Covalent Chromatography

PREPARATION OF FULLY ACTIVE PAPAIN FROM DRIED PAPAYA LATEX

By KEITH BROCKLEHURST, JAN CARLSSON,* MAREK P. J. KIERSTAN
and ERIC M. CROOK

*Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College,
University of London, Charterhouse Square, London EC1M 6BQ, U.K.*

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1. A Sepharose-(glutathione-2-pyridyl disulphide) conjugate has been prepared. 2. Its use in a new type of chromatography, covalent chromatography by thiol-disulphide interchange, is described. 3. With this technique, papain containing 1 intact catalytic site [thiol with high reactivity towards 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) at pH4] per mol of protein is readily prepared both from dried papaya latex and from commercial 2 $\times$ crystallized partially active papain. 4. The catalysis of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester at pH6.0, 25.0°C, $I = 0.3$ by fully active papain thus prepared is characterized by $K_m = 18.2 \pm 0.1$ mM and $k_{cat.} = 16.4 \pm 0.5$ s⁻¹.

Recent work in this laboratory (Brocklehurst & Little, 1970, 1973) has shown that 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) may be used to titrate active sites in papain even in the presence of low-molecular-weight thiols or denatured enzyme that still retains its thiol group.

Kinetic analysis of the reaction of papain with 2-Py-S-S-2-Py (Brocklehurst & Little, 1972) and of comparable reactions (Little & Brocklehurst, 1972; Brocklehurst *et al.*, 1972a) strongly suggests that the success of this titration depends on (i) the possession by the uncharged cysteine-25-histidine-159-asparagine-175 hydrogen-bonded system of papain of significant nucleophilic reactivity, (ii) the similarities of two of the apparent pK_a values of the 2-Py-S-S-2-Py-enzyme complex and (iii) the ability of 2-Py-S-S-2-Py to increase its electrophilicity by protonation on nitrogen. These factors result in a rate of reaction of papain with 2-Py-S-S-2-Py at pH values 3.5–4.0 that is at least 10² times greater than that of the reaction of 2-Py-S-S-2-Py with simple thiol groups at comparable concentrations. By contrast, at pH values greater than or equal to 8 where the reactions proceed mainly by the attack of thiolate ion on unprotonated disulphide, 2-Py-S-S-2-Py reacts with L-cysteine about 10–20 times faster than with papain.

The ability of monoprotonated 2-Py-S-S-2-Py to react specifically with the thiol group of an intact papain catalytic site suggested that suitable derivatives of this reagent might be of use as chromatography materials for the separation of papain containing intact catalytic sites from other forms of papain. Crystalline papain prepared by the method of Kimmel & Smith (1954) contains three types of protein: (i)

active papain in which the side chain of cysteine-25 contains a free thiol group, (ii) an inactive papain-L-cysteine mixed disulphide (see Sluyterman, 1967; Klein & Kirsch, 1969) and (iii) inactive protein which is not activated by treatment with thiol or KCN and which may be a sulphonic acid of papain (Glazer & Smith, 1965). The papain-L-cysteine mixed disulphide may be converted into active papain by treatment with a reducing agent such as a thiol or KCN. Separation from low-molecular-weight material, e.g. by chromatography on Sephadex G-25, yields a protein preparation composed usually of approx. 50% active papain and approx. 50% unactivatable protein. Separation of these components has been approached previously in two ways. Sluyterman & Wijdenes (1970) separated protein containing a thiol group from protein lacking a thiol group by means of an agarose mercurial column. Blumberg *et al.* (1970) also prepared active papain containing 1 mol of papain thiol/mol of protein by affinity chromatography by using a water-insoluble derivative of the papain inhibitor Gly-Gly-Tyr(Bzl)-Arg.

In the present work we report a facile synthesis of a Sepharose-(glutathione-2-pyridyl disulphide) conjugate (I).

In the present paper compound (I) and related compounds are represented as *N*-substituted imido-carbonates although they may exist in part in other forms, e.g. isourea derivatives and *N*-substituted carbamates (see Axen & Ernback, 1971).

Use of compound (I) as a chromatographic material is shown below to permit preparation of papain containing 1 intact catalytic site/mol of protein from dried papaya latex and from commercial 2 $\times$ crystallized papain. This is achieved both by reaction at pH4 involving attack by only the thiol groups of intact catalytic sites and also by reaction at pH8

*Present address: Institute of Biochemistry, University of Uppsala, Box 531, S-751 21, Uppsala 1, Sweden.

(I)

involving attack by protein thiolate ions. The Sepharose-glutathione conjugate prepared as an intermediate in the synthesis of compound (I) may be used as a thiol-containing chromatographic material. Some of the catalytic characteristics of papain prepared by the method described (covalent chromatography) are reported and compared with those reported previously for other papain preparations.

Materials

Papain

Three papain preparations were used for kinetic studies: (a) the partially active 2 × crystallized product of BDH Chemicals Ltd., Poole, Dorset, U.K., (b) fully active papain prepared from this product by covalent chromatography and (c) fully active papain prepared from dried papaya latex (BDH Chemicals Ltd.) by $(\text{NH}_4)_2\text{SO}_4$ fractionation and covalent chromatography (see below).

Other materials

Sepharose 2B, Sephadex G-25 and Sephadex G-100 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Cyanogen bromide and dithiothreitol were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Glutathione (reduced), Coomassie Brilliant Blue R250 and L-cysteine were the products of Sigma Chemical Co., St. Louis, Mo., U.S.A. 2-Py-S-S-2-Py and 4,4'-dipyridyl disulphide (4-Py-S-S-4-Py) were obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. Carrier ampholyte (pH7-10) was obtained from LKB-Produkter A.B., Bromma 1, Sweden. Celite, acid-washed sand, 5,5'-dithiobis-(2-nitrobenzoic acid) and α -N-benzoyl-L-arginine ethyl ester hydrochloride were obtained from BDH Chemicals Ltd. Other chemicals were of the highest commercial grade. Solutions were prepared in deionized water containing 1 mM-EDTA.

Methods

Preparation of the Sepharose-glutathione conjugate

(a) *Chemical activation of Sepharose.* This was carried out essentially as described by Axen &

Ernback (1971). Before use the Sepharose was washed with 1 mM-EDTA on a sintered-glass funnel. Freshly prepared CNBr solution (50 ml, 20 mg/ml) was added to 50 g of Sepharose 2B (50 g of swollen and vacuum-filtered Sepharose equivalent to 1 g dry weight equivalent to 60–70 ml bed volume). The pH of the stirred suspension was adjusted to 11.0 by addition of 4 M-NaOH and maintained at this value in a pH-stat at 25°C for 6 min. The activated gel was then washed rapidly (<15 min) with 0.1 M-NaHCO₃ (500 ml, 4°C) on a sintered-glass filter under suction. The activated gel was allowed to react with glutathione immediately after its preparation.

(b) *Reaction with glutathione.* After filtration, the activated gel was transferred to a 200 ml stoppered flask containing glutathione (1 g) dissolved in 20 ml of 0.1 M-NaHCO₃ adjusted to pH 8.5. To ensure good mixing 0.1 M-NaHCO₃ was added to give a total volume of 80–90 ml. The suspension was then mixed by shaking for 20 h at 22°C. The gel was washed with 0.1 M-NaHCO₃ (500 ml) on a sintered funnel and then packed in a column (1.8 cm × 30 cm) and washed by using the following solutions at a flow rate of 10 ml/h: (i) 0.1 M-NaHCO₃ buffer, pH 9.5, containing 1 M-NaCl and 1 mM-EDTA (24 h); (ii) 0.1 M-sodium acetate buffer, pH 4.3, containing 1 M-NaCl and 1 mM-EDTA (24 h); (iii) 1 mM-EDTA (24 h).

Determination of the thiol contents of the Sepharose-glutathione gels before and after reduction with dithiothreitol

(a) *Dry-weight determinations.* Samples of the unreduced and reduced gel (see below) (2 g wet weight) were suspended in deionized water (50 ml) to give suspensions of approx. 1 mg of gel/ml. Portions (10 ml) of the stirred stock suspensions were taken and the gel was isolated by filtration on Millipore filters of known weight. The filters were then dried *in vacuo* over P₂O₅ to constant weight. This usually required drying for 48 h.

(b) *Thiol determinations.* These were made by titration of the thiol-containing gel with 2-Py-S-S-2-Py. Samples of suspension containing 5–10 mg dry weight of gel were subjected to vacuum filtration in a column (1 cm × 12 cm) fitted with a fine glass sinter,

tap and stopper. To the gel was added 3 ml of a mixture containing 0.1 M-Tris-HCl buffer, pH 8.0, 1 mM-EDTA and 0.3 M-NaCl; 1 ml of 1.5 mM-2-Py-S-S-2-Py was added and the suspension was mixed by repeated inversion for 10 min. The mixture was subjected to vacuum filtration in the column and the concentration of 2-thiopyridone in the filtrate determined spectrophotometrically at 343 nm against an appropriate blank (Grassetti & Murray, 1967).

Preparation of the Sepharose-(glutathione-2-pyridyl disulphide) conjugate

The Sepharose-glutathione gel (approx. 50 g wet weight) was washed by vacuum filtration with 450 ml of 0.1 M-Tris-HCl buffer, pH 8.0, containing 0.3 M-NaCl and 1 mM-EDTA. The gel was then suspended in 60 ml of this buffer containing 20 mM-dithiothreitol and the mixture was stirred gently for 10 min. The gel was separated by vacuum filtration. The treatment with dithiothreitol and subsequent vacuum filtration was repeated twice more. The fully reduced gel was washed with each of the following solutions in turn: 1 M-NaCl (150 ml), 0.5 M-NaHCO₃ (150 ml) and 0.1 M-Tris-HCl buffer, pH 8.0 (150 ml), each of which contained 0.3 M-NaCl and 1 mM-EDTA. The final wash solution was shown to be free of dithiothreitol by its lack of reaction with 2-Py-S-S-2-Py. The thiol content of a sample of gel was determined as described above. After the washing procedure, the fully reduced gel was allowed to react with 1.5 mM-2-Py-S-S-2-Py in 0.1 M-Tris-HCl buffer, pH 8.0 (50 ml), containing 0.3 M-NaCl and 1 mM-EDTA with gentle stirring for 30 min. The gel was then washed as described above until no 2-Py-S-S-2-Py could be detected by measuring extinction at 281 nm (Grassetti & Murray, 1967).

Chromatography with the Sepharose-(glutathione-2-pyridyl disulphide) conjugate

The gel was equilibrated as required either in 0.1 M-Tris-HCl buffer, pH 8, or in 0.1 M-sodium acetate buffer, pH 4, both containing 0.3 M-NaCl and 1 mM-EDTA.

A batch of gel prepared as described above corresponds to a bed volume of 60–70 ml and is sufficient to prepare approx. 100 mg of papain. This amount of gel was packed into a column (1.8 cm × 30 cm) and flow rates of 30–60 ml/h were used. To prepare 1 g of papain, it is convenient to use a bed volume of 500 ml.

Gel regeneration

After each run, the gel was reconverted into the Sepharose-(glutathione-2-pyridyl disulphide) form

by reduction with dithiothreitol and reaction with 2-Py-S-S-2-Py as described above. Only one treatment with dithiothreitol was necessary to produce the fully reduced gel.

Preparation of fully active papain from dried papaya latex

Dried papaya latex (100 g) was ground at 22°C, with Celite (30 g) and sand (50 g) suspended in 20 mM-L-cysteine, pH 5.7 (250 ml).

Insoluble material was removed by vacuum filtration and the pH of the filtrate (approx. 250 ml) adjusted to pH 9.0 by addition of 1 M-NaOH. The precipitate that formed was removed by centrifugation at 14000 g for 30 min at 4°C. The pH of the clear supernatant was adjusted to pH 6.0 by addition of 1 M-HCl and, if the supernatant was still not clear, it was filtered as before. The clear solution was brought to 40% saturation with (NH₄)₂SO₄ and left at 4°C for 15 min.

The precipitate was removed by centrifugation as described above and then dissolved in 1 mM-EDTA (200 ml) and the solution brought to 35% saturation with respect to (NH₄)₂SO₄. The mixture was left at 4°C for 15 min, then the precipitate was removed by centrifugation as before. It was then dissolved in the minimum volume of either 0.1 M-Tris-HCl buffer, pH 8.0, or 0.1 M-sodium acetate buffer, pH 4.0, both containing 0.3 M-NaCl and 1 mM-EDTA, to give a clear solution. Usually approx. 200 ml was required. The solution containing 0.1–0.2 mol of thiol/mol of protein was applied to a chromatography column (1.8 cm × 30 cm) packed with Sepharose-(glutathione-2-pyridyl disulphide) gel at room temperature (approx. 22°C). The column was eluted with the buffer used to apply the protein to the column. Elution was continued until values of extinction at both 280 nm and 343 nm fell to less than 0.03 E unit. Then the gel was equilibrated with 0.1 M-Tris-HCl buffer, pH 8.0, containing 0.3 M-NaCl and 1 mM-EDTA. Covalently bonded material was removed from the Sepharose-(glutathione-papain disulphide) column by elution with 50 mM-L-cysteine in 0.1 M-Tris-HCl buffer, pH 8.0, containing 0.3 M-NaCl and 1 mM-EDTA. Fractions were monitored for extinction at 280 nm and 343 nm and for esterolytic activity towards α-N-benzoyl-L-arginine ethyl ester. The peak-activity fractions were bulked. At this stage the solution can be concentrated by precipitating the protein by 40% saturation with (NH₄)₂SO₄ and redissolving it in a minimum quantity of pH 8 buffer. The protein was then separated from low-molecular-weight materials by chromatography on Sephadex G-25 in 0.1 M-KCl containing 1 mM-EDTA as the eluent. To prevent the formation of a papain-L-cysteine mixed disulphide during gel filtra-

tion, dithiothreitol was added immediately before application to the Sephadex G-25 column to give a final concentration of 5 mM.

Preparation of fully active papain from commercial 2×crystallized partially activatable papain

Commercial 2×crystallized papain (200 mg) was treated with 5 mM-dithiothreitol in 0.1 M-Tris-HCl buffer, pH 8.0, and separated from low-molecular-weight material on Sephadex G-25. The protein (generally containing 0.4–0.6 mol of thiol/mol of protein) in 100 ml of the appropriate buffer was applied to a column (1.8 cm × 30 cm) of Sepharose-(glutathione-2-pyridyl disulphide) gel and subjected to covalent chromatography as described above.

Chromatography on Sephadex G-100

Samples of papain solution (2 ml containing 5 mg of protein) were applied to a column (1.6 cm × 65 cm) equilibrated at 4°C with 0.2 M-sodium acetate buffer, pH 4.5. Fractions (3.6 ml) were collected at a flow rate of 15 ml/h.

Other methods

Protein determination. The concentration of papain protein was determined from E_{280} by using $\epsilon_{280} = 5.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (R. Axen & J. Carlsson, unpublished work; see Table 2).

Thiol determination. This was carried out by titration with 2-Py-S-S-2-Py, 4-Py-S-S-4-Py and 5,5'-dithiobis-(2-nitrobenzoate) as described by Brocklehurst & Little (1970, 1973).

Esterolytic activity. This was measured as described previously (Brocklehurst *et al.*, 1972b).

Data processing. Values of V_{max} and K_m were obtained from $[S_0]$ and v data by using the computer program described by Cleland (1963). The standard errors on the parameters relate to the fit of the data to the hyperbolic form of the Michaelis-Menten equation.

Spectrophotometric measurements. These were made by using Cary 15 and Cary 16K spectrophotometers.

Isoelectric focusing. Isoelectric focusing of protein samples in polyacrylamide gel (pH 7–10) was carried out as described by Wrigley (1968) by using Coomassie Brilliant Blue R 250 as staining agent (see Vesterberg, 1971).

Results and Discussion

Covalent chromatography

This type of chromatography relies upon the scission and formation of covalent bonds during the interaction of the material of the chromatography

column with one of the components of a mixture. In principle, it could allow the separation, not only of molecules that differ from each other in their possession or otherwise of a given functional group, but also of molecules that all possess the particular functional group. This would be possible if conditions could be found in which the reactivity towards the column of a given group in one type of molecule differs significantly from that in another type of molecule.

While this work was being carried out, we were not aware of any instances in which this type of chromatography had been used, although, if the affinity column of Blumberg *et al.* (1970) acts as a virtual substrate and reacts with papain to form an acyl-enzyme, their method would constitute a form of covalent chromatography. When the work had been completed, we became aware of two systems in which covalent chromatography had recently been employed. Ashari & Wilson (1972) allowed acetylcholinesterase to react with a column containing a bound 'irreversible' cholinesterase inhibitor, 2-aminoethyl *p*-nitrophenylmethyl phosphonate, and subsequently eluted the enzyme with 2-(hydroxyiminomethyl)-1-methylpyridinium iodide. Blumberg & Strominger (1972) reported the isolation of five penicillin-binding components present in *Bacillus subtilis* membranes by 'covalent affinity chromatography'. The penicillin-binding components were covalently linked to a Sepharose column containing bound 6-aminopenicillanic acid and subsequently eluted with hydroxylamine, which cleaves the penicilloyl-enzyme bond. Presumably in this case the reactive column cannot readily be regenerated.

Purification of thiol-containing proteins by covalent chromatography: general principle

As a general approach, it was decided to prepare a polymer containing aliphatic thiol groups (II) and subsequently to let these thiol groups react with 2-Py-S-S-2-Py to produce a polymer containing mixed disulphide residues (III) (Scheme 1a). By using the 'activated' polymer (III) as a chromatographic material, it was hoped to attach to the column only protein containing thiol groups (PSH) as shown in Scheme 1b. Subsequent elution with a solution of a low-molecular-weight thiol (RSH) would be expected to produce protein containing 1 mol of thiol/mol of protein, the thiolated column (II) and the disulphide derivative of the low-molecular-weight thiol (IV) (Scheme 1c). If the thiol-containing protein to be purified is an enzyme containing an essential thiol group, all the protein molecules containing thiol groups may not be catalytically active. Separation of catalytically active protein from catalytically inactive protein would be achieved only if conditions could be found in which the reactivities of the active

Scheme 1. Covalent chromatography by thiol-disulphide interchange: general principle

For details see the text.

and inactive thiol-containing proteins towards the mixed-disulphide column are sufficiently different.

It was considered possible that such conditions could be found for papain. Thus if the mixed-disulphide column (III) possessed the high reactivity in acidic media towards the thiol group of active papain but not towards other thiol groups as is found with 2-Py-S-S-2-Py, it was expected that this type of chromatography would permit separation of active papain from other thiols including thiol-containing but inactive protein.

Choice of the polymer-thiol conjugate

It was decided not to use the two thiol-containing polymers that are available commercially, namely Enzacryl Polythiol (Koch-Light Laboratories Ltd.) and Insolubilized Cysteine [Miles-Seravac (Pty.) Ltd., Maidenhead, Bucks., U.K.] for two reasons. First, the thiol groups of both of these products are those of cysteine residues that are attached to the polymer chain by their N atoms. It was considered desirable to create a situation in which the thiol group may be situated at a greater distance from the polymer chain than obtains in these products. This would be expected to decrease steric interactions of the proteins to be chromatographed with the polymer.

Secondly, it was decided to make use of the excellent packing and flow properties and low ion-exchange capacity of Sepharose which is not used as the supporting polymer in either of the commercial products.

Cyanogen bromide 'activation' constitutes a simple and convenient one-step synthesis of reactive (electrophilic) sites (VI) in polysaccharides such as Sepharose (V) (Scheme 2) (Axen *et al.*, 1967; Porath *et al.*, 1967; Axen & Ernback, 1971). Reaction of a suitable amino-thiol with (VI) could result in the production of a thiol-containing polymer (VII) (Scheme 2) in which steric hindrance of the thiol groups by the polymer may not be a serious problem. It was decided to use the readily available amino-thiol, glutathione (γ -L-glutamyl-L-cysteinylglycine). Since it is possible that the product (VII) may exist predominantly as the *N*-substituted imidocarbonate as shown or alternatively that this may be an intermediate in the formation of the *N*-substituted carbamate, we decided not to protect the thiol group in glutathione for the reaction with compound (VI). If either of these situations obtained, the possibility of the reaction of the thiol group with compound (VI) proceeding further than the reversible formation of a tetrahedral adduct was considered unlikely.

It was decided not to synthesize a thiol-containing Sepharose by the method of Cuatrecasas (1970), i.e. reaction of an ω -aminoalkyl-Sepharose with *N*-acetyl

Scheme 2. *Synthesis of a thiol-containing Sepharose*

For details see the text.

homocysteine thiolactone, because this route involves an extra step.

Thiol content of the Sepharose–glutathione gel

The sepharose–glutathione conjugate (VII) prepared as described in the Methods section contained 1.0 ± 0.2 mg of reduced glutathione/100 mg of dry gel (0.55 ± 0.05 μ mol of thiol/ml of swollen gel; standard errors based on three preparations as determined by titration with 2-Py-S-S-2-Py at pH 8.0).

After treatment of the gel with dithiothreitol as described in the Methods section (20 mM-dithiothreitol, pH 8.0, 22°C for 30 min) the reduced glutathione content increased by 100%, i.e. to 2.0 mg of reduced glutathione/100 mg of dry gel (1.1 ± 0.1 μ mol of thiol/ml of swollen gel). No further increase in the thiol content of the gel was achieved by increasing the concentration of dithiothreitol to 100 mM or by using NaBH_4 as reducing agent. The fully reduced gel has been stored as a suspension in 1 mM-EDTA solution for 4 weeks at 4°C without a detectable fall in its thiol content. The thiol content of the fully reduced gel is somewhat higher than that of the thiolated agarose reported by Cuatrecasas (1970) (0.58 μ mol of thiol/ml of swollen gel).

Capacity of the Sepharose–(glutathione–2-pyridyl disulphide) column with respect to papain

The theoretical capacity of a Sepharose–(glutathione–(2-pyridyl disulphide) column acting as a thiol–disulphide exchanger is its content of mixed disulphide residues. This is measured during its preparation by monitoring the 2-thiopyridone released in the reaction of the reduced Sepharose–glutathione gel with 2-Py-S-S-2-Py. The practical capacity of the column for a given thiol may be less than the theoretical capacity and will depend on the nature

of the molecule containing the reactive thiol group. The results presented below demonstrate that the capacity of the column for papain is $10 \pm 1\%$ of theoretical and for L-cysteine is probably essentially 100% of theoretical. The capacity for papain (substantially less than 100%) presumably arises from the inaccessibility of some of the mixed disulphide residues to the papain thiol group occasioned either by their location in hindered regions of the gel or more likely by steric shielding of some glutathione–2-pyridyl disulphide residues by glutathione–papain disulphide residues.

To determine the capacity of the column with respect to active papain it was loaded with activated, activator-free papain (0.5 mol of thiol/mol of protein) prepared from the 2 $\times$ crystallized product of BDH Chemicals Ltd. This enzyme preparation consists of 50% of papain containing 1 mol of thiol/mol of protein (that of cysteine-25) and 50% of protein that lacks a thiol group (see the introduction). A solution of this mixture of proteins was applied to the column [either in Tris–HCl buffer, pH 8.0, or in sodium acetate buffer, pH 4.0 (Sephacrose is stable in aqueous suspension in the pH range 4–9. Prolonged storage of the gel at pH 4, the low pH limit of its stability, should be avoided.)] until the concentration of thiol in the eluate became equal to the concentration of thiol in the solution applied to the column. This occurred after $10 \pm 1\%$ of the available mixed disulphide residues had released 2-thiopyridone and the thiol–disulphide interchange between the column and active papain was then considered to be complete. The attached protein was then eluted from the column by 20 mM-L-cysteine solution in 0.1 M-Tris–HCl buffer, pH 8.0, containing 0.3 M-NaCl. In the cases in which the reaction of papain with the column was allowed to proceed at pH 4, the pH of the column was changed to 8.0 by washing with 0.1 M-Tris–HCl buffer, pH 8.0, before the L-cysteine solution was

Fig. 1. Covalent chromatography of papain

Papain (20mg of the activated activator-free $2 \times$ crystallized product of BDH Chemicals Ltd., containing 0.47 mol of thiol/mol of protein, in 15 ml of 0.1M-Tris-HCl buffer, pH 8.0, containing 0.3M-NaCl and 1mM-EDTA) was applied to a column (25cm $\times$ 1.2cm; bed volume 28ml) containing 25g wet weight (500mg dry weight) of Sepharose-(glutathione-2-pyridyl disulphide) gel at room temperature (22°C). Elution patterns: $\circ$, E_{343} ; $\blacksquare$, E_{280} due to protein; $\bullet$, E_{280} ; $\triangle$, relative activity towards α -N-benzoyl-L-arginine ethyl ester at pH 6.0, $I = 0.3$, 25.0°C, $[S_0] = 25$ mM, $[L\text{-cysteine}] = 5$ mM. The contribution of E_{280} owing to protein in protein-2-thiopyridone mixtures was calculated from the extinction coefficients of 2-thiopyridone: $\epsilon_{343} = 7.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{280} = 7.7 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; extinction at 343 nm due to protein is negligible at the concentrations in the eluates. (a) Elution with 0.1M-Tris-HCl buffer, pH 8.0, containing 0.3M-NaCl and 1mM-EDTA. (b) Elution with 0.1M-Tris-HCl buffer, pH 8.0, containing 0.3M-NaCl, 1mM-EDTA and 50mM-L-cysteine.

introduced. Typical elution patterns are shown in Fig. 1. Spectral scanning of the L-cysteine eluate showed that it contained both protein and 2-thiopyridone. When the elution of protein was complete the eluate contained protein in amount equivalent to $10 \pm 1\%$ of the theoretical capacity of the column and 2-thiopyridone in amount equivalent to 90% of the theoretical capacity of the column. The protein was separated from low-molecular-weight material by chromatography on Sephadex G-25. Immediately before the Sephadex chromatography, dithiothreitol was added to give a concentration of 5mM. This is necessary when using aerobic solutions to prevent oxidation of the enzyme's thiol group in the presence of low concentrations of L-cysteine to provide the

papain-L-cysteine mixed disulphide during the latter stages of the Sephadex chromatography. When this precaution is taken, the protein emerging from the Sephadex column contains 1.04 ± 0.05 mol of thiol/mol of protein and its esterolytic activity towards α -N-benzoyl-L-arginine ethyl ester is not increased by preincubation with L-cysteine or with dithiothreitol (see below). When 5mM-dithiothreitol is omitted, the thiol content of the protein is generally 0.75 ± 0.05 mol of thiol/mol of protein and its esterolytic activity is proportionately less than that of the enzyme containing 1.0 mol of thiol/mol of protein when assayed in the absence of reducing agent. When 5mM-L-cysteine is added to such assay mixtures full activity is regained.

Preparation of fully active papain by covalent chromatography of the commercially available 2×crystallized protein

For routine preparation of papain containing 1 mol of thiol/mol of protein from the activated activator-free 2×crystallized product of BDH Chemicals Ltd., a procedure similar to the one described above was used except that the column was operated below the limit of its practical capacity. This was done to obtain maximum yield of active papain from a given batch of expensive commercial enzyme without having to recycle the protein. This procedure also allowed the demonstration that the Sepharose-(glutathione-2-pyridyl disulphide) column is not acting to an appreciable extent as an ion-exchanger in addition to acting as a thiol-disulphide exchanger. Spectrophotometric analysis of the eluate resulting from loading the column with the mixture of active and inactivatable papain under non-saturating conditions showed that all of the thiol-containing protein becomes attached to the column and all the protein lacking a thiol group passes through. Washing the loaded column with solutions of KCl up to 1 M in concentration at pH values of 4 and 8 failed to remove protein from the column. The possibility that the column is acting as a conventional affinity column, i.e. that the glutathione-2-pyridyl disulphide moiety binds active papain without thiol-disulphide interchange is discounted because of the stoichiometric release of 2-thiopyridone when the thiol-containing protein binds to the column.

Preparation of fully active papain from dried papaya latex

Twice-crystallized papain, containing after activation approx. 0.5 mol of thiol/mol of protein, is commercially prepared by the method of Kimmel & Smith (1954, 1958) (see also Arnon, 1970). This method consists of (1) preparation of a crude aqueous extract, (2) removal of material insoluble at pH 9 which may contain papain inhibitors, (3) $(\text{NH}_4)_2\text{SO}_4$ fractionation which removes another thiol-protease chymopapain (EC 3.4.4.11), (4) NaCl precipitation, (5) crystallization and (6) recrystallization from NaCl solution.

The first three steps of this procedure perform functions that covalent chromatography may not necessarily perform and these steps therefore were retained. The $(\text{NH}_4)_2\text{SO}_4$ -fractionation step was modified in that the second precipitation was carried out at 35% instead of 40% saturation to minimize the chymopapain content of the preparation before the covalent chromatography step. A solution of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated protein was applied to the Sepharose-(glutathione-2-pyridyl disulphide) column and subjected to covalent chromatography as de-

scribed in the Methods section. Separation from low-molecular-weight material by chromatography on Sephadex G-25 in the presence of 5 mm-dithiothreitol (see above) yielded active papain containing 1.04 ± 0.06 mol of thiol/mol of protein.

Separation of papain from L-cysteine by covalent chromatography

When a mixture of fully active papain (0.1 mM) and L-cysteine (up to approx. 5 mM) was subjected to covalent chromatography at pH 4 under rigorously anaerobic conditions to prevent formation of the papain-L-cysteine mixed disulphide, all of the papain reacted with the mixed-disulphide gel and the L-cysteine passed through the column without reacting with it to an appreciable extent. This result supports the view, discussed above, that the reaction of papain with the gel at pH values about 4 results mainly from reaction of the unionized thiol group of papain in the cysteine-25-histidine-159-asparagine-175 hydrogen-bonded system with the mixed-disulphide gel protonated on the nitrogen atom of the pyridine ring. As the concentration of L-cysteine in the mixture to be chromatographed is increased from 5 mM, an increasing proportion of L-cysteine reacts with the gel. This is because at such concentrations there is a sufficiently large concentration of the L-cysteine thiolate ion to make its rate of reaction with the protonated gel comparable with that of the un-ionized thiol group of papain. By using a thiol with a higher pK_a value (e.g. 2-mercaptoethanol) or with a low intrinsic reactivity, it should be possible to attach papain to the gel at pH 4 in the presence of even higher concentrations of such a thiol. This would be predicted from the above discussion and from our previous observation that it is possible to titrate papain active sites by using 2-Py-S-S-2-Py at pH values approx. 4 in the presence of up to a 10-fold molar excess of L-cysteine and up to a 100-fold molar excess of 2-mercaptoethanol (Brocklehurst & Little, 1970, 1973).

Properties of fully active papain prepared by covalent chromatography

Fully active papain prepared from dried papaya latex (see above) is identical with fully active papain prepared by covalent chromatography of the 2×crystallized product of BDH Chemicals Ltd., as assessed by its catalytic activity towards α -N-benzoyl-L-arginine ethyl ester and by the reactivity characteristics of its thiol group towards 2-Py-S-S-2-Py (see below). Analytical chromatography on Sephadex G-100 produced protein that emerged as a single symmetrical peak. Isoelectric focusing in polyacrylamide gel (pH range 7–10) of papain prepared from the latex by covalent chromatography and of the

2×crystallized product of BDH Chemicals Ltd. gave closely similar results. This technique would not show the presence of chymopapain in the preparation because of its high (10.1–10.4) isoelectric point (Kunimitsu & Yasunobu, 1970). The close similarities of the values of the parameters that characterize the catalysis of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester by fully active papain prepared from (a) 2×crystallized commercial product and (b) dried papaya latex, however, demonstrate that the chymo-papain contents (if any) of these preparations are closely similar.

It is well known that a free thiol group in the side chain of cysteine-25 in papain is a necessary requirement for enzymic activity. The presence of a thiol group, however, may not be sufficient. It is possible that some papain molecules may possess a reactive thiol group but not the active-site conformation required for catalytic activity.

The successful separation of papain from L-cysteine by covalent chromatography at pH4 (see above) suggests that the high reactivity that the thiol group of catalytically active papain exhibits towards 2-Py-S-S-2-Py in acidic media is exhibited also towards the mixed-disulphide gel and that this high reactivity is not exhibited by simple thiol groups. This is confirmed by preliminary kinetic experiments on reactions of papain and other thiols with samples of the mixed-disulphide gel in stirred suspension. In view of our previous observation that usually less than 10% of the papain molecules containing a thiol group do not exhibit high reactivity towards 2-Py-S-S-2-Py in acidic media (Brocklehurst & Little, 1970), it was not surprising that papain containing 1 mol of thiol/mol of protein prepared by covalent chromato-

graphy at pH4 has essentially the same characteristics as papain prepared by this technique at pH8. Thus for papain, enzyme containing 1 mol of thiol/mol of protein usually contains 1 intact catalytic site/mol of protein. This may not be true for other thiol enzymes or for papain that has been subjected to certain denaturing procedures and in these cases covalent chromatography may provide a means of separating active from inactive protein, both of which contain a thiol group.

Thiol content of papain and reactivity of the thiol group of cysteine-25 towards 2-Py-S-S-2-Py

The high reactivity of the papain thiol group towards 2-Py-S-S-2-Py in acidic media observed with partially active papain preparations (Brocklehurst & Little, 1970, 1972) is observed also with fully active papain.

At 25.0°C, $I = 0.1$ and pH values both 4.2 and 8.0 (buffers in Table 1) the values of the second-order rate constant (k) obtained with fully active papain from both sources (see above) at a particular pH are closely similar to each other and to the value obtained with the partially active 2×crystallized papain preparation (at pH4.2, $k = 1 \times 10^4 \pm 2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$; at pH8.0, $k = 900 \pm 100 \text{ M}^{-1} \cdot \text{s}^{-1}$). At both pH values the yields of 2-thiopyridone obtained with the preparations of fully active papain are stoichiometric with the protein and about double the yield obtained with partially active papain (see Table 1).

The thiol content of papain prepared by covalent chromatography at pH4 as determined by titration with 2-Py-S-S-2-Py (1.0 mol of thiol/mol of protein) was confirmed by titration with two other titrants

Table 1. Titration of the papain thiol group with 2-Py-S-S-2-Py at 25°C and $I = 0.1$

Standard errors are based on three determinations.

Papain preparation	Buffer ($I = 0.1$)	Yield of product (number of mol of 2-thiopyridone produced/mol of protein at infinite time)
Papain prepared from dried papaya latex by covalent chromatography at pH4.0	Sodium acetate, pH4.2	1.05 ± 0.05
	Tris-HCl, pH8.0	1.05 ± 0.05
Papain prepared from dried papaya latex by covalent chromatography at pH8.0	Sodium acetate, pH4.2	1.03 ± 0.05
	Tris-HCl, pH8.0	1.03 ± 0.05
Papain prepared from the 2×crystallized product of BDH Chemicals Ltd. by covalent chromatography at pH8.0	Sodium acetate, pH4.2	1.04 ± 0.05
	Tris-HCl, pH8.0	1.04 ± 0.05
Partially active papain: the 2×crystallized product of BDH Chemicals Ltd. activated by treatment with dithiothreitol and subsequently separated from low-molecular-weight material by chromatography on Sephadex G-25	Sodium acetate, pH4.2	0.53 ± 0.03
	Tris-HCl, pH8.0	0.58 ± 0.03

Table 2. *Catalytic characteristics of some preparations of papain*

In preparation no. (6) the latex was from Wallenstein Co., Staten Island, N.Y., U.S.A.

No.	Papain preparation	$10^{-4} \times \epsilon_{280}$ used to calculate protein concn. ($M^{-1} \cdot cm^{-1}$)	Michaelis parameters for the catalysis of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester at pH 6.0, $I = 0.3$ and 25°C			Reference
			K_m (mm)	k_{cat} (s^{-1}) calculated from V_{max} and the protein concentration	k_{cat} (s^{-1}) calculated from V_{max} and the active-site concn.	
(1)	Fully active papain prepared by covalent chromatography. Starting material either as in no. (4) or dried papaya latex of BDH Chemicals Ltd.	5.6	$18.2 \pm <0.1$	16.4 ± 0.5	$16.4 \pm 0.5^*$	Present paper
(2)	Fully active papain prepared by chromatography on an agarose mercurial column. Starting material as in no. (7)	5.75	18	26	26†	Sluyterman & Wijdenes (1970)
(3)	Fully active papain prepared by affinity chromatography. Starting material as in no. (5)	5.75	18	28.5	28.5‡	Blumberg <i>et al.</i> (1970)
(4)	Partially active papain: the 2 × crystallized product of BDH Chemicals Ltd.	5.6	$18.5 \pm <0.1$	8.5 ± 0.2	$16.1 \pm 0.5^*$	Present paper
(5)	Partially active papain: the 2 × crystallized product of Worthington Biochemicals Inc.	5.1	13.3 ± 0.33 15.2 ± 0.33	—	$16.1 \pm 1.8§$ $16.5 \pm 0.7§$	Whitaker & Bender (1965)
(6)	Partially active papain prepared from the dried papaya latex of the Wallenstein Co. by the method of Kimmel & Smith (1954)	5.75 —	18 22	13 9 (12 at 37°C)	— —	Blumberg <i>et al.</i> (1970) Smith & Parker (1958)
(7)	Partially active papain prepared by the method of Kimmel & Smith (1954), presumably from dried papaya latex (of unstated origin)	5.75	18	16	—	Sluyterman & Wijdenes (1970)

Determined by titration with : *2-Py-S-S-2-Py; †HgCl₂; ‡5,5'-dithiobis-(2-nitrobenzoate); §N-benzoyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.

(see the Methods section), which gave the following results: 5,5'-dithiobis-(2-nitrobenzoate), 0.99 and 1.01 mol of thiol/mol of protein; 4-Py-S-S-4-Py, 1.01 mol of thiol/mol of protein.

Esterolytic activity: Michaelis parameters for the catalysis of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester by papain at 25.0°C, pH 6.0 and $I = 0.3$

Over the years these parameters have been determined by a number of workers with both partially active papain preparations and more recently preparations containing 1.0 mol of thiol/mol of protein. These values of the parameters together with those obtained in the present study with fully active papain prepared by covalent chromatography are collected in Table 2.

The results of most of the studies are in reasonable accord about the K_m value. In the three most recent studies (Sluyterman, 1968; Blumberg *et al.*, 1970; the present paper) essentially the same K_m value (18–19 mM) is reported. There are much more marked discrepancies, however, in the various values reported for $k_{cat.}$. This parameter is calculated by dividing the value of $V_{max.}$ by the value of the enzyme concentration.

Table 2 shows that when allowance is made for the fact that some papain preparations are only 50–60% active, the values of $k_{cat.}$ fall into two groups. The present work is in agreement with the reports of Whitaker & Bender (1965) and Smith & Parker (1958) that the catalysis by active papain under the stated conditions is characterized by a $k_{cat.}$ value of approx. $16\text{--}17\text{ s}^{-1}$. By contrast, Sluyterman & Wijdenes (1970) and Blumberg *et al.* (1970) reported that $k_{cat.}$ is approx. 27 s^{-1} ($26\text{--}28.5\text{ s}^{-1}$), i.e. some 50% higher.

Smith & Parker (1958) reported a $k_{cat.}$ value of 9.0 s^{-1} assuming that their papain preparation (prepared by the procedure of Kimmel & Smith, 1954) was 100% active. Whitaker & Bender (1965) used an active-site titration to estimate the concentration of active papain in their partially active preparation and by using these data calculated $k_{cat.}$ to be $16\text{--}17\text{ s}^{-1}$. Bender *et al.* (1966) pointed out that these two values of $k_{cat.}$ may be considered to be in accord with each other if the protein preparation used by Smith & Parker (1958) contained only 56%-active papain. In view of what is now known about the composition of the mixture of proteins prepared by the method of Kimmel & Smith (1954) (see Brocklehurst *et al.*, 1972b; Sluyterman, 1967; Klein & Kirsch, 1969) this is entirely reasonable. In the work reported in the present paper, a value of $k_{cat.}$ of $16.4 \pm 1.5\text{ s}^{-1}$ was obtained by using fully active papain containing 1 mol of thiol with high reactivity towards 2-Py-S-S-2-Py in acidic media/mol of protein. By using the activated 2 $\times$ crystallized product of BDH Chemicals Ltd., values of $k_{cat.}$ of $8.5 \pm 0.2\text{ s}^{-1}$ and $16.1 \pm 0.5\text{ s}^{-1}$

Fig. 2. Demonstration of the adherence to Michaelis-Menten kinetics of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester at pH 6.0, $I = 0.3$, 25.0°C, catalysed by the 2 $\times$ crystallized papain of BDH Chemicals Ltd. (○ and ●) and by fully active papain containing 1 mol of thiol/mol of protein prepared by covalent chromatography (□ and ■)

Fully active papain was prepared as described in the Methods section from dried papaya latex; covalent chromatography was carried out at pH 8. Buffer: phosphate ($\text{KH}_2\text{PO}_4 + \text{NaOH}$), pH 6.0, containing 1 mM-EDTA and 5 mM-L-cysteine. Values of $[E_T]$ (total enzyme concentration) were calculated from measurement of E_{280} by using $\epsilon_{280} = 5.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and were in the range $0.3\text{--}1 \mu\text{M}$. The lines were drawn by using the Michaelis parameters obtained by computer fit to the hyperbolic form of the Michaelis-Menten equation (see the Methods section), i.e. for ○ and ●, $K_m = 18.5 \text{ mM}$, $k_{cat.} = 8.5 \text{ s}^{-1}$; for □ and ■, $K_m = 18.2 \text{ mM}$, $k_{cat.} = 16.4 \text{ s}^{-1}$.

were obtained by using a protein determination (E_{280}) and active-site titration (reaction with 2-Py-S-S-2-Py at pH 4.2) respectively to estimate the enzyme concentration. That the catalysis by both the commercial, partially active enzyme and the fully active enzyme prepared by covalent chromatography obey Michaelis-Menten kinetics is demonstrated in Fig. 2. The $k_{cat.}$ value of $16\text{--}17\text{ s}^{-1}$ discussed above is considerably lower than the values that have been reported previously to characterize the catalysis by preparations of papain containing 1 mol of thiol/mol of protein. Sluyterman & Wijdenes (1970) used an agarose mercurial column to prepare fully active papain. They reported that whereas the partially active papain used as the starting material was

characterized by $k_{\text{cat.}} = 16\text{s}^{-1}$, fully active papain was characterized by $k_{\text{cat.}} = 26\text{s}^{-1}$; both values were calculated by using total protein concentration as enzyme concentration. Blumberg *et al.* (1970) prepared fully active papain by affinity chromatography and reported a value of $k_{\text{cat.}} = 28.5\text{s}^{-1}$. They also reported that with a partially active commercial papain preparation and the total protein concentration as enzyme concentration, $k_{\text{cat.}} = 13.0\text{s}^{-1}$.

We cannot explain the much higher values of $k_{\text{cat.}}$ reported by Sluyterman & Wijdenes (1970) and by Blumberg *et al.* (1970) for their fully active papain preparations. It is interesting that the values of $k_{\text{cat.}}$ reported by these authors as characteristic of the catalysis by partially active papain preparations are also much higher (again by approx. 50%) than those reported by Smith & Parker (1958), Whitaker & Bender (1965) and in the present paper for presumably analogous preparations. Both Whitaker & Bender (1965) and Blumberg *et al.* (1970) used the 2× crystallized product of Worthington Biochemicals Inc., Freehold, N.J., U.S.A., as their source of partially active papain. The discrepancy between the results of Sluyterman & Wijdenes (1970) and of Blumberg *et al.* (1970) on the one hand and those of Smith & Parker (1958), of Whitaker & Bender (1965) and of ourselves on the other, could reflect a real difference in the papain preparations. In this event, the difference would have to occur in the partially active papain preparations as well as in the fully active ones and therefore could not be attributed to a differential effect in the methods of preparing fully active papain.

If the discrepancy does not reflect a real difference in the enzyme preparations, it must presumably reflect an undiscovered systematic error in some measurement (probably that of initial rates).

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