is assumed, the value of ε_{280} for ficin prepared by this method is calculated to be 46000 litre·mol^{-1}·cm^{-1}, which is the value reported by Hollaway (1967) (see Hollaway *et al.*, 1971) for ficin prepared by the method of Sgarbieri *et al.* (1964).

In view of the other values for ε_{280} ranging from 32400 to 54000 litre·mol^{-1}·cm^{-1} reported for ficin (see Table 2), it is useful to assess the possibility that ficin prepared by covalent chromatography does not contain 1 mol of thiol/mol of protein. A value of ε_{280} greater than 46000 litre·mol^{-1}·cm^{-1} would require a thiol content of over 1.0 mol/mol of protein, and vice versa. An apparent thiol content over 1.0 mol/mol of protein could be obtained either by incomplete separation of ficin from low-molecular-weight thiol or by cleavage of a cystine disulphide bond in some of the ficin molecules. Both of these possibilities are unlikely, however, in view of the high reactivity of the ficin thiol group towards 2-Py–S–S–2-Py at pH approx. 4. This type of reactivity is characteristic of the intact ficin active centre (see below). A thiol content less than 1.0 mol/mol of protein would be obtained if some of the ficin thiol had undergone oxidation. The most likely type of oxidation is mixed-disulphide formation with L-cysteine, and this possibility is discounted by the observation that the catalysis of the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester by the present ficin preparation is not enhanced by pre-incubation of the enzyme with 20 mM-L-cysteine at pH 6 for 15 min. This treatment would regenerate any reversibly oxidized ficin. Irreversible oxidation of the ficin thiol to sulphinic acid and sulphonic acid would, by analogy with the papain thiol (Sluyterman, 1967) be expected to be slow, and this should not have occurred to an appreciable extent in samples of ficin freshly prepared by covalent chromatography.

The Michaelis parameters for the hydrolysis of *p*-nitrophenyl hippurate and of α-*N*-benzoyl-L-arginine ethyl ester at pH values around 6–7 catalysed by the various preparations of ficin are collected in Table 2. For the hydrolysis of *p*-nitrophenyl hippurate there is general agreement that the value of $k_{cat.}$ is 6–7 s^{-1}. There is less agreement about the value of $k_{cat.}$ for the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester. Our value of 4 s^{-1} lies between that reported by Hollaway (1968) (2.4 s^{-1}) and that reported by Whitaker (1969) (9.4 s^{-1}, or 8 s^{-1} if a value of 46000 litre·mol^{-1}·cm^{-1} is used instead of 54000 litre·mol^{-1}·cm^{-1} for ε_{280}, see Table 2). Our value of K_m for this catalysis is in good agreement with that reported by Hollaway (1968) but is considerably different from that reported by Whitaker (1969).

Chromatography on CM-cellulose of ficin prepared by covalent chromatography

It is well known that ficin prepared by other methods can be fractionated into a number of components by chromatography on CM-cellulose (see, e.g., Englund *et al.*, 1968). Fig. 1(*a*) is a typical elution pattern on CM-cellulose of partially active ficin prepared by the method of Englund *et al.* (1968), and shows the presence of the components noted by Englund *et al.* (1968) and Sgarbieri *et al.* (1964), i.e. ficins I–IV and ficin G, and an inactive component appearing between ficins II and III. Fig. 1(*b*) is an elution pattern from CM-cellulose for fully active ficin prepared by covalent chromatography. The preparation is shown to contain ficins I–IV and ficin G, but the inactive component between ficins II and III appears to be absent. This absence would be predicted if the inactive component does not possess a thiol group reactive towards the mixed-disulphide gel at pH 4.5. The difference in the relative proportions of the components shown in the elution patterns of Figs. 1(*a*) and 1(*b*) may be due in part to the

Table 2. *Michaelis parameters for the hydrolysis of α-N-benzoyl-L-arginine ethyl ester and p-nitrophenyl hippurate at 25°C by various preparations of ficin from Ficus glabrata*

In the present work, values of $k_{cat.}$ were calculated by using active-site concentrations obtained from titrations with 2-Py–S–S–2-Py at pH4.5 and $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1} (see the text); other values of $k_{cat.}$ were obtained by using: (*a*) $\varepsilon_{280} = 32400$ litre·mol^{-1}·cm^{-1}; (*b*) *N*-ethylmaleimide-titration data and $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1}; (*c*) $\varepsilon_{280} = 54000$ litre·mol^{-1}·cm^{-1}; (*d*) $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1}; (*e*) $\varepsilon_{280} = 53750$ litre·mol^{-1}·cm^{-1} and relative-specific-activity data; (*f*) $\varepsilon_{280} = 60100$ litre·mol^{-1}·cm^{-1} and relative-specific-activity data. Sources of data: (*a*) Kramer & Whitaker (1969); (*b*) Hollaway (1967), quoted in Hollaway (1968); (*c*) Williams (1968), quoted in Whitaker (1969); (*d*) Hollaway (1967), quoted in Hinkle & Kirsch (1971); (*e*) and (*f*), calculated from absorbance and molecular-weight data given in Kortt *et al.* (1974*b*).

Ficin preparation	Assay conditions: pH	*I* (M)	CH_3CN content (%, v/v)	$k_{cat.}$ (s^{-1})	K_m (mM)	Reference
Assays with α-*N*-benzoyl-L-arginine ethyl ester						
Ficin prepared by covalent chromatography	6.6	0.1	—	3.94±0.12	24.3±2.2	Present work
Ficin III prepared by the method of Englund *et al.* (1968)	6.6	0.1	—	4.08±0.42	17.2±4.5	Present work
Ficin D* prepared by the method of Sgarbieri *et al.* (1964)	†	0.3	—	5.20[a]	33.2	Kramer & Whitaker (1969)
Ficin III or G prepared by a modification of the method of Sgarbieri *et al.* (1964)	6.6	0.1	—	2.40±0.32[b]	23.1±3.4	Hollaway (1968)
Ficin G prepared by a modification of the method of Sgarbieri *et al.* (1964)	6.0	0.3	—	9.40±0.3[c] (8.00)‡	41.7±1.6	Whitaker (1969)
	7.0	0.3	—	9.91±0.58[c] (8.44)‡	38.5±2.5	Whitaker (1969)
Assays with *p*-nitrophenylhippurate						
Ficin prepared by covalent chromatography	5.90	0.1	8.2	5.87±0.25	0.0234±0.002	Present work
Ficin III prepared by the method of Englund *et al.* (1968)	5.90	0.1	8.2	5.71±0.15	0.0258±0.0005	Present work
	7.80	0.45	9.75	7.11±0.28[d]	0.068 ±0.0017	Hinkle & Kirsch (1971)
Ficins prepared by covalent chromatography and subsequently fractionated by chromatography on CM-cellulose:	5.90	0.1	8.2			
Ficin I				2.60±0.09	0.0219±0.0007	Present work
Ficin II				5.54±0.13	0.0238±0.001	Present work
Ficin III				5.92±0.02	0.0248±0.0009	Present work
Ficins prepared by NaCl precipitation followed by chromatography on CM-cellulose:	6.0	—	3.17			
Ficin II				5.0 ±0.2[e] (4.28)‡	0.0181	Kortt *et al.* (1974*b*)
Ficin III				7.47±0.1[f] (5.72)‡	0.0197	Kortt *et al.* (1974*b*)
Ficin III or Ficin G prepared by a modification of the method of Sgarbieri *et al.* (1964)	5.90	0.1	3.3	7.1 ±0.30[d]	0.053 ±0.004	Hollaway *et al.* (1969)

* Prepared from *Ficus carica*.

† These parameters are the pH-independent parameters and thus can be compared with values obtained in the pH region around the optimum (pH6–7).

‡ Recalculated by using $\varepsilon_{280} = 46000$ litre·mol^{-1}·cm^{-1}.

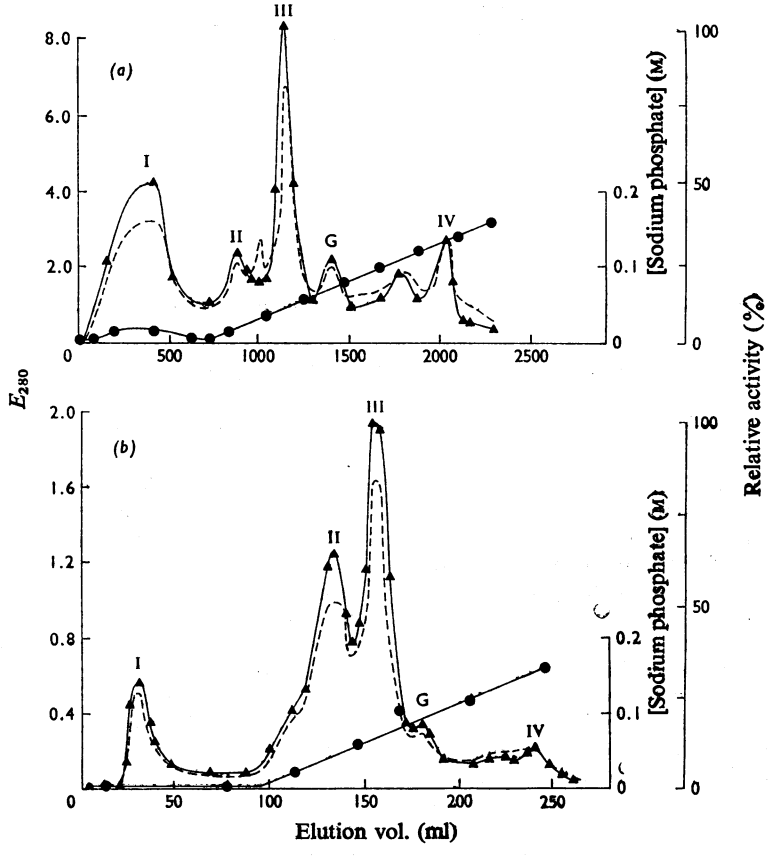


Fig. 1. *Chromatography on CM-cellulose* (1.0 *mequiv./g*) *of* (*a*) *ficin prepared by the method of Englund et al.* (1968) *on a* 2.6 *cm* × 32 *cm column, and* (*b*) *ficin prepared by covalent chromatography on a* 1.5 *cm* × 13 *cm column*

▲, Relative activity towards α-*N*-benzoyl-DL-arginine *p*-nitroanilide: the highest activity in each elution pattern is taken as 100%; ----, E_{280}; ●, concn. of sodium phosphate in the elution buffer. For further details, see the text. Fractions I–IV correspond to those reported by Englund *et al.* (1968) and fraction G to that reported by Sgarbieri *et al.* (1964).

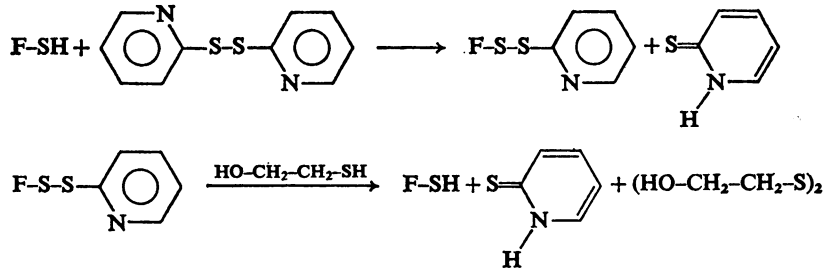
$(NH_4)_2SO_4$ precipitation that is used to concentrate the solutions of ficin prepared by covalent chromatography before application to the CM-cellulose column (see the Materials and Methods section). $(NH_4)_2SO_4$ precipitation has been shown to exhibit some selectivity in favour of ficin II (see Sgarbieri *et al.*, 1964).

The relative values of $k_{cat.}$ for ficins I–III towards *p*-nitrophenyl hippurate (see Table 2), I/II/III = 1:0.94:0.44, correlate reasonably well with the relative specific activities of these enzymes towards α-*N*-benzoyl-L-arginine *p*-nitroanilide found in the present work (1:1.05:0.60), towards *p*-nitrophenyl hippurate as reported by Kortt *et al.* (1974*a*) (1:0.95:0.69) and towards casein (1:0.90:0.59; Sgarbieri *et al.*, 1964).

Reaction of ficin with 2-Py–S–S–2-Py

(*i*) *At pH4 and above.* The reaction of 1 mol of 2-Py–S–S–2-Py per mol of ficin (see Fig. 2) abolishes the hydrolytic activity of the latter towards α-*N*-benzoyl-L-arginine ethyl ester. Treatment of the inactive ficin derivative with 2-mercaptoethanol results in complete regain of hydrolytic activity concomitant with release of 1 mol of Py-2-SH/mol of protein. The reactions therefore may be represented by Scheme 1.

(*ii*) *At pH values below* 4. Scheme 1 fairly describes the reaction of ficin with 2-Py–S–S–2-Py in the pH range 4–10.5. At pH values below 4, however, the rapid reaction of the essential thiol group with 2-Py–S–S–2-Py is followed by a much slower reaction



Scheme 1. *Reaction of ficin with 2-Py–S–S–2-Py and subsequent reaction of the mixed-disulphide product with 2-mercaptoethanol*

F–SH = active ficin; the thiol group depicted is the essential thiol group.

that also releases Py-2–SH (2-thiopyridone). When the reaction is studied by the stopped-flow technique, the stoicheiometry of the fast phase of the reaction is found to be 1.0mol of Py-2–SH released/mol of protein at pH values ≥2.5. Below this pH value the stoicheiometry decreases, presumably owing to conformational changes that destroy the interaction in the thiol–imidazole system of the active centre (see below). When the reaction is followed at pH2.06 by using conventional spectral analysis (see Fig. 2), the very fast reaction that releases 0.5mol of Py-2–SH/mol of protein is seen to be followed by a much slower reaction that releases a further 1.5mol of Py-2–SH/mol of protein. This slower reaction presumably results from reaction of the thiol group in those molecules that now possess distorted active centres (0.5mol/mol), together with reaction of the buried thiol group of ficin (1.0mol/mol). This thiol group has been detected previously by unfolding the protein in sodium dodecyl sulphate (Liener, 1961) or in guanidinium chloride (Englund *et al.*, 1968), and Husain & Lowe (1970) have suggested that it may be in the side chain of the residue analogous to serine-206 in papain.

The apparent rate of the reaction of the inessential thiol group of ficin is always at least 2500 times less than that of the reaction of the essential thiol group, and thus its reaction is not a complicating factor in the kinetic analysis of the reactions of the latter. The rate of production of Py-2–SH could of course be determined at least in part by the rate of exposure of the inessential thiol group. The conformational stability of ficin as indicated by its activity towards α-*N*-benzoyl-L-arginine ethyl ester at pH6.6 may be assessed from the data in Fig. 3. These data suggest that over the time-course of a stopped-flow kinetic experiment on the reaction of 2-Py–S–S–2-Py with the essential thiol group of ficin (≤0.5s in the pH range 2–4), this type of 'irreversible' conformational instability is unlikely to affect seriously the kinetics at pH ≥3, and there is a good chance that the kinetics are unaffected also even in the pH region around 2.

Thus although at pH values <2.5 a proportion of the enzyme molecules lose their original active-centre geometry, as evidenced by the decrease in stoicheiometry of the fast phase of the reaction, the rate constant of the residual fast phase probably characterizes intact active centres. The possibility of freely reversible pH-dependent conformational changes in ficin in acidic media is considered below.

The high electrophilicity of the 2-Py–S–S–2-PyH$^+$ monocation makes this reagent particularly valuable for direct monitoring of the exposure of the buried thiol group of ficin in acidic media. At pH values around 2, most thiol groups would be expected to exist to only a very small extent in their nucleophilic (thiolate ion) state, and reaction with many commonly used thiol reagents would occur too slowly to be of use. The high pK_a value of Py-2–SH (9.8; see Brocklehurst & Little, 1973) makes the reactions of 2-Py–S–S–2-Py with thiols in acidic media essentially stoicheiometric. This contrasts with the analogous reactions of 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2), which could not easily be used for the direct detection of the acid-promoted exposure of the buried thiol group of ficin. This is because the low pK_a value of 2-nitro-5-mercaptobenzoic acid (pK_{aII} = 4.41; see Brocklehurst & Little, 1973) leads to an unfavourable equilibrium position for reactions of thiols with Nbs_2 in acidic media.

Characterization of the ficin active centre by using 2-Py–S–S–2-Py as a reactivity probe

Byers & Koshland (1975) have emphasized the usefulness of reactivity probes compared with spectroscopic probes for the study of conformational states of proteins, and we have pointed out the particular value of disulphide reagents containing the 2-pyridyl moiety in the delineation of molecular environments

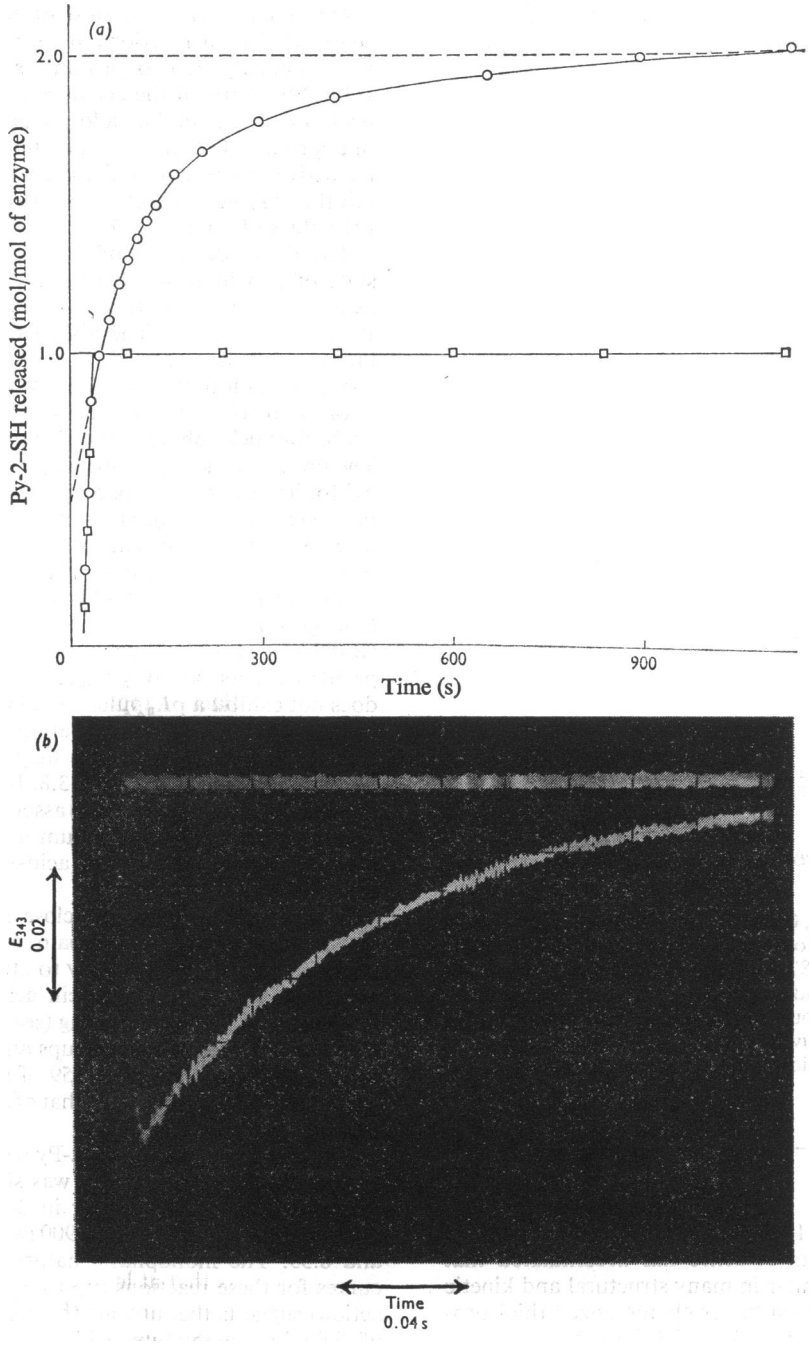


Fig. 2. *Progress curves for the reaction of ficin with 2-Py–S–S–2-Py at pH4.00 and pH2.96 at 25.0°C and I = 0.1mol/litre*

(*a*) Progress curves recorded by using conventional spectrophotometry: ficin (7.5 μM) was allowed to react with 2-Py–S–S–2-Py (248 μM), □, at pH4.00 and ○, at pH2.06. The points are taken from the continuous trace provided by a Cary 16K spectrophotometer. The recorder was started and the reactants were mixed as the pen crossed a mark on the chart (zero time). The reaction cell was placed in the cell compartment approx. 15s later. The reaction at pH4 and the fast phase of the reaction at pH2.06 were complete within the response time of the pen. (*b*) Progress curve for the reaction at pH4.00 recorded by using a Durrum stopped-flow spectrophotometer and a Tektronix oscilloscope.

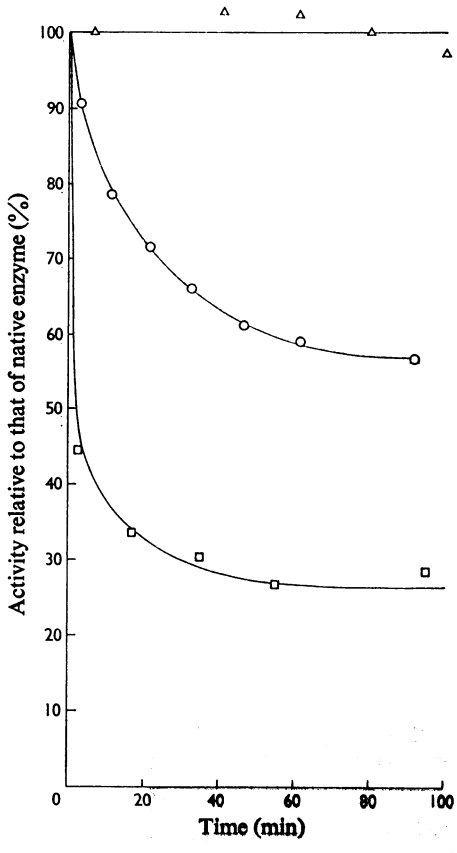


Fig. 3. *Progress curves for the denaturation of ficin in acidic media*

Ficin (45 μM) in: △, 0.02M-sodium formate buffer, pH3.9; ○, 0.02M-sodium formate buffer, pH2.75; and □, 0.1M-HCl/KCl buffer (87mM-KCl+13mM-HCl), pH2.0, was kept at 25.0°C, and samples (0.25ml or 0.5ml) were removed after various time-intervals and assayed titrimetrically for activity towards α-*N*-benzoyl-L-arginine ethyl ester by addition to 4.5–4.75ml of substrate solution (80mM) at pH6.6.

of thiol groups (see, e.g., Brocklehurst, 1974; Stuchbury *et al.*, 1975).

For many years evidence has accumulated that ficin is closely similar in many structural and kinetic respects to the much better characterized thiol proteinase, papain (see the introduction).

One of the pK_a values that characterizes the pH-dependence of the kinetic parameters of reactions catalysed by both enzymes is near to 4, and for papain evidence has been presented that this pK_a value probably characterizes proton loss from the thiol–imidazolium ion system of the active centre (see Brocklehurst, 1974; Polgar, 1974; Shipton *et al.*, 1975).

One characteristic feature of the papain active centre is that in reactions at its S atom it exhibits nucleophilicity in two protonic states, one at pH values$\gg$9 where all the active-centre side chains are proton-deficient, and an additional one at pH values of approx. 6. The existence of a nucleophilic state of the active centre that predominates at pH6 may permit the enzyme to function as an efficient catalyst at pH values of approx. 5–7.

The direct detection of this second nucleophilic state of papain is possible by using 2-Py–S–S–2-Py as a reactivity probe (Brocklehurst & Little, 1970; Brocklehurst, 1974). The ability of this reagent to increase its electrophilicity by approx. 1000 times consequent on protonation or on hydrogen-bonding to one of its N atoms makes it a particularly sensitive probe for nucleophilic states of thiols in acidic media. The profile of the second-order rate constant against pH for its reaction with papain exhibits three reactive protonic states, including a striking rate maximum at pH3.8 (see Fig. 4). The existence of three reactive protonic states in this type of reaction is characteristic of interaction of a thiol group with another acid/base group leading to a second nucleophilic state (Brocklehurst, 1974; Stuchbury *et al.*, 1975). That the profile exhibits two pK_a values of approx. 3.8 and does not exhibit a pK_a value of 2.45 characteristic of the 2-Py–S–S–2-PyH$^+$ monocation led us to suggest that the papain active centre may be characterized by two pK_a values of approx. 3.8. It is proposed that these molecular pK_a values are associated with proton loss from the thiol–imidazolium ion system and the carboxyl group of aspartic acid-158 (see Shipton *et al.*, 1975).

In view of the supposed close similarity of the active-centre regions of papain and ficin, it was decided to use 2-Py–S–S–2-Py to attempt to demonstrate the presence in the ficin active centre of (i) interaction of the neighbouring (see Husain & Lowe, 1968) thiol and imidazole groups analogous to those of cysteine-25 and histidine-159 of papain and (ii) a carboxyl group analogous to that of aspartic acid-158 of papain.

The reaction of 2-Py–S–S–2-Py with ficin prepared by covalent chromatography was shown to be first-order in ficin and first-order in 2-Py–S–S–2-Py by using [2-Py–S–S–2-Py] up to 900 μM at pH3.56, 6.56 and 8.35. The monophasic nature of the progress curves for these reactions (up to at least 80% of reaction) suggests that at least the predominant forms of ficin in the mixture exhibit similar reactivities towards 2-Py–S–S–2-Py. This view is supported by the additional kinetic data obtained using two of the individual forms of ficin (see Fig. 4). The pH-dependence of the second-order rate constant (k) for this reaction is compared with the analogous profile for the reaction of papain with 2-Py–S–S–2-Py in Fig. 4. The profiles are similar in the plateau region at pH

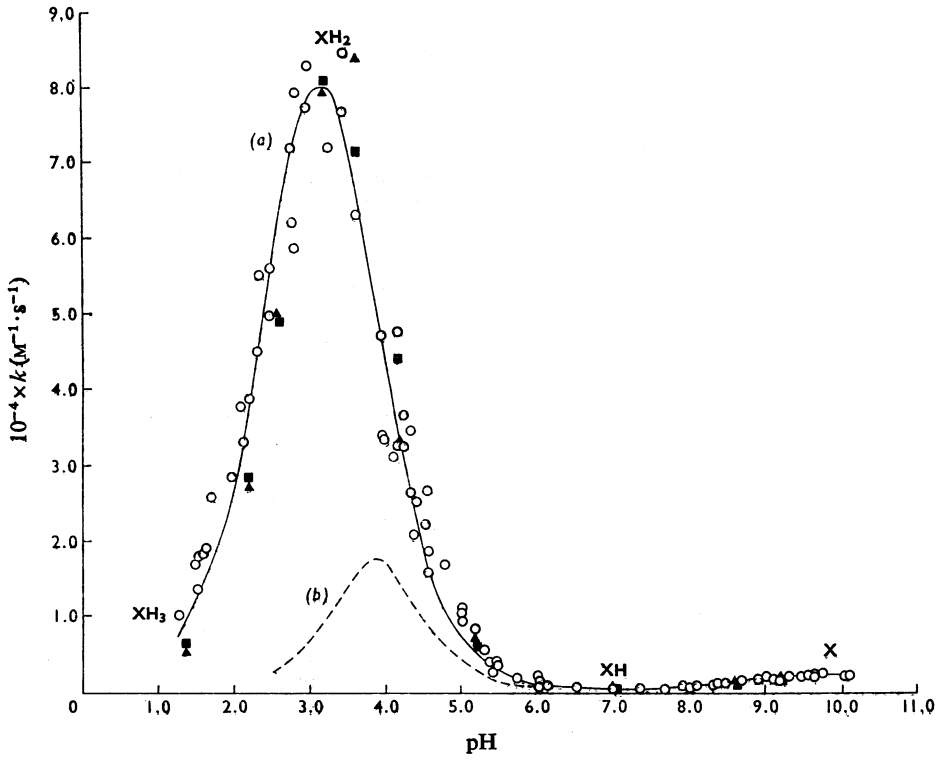


Fig. 4. *Dependence on pH of the apparent second-order rate constant, k, for the reaction of 2-Py–S–S–2-Py with (a) ficin and (b) papain at 25.0°C and I = 0.1 M*

(*a*) Ficin reaction; the points are experimental: ○, ficin prepared by covalent chromatography; ▲, ficin II, ■, ficin III; the solid line is theoretical for

$$k = \frac{\bar{k}_1}{\left(1+\frac{[H^+]}{K_I}+\frac{K_{II}}{[H^+]}\right)} + \frac{\bar{k}_2}{\left(1+\frac{[H^+]}{K_{II}}+\frac{K_{III}}{[H^+]}\right)} + \frac{\bar{k}_3}{\left(1+\frac{[H^+]}{K_{III}}\right)}$$

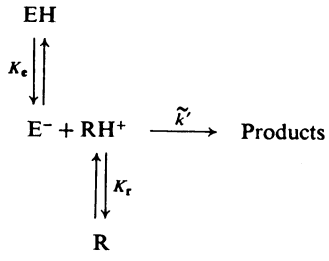
(see Brocklehurst, 1974), in which $\bar{k}_1 = 1.11 \times 10^5 M^{-1} \cdot s^{-1}$, $\bar{k}_2 = 400 M^{-1} \cdot s^{-1}$, $\bar{k}_3 = 2.23 \times 10^3 M^{-1} \cdot s^{-1}$, $pK_I = 2.42$, $pK_{II} = 3.82$ and $pK_{III} = 8.61$. (*b*) Papain reaction; the broken line is theoretical for the equation given in (*a*) in which $\bar{k}_1 = 5 \times 10^4 M^{-1} \cdot s^{-1}$, $\bar{k}_2 = 700 M^{-1} \cdot s^{-1}$, $\bar{k}_3 = 1.7 \times 10^3 M^{-1} \cdot s^{-1}$, $pK_I = pK_{II} = 3.8$ and $pK_{III} = 8.8$ (Brocklehurst & Little, 1972). Only the portion of this profile in the pH range 2.5–6 is shown.

values ≫9 and at approx. 6. Unexpectedly, however, the profile for the ficin reaction is strikingly different in the region of the rate optimum in acidic media.

Although most data were collected by using ficin prepared by covalent chromatography, Fig. 4 shows that similar rate constants are obtained also, in this pH region, by using ficin II and ficin III separately.

The three reactive protonic states in each of the profiles of Fig. 4 are designated X–XH_2 to indicate their relative stoicheiometries in protons, and XH_3 is the unreactive protonic state at low pH.

The general shape of the pH–*k* profile for the ficin reaction is like that of the papain reaction, and the existence of three reactive protonic states suggests that as in the papain active centre, the essential thiol and imidazole groups of ficin are interactive (see Brocklehurst, 1974). The sets of parameters that characterize the bell-shaped components of the two profiles at low pH, however, are considerably different. The bell-shaped components are each characterized by two molecular pK_a values (for papain $pK_{aI} = pK_{aII} = 3.8$, and for ficin $pK_{aI} = 2.42 \pm 0.07$ and $pK_{aII} = 3.82 \pm 0.06$) and an apparent [see below and eqn. (2)] pH-independent rate constant, $\bar{k}'$ (for papain $\bar{k}' = 5 \times 10^4 M^{-1} \cdot s^{-1}$ and for ficin $\bar{k}' = 1.11 \times 10^5 \pm 0.01 \times 10^5 M^{-1} \cdot s^{-1}$). The experimentally ob-



Scheme 2. *One-step kinetic model for the XH_2 state of the reaction of ficin with 2-Py–S–S–2-Py*

Free protons are omitted; the parameters are defined in the text.

served rate constants at the respective pH optima are for papain approx. $1.75\times10^4 M^{-1}\cdot s^{-1}$ and for ficin approx. $8\times10^4 M^{-1}\cdot s^{-1}$.

The use of 2-Py–S–S–2-Py as a reactivity probe therefore reveals a structural difference between ficin and papain that is expressed as (i) a much greater rate of reaction of 2-Py–S–S–2-Py with ficin than with papain in the XH_2 state and (ii) a broader bell-shaped component of the pH–k profile for the XH_3–XH-state regions in the ficin reaction.

In contrast with the papain profile, pK_{aI} for the ficin profile corresponds closely to the molecular pK_a value associated with proton loss from the 2-Py–S–S–2-PyH$^+$ monocation ($pK_{aII} = 2.45$; Brocklehurst & Little, 1973).

The simplest interpretation of the XH_2 state of the ficin–2-Py–S–S–2-Py reaction is therefore that the nucleophilic thiol–imidazole interactive system of ficin (see below) reacts with the 2-Py–S–S–2-PyH$^+$ monocation. A low-molecular-weight model for this type of reaction which provides a profile like that shown in Fig. 4 has been described (Stuchbury *et al.*, 1975). This suggests that ficin may not contain a carboxyl group functionally equivalent to that of aspartic acid-158 of papain, which we suggested might be associated with one of the pK_a values of 3.8 in the papain profile (Shipton *et al.*, 1975). If the limb characterized by pK_a of 2.5 were complicated by rapidly reversible conformational changes in ficin, an unsymmetrical bell with a steeper acid limb might be predicted. In the accompanying paper (Shipton *et al.*, 1976) the lack of acid catalysis in the reaction of ficin with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (Nbd chloride) is demonstrated. This is in marked contrast with the acid catalysis observed in the analogous papain reaction, which is dependent on a group associated with pK_a approx. 3.5 and strongly supports the postulate that ficin lacks a residue conformationally equivalent to aspartic acid-158 in papain. The lack of this residue in ficin would be expected to lead to the appearance of the pK_a of 2-Py–S–S–2-PyH$^+$ in the profile given in Fig. 4 for the ficin reaction.

XH_2 state of the reaction of ficin with 2-Py–S–S–2-Py

In terms of the interpretation suggested above, the bell-shaped component of the profile shown in Fig. 4 for the ficin reaction can be described using the kinetic model of Scheme 2. Since the reaction is first-order in 2-Py–S–S–2-Py up to a concentration of 900 μM, a simple one-step model was assumed, although this does not exclude the possibility of a two-step (intermediate-complex) mechanism with a pre-equilibrium or steady-state constant >900 μM. Some of the considerable difficulties that can be encountered in attempting to interpret pH-dependent rate data in terms of a non-equilibrium intermediate-complex model when non-saturating reagent concentrations have to be used have been discussed by Brocklehurst & Dixon (1976).

Conventional analysis of the model of Scheme 2 provides the rate equation, eqn. (1), in which k is the second-order rate constant determined at a particular pH value by measurement either of initial rates or of pseudo-first-order rate constants and the appropriate total concentrations, K_e is the acid dissociation constant associated with provision of the nucleophilic form of the enzyme, E^-, from a protonated, non-nucleophilic form, EH, K_r is the acid dissociation constant associated with the loss of the highly electrophilic form of the reagent, RH^+, by deprotonation, and $\tilde{k}$ is the 'true' rate constant for the reaction of the specific ionic forms E^- and RH^+.

$$k = \frac{\tilde{k}\cdot\dfrac{K_e}{K_e+K_r}}{1+\dfrac{[H^+]}{(K_e+K_r)}+\dfrac{K_e K_r/(K_e+K_r)}{[H^+]}} \qquad (1)$$

Eqn. (1) is the well-known form that describes a bell-shaped curve. Even when the ionizations are in different molecules, the apparent pK_a values are mixed constants and the pH-independent rate constant obtained by conventional analysis ($\tilde{k}'$) is the 'true' rate constant multiplied by an assembly of the two acid dissociation constants (eqn. 2).

$$\tilde{k}' = \tilde{k}\cdot\frac{K_e}{K_e+K_r} \qquad (2)$$

Eqn. (2) provides for the possibility, in the general case, that intrinsic ionizations may be 'crossed over' (see Dixon & Webb, 1964).

If K_e and K_r differ by more than 1 pH unit the two apparent pK_a values will provide good approximations of K_e and K_r separately. If $K_e \gg K_r$, $\tilde{k}' \simeq \tilde{k}$ (see eqn. 2). If, however, as in the reaction of ficin and 2-Py–S–S–2-Py, $K_e \ll K_r$, $\tilde{k}'$ does not provide directly a good estimate of $\tilde{k}$. Rather, it provides a low estimate

and has to be multiplied by the ratio K_r/K_e [or strictly $(K_e+K_r)/K_e$] to provide $\tilde{k}$. For the ficin–2-Py–S–S–2-PyH$^+$ reaction, $\tilde{k}$ is calculated to be $1.11\times10^5\times(3.8\times10^{-3}+1.51\times10^{-4}/1.51\times10^{-4})=2.90\times10^6\,M^{-1}\cdot s^{-1}$. This value of $\tilde{k}$ is comparable with values for the analogous rate constants that characterize reactions of 2-Py–S–S–2-PyH$^+$ with low-molecular-weight thiolate ions. After correction for differences in pK_a by using 0.25 as the value of the Brönsted coefficient, such rate constants have values approx. $5\times10^6\,M^{-1}\cdot s^{-1}$ (T. Stuchbury & K. Brocklehurst, unpublished work).

The value of $pK_e = 3.8$

In the above interpretation of the bell-shaped component at low pH of the ficin–2-Py–S–S–2-Py profile of Fig. 4, the ionizations are 'crossed-over'. Thus chemical experience suggests that protonation of R (i.e. 2-Py–S–S–2-Py) (pK_{aII} = 2.45; see Scheme 2) would increase its electrophilicity and thus lead to a rate enhancement. The pK_a value of 3.8, therefore, which must be associated with the enzyme, relates to loss of activity consequent on protonation.

By analogy with papain (see Shipton *et al.*, 1975), the simplest interpretation of this pK_a value is that it characterizes proton loss from the thiol–imidazolium ion system of the ficin active centre, which leads to the production of the nucleophilic interactive thiol–imidazole and/or thiolate–imidazolium pair. The problem involved in making decisions about the structure of this interactive pair from spectroscopic data such as that of Polgar (1974) was pointed out by Shipton *et al.* (1975) and Lowe (1976) and discussed more generally by Dixon (1976). In an attempt to assess the effect of ion-pair formation on the u.v. spectrum of a thiolate ion, T. Stuchbury & K. Brocklehurst (unpublished work) examined the spectra of some low-molecular-weight thiolate ions in aqueous NaOH and in saturated solutions of tetramethylammonium chloride. The two sets of spectra were not significantly different, but the result is not conclusive because (*a*) ion-pair formation could not necessarily be assumed and (*b*) this system does not provide 'hydrogen-bonding' within the ion pair. Polgar's (1974) data might mean that the interactive system of papain consists of at least 50% ion pair, but the possibility remains that the system might contain the proton in a single potential well (see Jencks, 1969) and be characterized by an extinction coefficient that is 50% of that of the uncomplicated thiolate ion.

If the pK_a value of 3.8 relates not to this active-centre assembly of ficin but to some other group which, when protonated, permits a conformational change leading to loss of reactivity, then the first molecular pK_a value of the ficin active-centre system must be less than 3.8.

pK_a value of 8.6 *in the ficin–2-Py–S–S–2-Py profile of Fig.* 4

In terms of a single-step model for the reaction in this pH region analogous to that of Scheme 2, the pK_a value of 8.6 in the ficin–2-Py–S–S–2-Py profile of Fig. 4 is the second molecular pK_a value of the thiol–imidazolium system, which characterizes the formation of the uncomplicated thiolate ion of ficin. This value is closely similar to that which characterizes the pH-dependence of the reaction of ficin with chloroacetamide and of $k_{cat.}/K_m$ for ficin-catalysed hydrolyses (see Hollaway *et al.*, 1964, 1971).

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