Affinity purification of the novel cysteine proteinase papaya proteinase IV, and papain from papaya latex

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A procedure is described for the purification of a previously undetected cysteine proteinase, which we have called papaya proteinase IV, from spray-dried latex of the papaya (*Carica papaya*) plant. The purification involves affinity chromatography on Gly-Phe-aminoacetonitrile linked to CH-Sepharose 4B, with elution by 2-hydroxyethyl disulphide at pH 4.5. The product thus obtained is a mixture of almost fully active papain and papaya proteinase IV, which are then separated by cation-exchange chromatography. A preliminary characterization of papaya proteinase IV showed it to be very similar to chymopapain in both molecular size and charge. However, the new enzyme is immunologically distinct from the previously characterized cysteine proteinases of papaya latex. It also differs in its lack of activity against the synthetic substrates of the other papaya proteinases, in its narrow specificity against protein substrates and its lack of inhibition by chicken cystatin. Papaya proteinase IV is abundant, contributing almost 30 % of the protein in spray-dried papaya latex, and contamination of chymopapain preparations with this enzyme may account for some of the previously reported heterogeneity of chymopapain.

INTRODUCTION

Three immunologically distinct cysteine proteinases have already been discovered in the latex of the papaya (*Carica papaya*) fruit: papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6) and papaya proteinase III (Schack, 1967; Buttle & Barrett, 1984; Zucker *et al.*, 1985; Barrett & Buttle, 1985). Each of these enzymes can occur in immunologically identical multiple forms differing in molecular charge; these are particularly marked for chymopapain (Kunimitsu & Yasunobu, 1967; Buttle & Barrett, 1984). There have been preliminary reports suggesting the presence of other cysteine proteinases in papaya latex (Schack, 1967; Clagett *et al.*, 1974; Lynn, 1979). To date these have not been followed up.

In the course of experiments with new ligands for the affinity purification of cysteine proteinases, we have now discovered a fourth cysteine proteinase in papaya latex. We have named it papaya proteinase IV, in accordance with a principle applied earlier (Barrett & Buttle, 1985).

EXPERIMENTAL

Materials

Many of the reagents (including papain, chymopapain and papaya proteinase III) were obtained as described previously (Buttle & Barrett, 1984). Activated CH-Sepharose 4B was from Pharmacia Fine Chemicals. Z-Phe-Cit-NHMec and Boc-Gly-NHMec were custom-synthesized by Cambridge Research Biochemicals (Cambridge, U.K.). Z-Gly-Phe-Cit-NHMec was given

by Dr. C. J. Gray (Department of Chemistry, University of Birmingham, Birmingham, U.K.). All other 7-(4-methyl)coumarylamide substrates were from Bachem Feinchemikalien (Bubendorf, Switzerland), who also supplied the *p*-nitroanilide and *p*-nitrophenyl ester substrates. Casein (Hammarsten) was supplied by Fisons. Hide Powder Azure and compound E-64 were from Sigma Chemical Co. Bovine haemoglobin was prepared as described previously (Barrett, 1970). Chicken cystatin was a mixture of forms 1 and 2 purified by affinity chromatography (Anastasi *et al.*, 1983), titrated with papain that had previously been standardized by titration with compound E-64.

Sepharose–Ahx–Gly–Phe–NHCH₂CN

To a stirred mixture of aminoacetonitrile hydrochloride (20 mmol) and ethyldi-isopropylamine (20 mmol) in dimethylformamide (20 ml) was added solid Boc-L-Phe-OPhNO₂ (10 mmol). The mixture was stirred at 20 °C for 2 h. It was then diluted with 100 ml of ethyl acetate, washed with water (2 ×), dilute aqueous triethylamine (5 ×), water (3 ×), dilute KHSO₄ (2 ×) and water (3 ×), dried and evaporated. The product (2.46 g, 81 %) was recrystallized from ethyl acetate/hexane: m.p. 134.5–135 °C; [α]_D –4.1° (*c* 1.1 in chloroform); n.m.r. (200 MHz), δ (p.p.m.) 1.40 (9H, s), 3.08 (2H, d, *J* = 8 Hz), 4.10 (2H, d, *J* = 5.5 Hz), 4.38 (1H, m), 5.05 (1H, d, *J* = 8 Hz), 6.70 (1H, t, *J* = 5.5 Hz) and 7.26 (5H, m) (Found: C, 63.26; H, 7.25; N, 13.71; calc. for C₁₆H₂₁N₃O₃: C, 63.35; H, 6.98; N, 13.85 %).

To a solution of Boc-Phe–NHCH₂CN (above)

Abbreviations used: Ahx-, 6-aminohexanoyl; Z-, benzyloxycarbonyl; Boc-, t-butyloxycarbonyl; Bz-, benzoyl; Suc-, 3-carboxypropionyl; -NHPhNO₂, *p*-nitroanilide; -NHMec, 7-(4-methyl)coumarylamide; -OPhNO₂, *p*-nitrophenyl ester; Cit, citrulline; compound E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane; *k*_{2(app.)}, apparent second-order rate constant for inactivation; *K*_{i(app.)}, apparent inhibition constant in the presence of substrate.

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(5 mmol) in dichloromethane (10 ml) was added 10 ml of ice-cold trifluoroacetic acid. The reaction mixture was stored at 20 °C for 30 min, after which time the solvents were evaporated at 40 °C. The residue was taken up in chloroform and re-evaporated, and the procedure was repeated twice more. The resulting crude trifluoroacetate salt (5 mmol) was taken up in 10 ml of dimethylformamide containing ethyldi-isopropylamine (7.5 mmol). To this solution was added solid Boc-Gly-OPhNO₂ (6.25 mmol), solid *N*-hydroxybenzotriazole monohydrate (6.25 mmol) and sufficient ethyldi-isopropylamine, dropwise, to generate the characteristic golden colour of *p*-nitrophenol. The reaction mixture was stirred at room temperature for 2 h, then quenched by addition of *NN*-diethylethylenediamine (1.5 ml) followed after 15 min by ethyl acetate (60 ml). The mixture was washed with water, dilute aqueous triethylamine, water and dilute KHSO₄, water, dried and evaporated. The residue was chromatographed on silica with ethyl acetate/hexane (20:1, v/v) to yield the product (0.95 g) as a foam: *R_F* (ethyl acetate/toluene, 20:1, v/v) 0.30, (ethyl acetate/methanol, 15:1, v/v) 0.57; [α]_D -41.0° (c 1.3 in chloroform); n.m.r. (200 MHz), δ (p.p.m.) 1.40 (9H, s), 3.07 (1H, dd, *J* = 14 and 6 Hz), 3.25 (1H, dd, *J* = 14 and 6 Hz), 3.71 (2H, d, *J* = 5.5 Hz), 3.95 (1H, dd, *J* = 18 and 5 Hz), 4.18 (1H, dd, *J* = 18 and 6 Hz), 4.79 (1H, m), 5.31 (1H, t, *J* = 5.5 Hz), 6.77 (1H, d, *J* = 9 Hz), 7.27 (5H, m) and 7.50 (1H, t, *J* = 5.5 Hz) (Found: C, 59.67; H, 6.83; N, 15.51; calc. for C₁₈H₂₄N₄O₄: C, 59.99; H, 6.71; N, 15.54%). Boc-Gly-Phe-Gly-*t*-butylamide (0.3 g) was also recovered from the column, as an oil, with ethyl acetate as solvent after elution of the desired product.

Boc-Gly-Phe-NHCH₂CN (30 mg) (above) was dissolved in 1 ml of 25% (v/v) trifluoroacetic acid in dichloromethane containing 10% (v/v) anisole, and incubated at 0 °C for 30 min. The mixture was then dried on a rotary evaporator at 34 °C and taken up in 1.5 ml of methanol to which 1.5 ml of 0.1 M-NaHCO₃, pH 8.0, was added.

Activated CH-Sepharose 4B (3 g dry wt.) was hydrated overnight in 75 ml of 1 mM-HCl at 4 °C, and washed in 600 ml of 1 mM-HCl followed by 300 ml of 0.1 M-NaHCO₃, pH 8.0, on a sintered-glass filter. The gel was suspended in 30 ml of 0.1 M-NaHCO₃, pH 8.0. To this was added the ligand (see above). The mixture was agitated gently overnight at 20 °C. The gel was collected on a sintered-glass filter, washed with 50% (v/v) methanol and then water, and suspended in 30 ml of 0.1 M-ethanolamine adjusted to pH 9.0 with HCl. This was shaken for 4 h at 20 °C. Finally the gel was collected, washed in water and stored in application buffer (see below) at 4 °C.

Enzyme purification

A column (4 ml bed volume) of Sepharose-Ahx-Gly-Phe-NHCH₂CN was prewashed with 12 ml of 50 mM-sodium citrate buffer in water/ethanediol (2:1, v/v), pH 4.5 (elution buffer), followed by 12 ml of 50 mM-sodium phosphate buffer containing 1 mM-EDTA in water/ethanediol (2:1, v/v), pH 6.8 (application buffer).

Spray-dried latex (0.5 g) was dissolved in 10 ml of application buffer. The solution was clarified by filtration (0.22 μm pore size), and the protein concentration was determined. Dithiothreitol was added to 2 mM final concentration, and the mixture was kept at 0 °C for 20 min. A 80 mg portion of the latex protein was applied

to the column (38 ml/h per cm²) at 20 °C, followed by 8 ml of application buffer and then 8 ml of elution buffer. 4 ml (1 bed volume) of elution buffer containing 50 mM-hydroxyethyl disulphide was applied, the flow was stopped and the column was left overnight at 20 °C. Elution with the hydroxyethyl disulphide solution was then resumed for a further 10 ml, before the column was re-equilibrated in application buffer. Fractions (1 ml) were collected throughout.

Active fractions from the affinity column (see above) were combined into two pools; material unretarded by the column, and material eluted in hydroxyethyl disulphide. These were applied separately to the Mono S HR 5/5 (cation-exchange) column without any further treatment. The column had been pre-equilibrated in 50 mM-sodium acetate/acetic acid buffer, pH 5.0, containing 1 mM-EDTA, and was washed with the same buffer (1 ml/min) after application of the sample until the *A*₂₈₀ returned to zero. A gradient (21.5 mM-Na⁺/ml) to 1 M-sodium acetate was then applied to the column (Buttle & Barrett, 1984) and 1 ml fractions were collected.

Protein determination

Column fractions and pools from the affinity column were assayed by the Bio-Rad dye-binding assay (Bio-Rad Laboratories, Watford, Herts., U.K.) with the use of filtered spray-dried latex solutions (*A*₂₈₀^{1.0} = 20.0) as standard. This assay showed less interference with dithiothreitol and hydroxyethyl disulphide than did *A*₂₈₀. The *A*₂₈₀^{1.0} was determined for papaya proteinase IV. Pure enzyme was dialysed exhaustively against water, freeze-dried, weighed and redissolved at a known concentration, and the *A*₂₈₀ was determined. The value of 16.5 thus obtained was used in calculating the concentrations of solutions of pure papaya proteinase IV from their *A*₂₈₀ values.

Enzyme assays

Assays of hydrolysis of Bz-Arg-NHPhNO₂ were performed as previously described (Buttle & Barrett, 1984). For stopped assays of hydrolysis of peptide-NHMe derivatives, azocasein and Hide Powder Azure, the methods described by Rowan *et al.* (1988) were used. Activities against casein and haemoglobin were measured by substituting these substrates in the azocasein protocol and measuring the *A*₂₈₀ of the supernatant. For assay of hydrolysis of *p*-nitrophenyl ester substrates, the sample was added to 0.10 M-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA and 0.2 mM-dithiothreitol (final volume 0.975 ml). The enzyme was allowed to activate for 10 min at 40 °C before the reaction was started by the addition of 25 μl of 10 mM substrate in acetonitrile or propan-2-ol. After 5 min the enzyme was inactivated by the addition of 0.10 M-sodium chloroacetate/0.04 M-sodium phosphate buffer, pH 6.0. Released *p*-nitrophenol was determined by immediate measurement of *A*_{347.5} (ε = 5500 M⁻¹·cm⁻¹; Lawson & Schramm, 1965).

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was done with reduction, in slab gels containing 12.5% (w/v) total acrylamide, as described by Bury (1981).

Multizonal cathodal electrophoresis, with the buffer system of Thomas & Hodes (1981) and 12.5% (w/v) total acrylamide, was otherwise as described by Zucker *et al.* (1985).

Titration with compound E-64

Papain was titrated with compound E-64, with Bz-Arg-NHPhNO₂ as substrate, as previously described (Zucker *et al.*, 1985). Papaya proteinase IV was titrated with compound E-64 by an adaptation of this method for the use of azocasein as substrate.

Immunological methods

The antisera to papain and papaya proteinase III were those described previously (Zucker *et al.*, 1985). Antisera were raised against a chymopapain preparation and papaya proteinase IV. The antigens were mildly carboxymethylated (Zucker *et al.*, 1985) before injection. The antiserum to the chymopapain preparation was raised in a sheep by one intramuscular injection of 100 µg of antigen in Freund's complete adjuvant followed by two of 50 µg at fortnightly intervals. The antiserum to papaya proteinase IV was raised in a rabbit by intramuscular injection of 360 µg of the enzyme in Freund's complete adjuvant followed after a fortnight by a subcutaneous injection of 100 µg in incomplete adjuvant.

IgG was partially purified from the antisera by (NH₄)₂SO₄ fractionation (Heide & Schwick, 1978), followed by dialysis into 0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.3.

Double-immunodiffusion analysis was done as previously described (Buttle & Barrett, 1984). Single radial immunodiffusion was based on the method of Mancini *et al.* (1965), as follows. Agarose (1%, w/v) in 0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.3, containing the appropriate IgG preparation, was poured on to GelBond (FMC Corp., Rockland, ME, U.S.A.) and a rectangular pattern of wells ($r = 1$ mm) 1.5 cm apart was cut. The reference and unknown samples were randomly distributed among the wells to minimize errors due to edge effects. Following application of antigen to the wells, the plates were left for 24 h for the precipitin rings to develop. They were then washed, dried and stained. Pure mildly carboxymethylated papaya proteinase IV was used to generate standard curves, which were plotted as antigen concentration versus the square of ring diameter. The useful range of the assay was 25–300 ng of antigen/well.

pH optimum of azocasein hydrolysis

The rate of azocasein hydrolysis by papaya proteinase IV was examined at pH 4.0, 5.0 and 6.0 (0.10 M-sodium citrate/citric acid buffer), 6.0 and 7.0 (0.10 M-sodium phosphate buffer), 7.0 and 8.0 (0.10 M-Tris/HCl buffer), 8.0, 9.0 and 10.0 (0.10 M-sodium borate buffer). All incubation mixtures also contained 1 mM-EDTA and 2 mM-dithiothreitol.

Inactivation by compound E-64

The rate constant for inactivation of papaya proteinase IV by compound E-64 was determined by incubating the enzyme and inhibitor in equimolar concentration. Pre-activated papaya proteinase IV and compound E-64 were set up in 0.10 M-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 2 mM-dithiothreitol at 40 °C. At various times, samples were removed, diluted 20-fold and assayed for activity against azocasein. The results were analysed to obtain $k_{2(\text{app.})}$ by using the relationship:

$$k_{2(\text{app.})} = (1/t)[(a_0 - a_t)/a_0 \cdot a_t]$$

where a_0 is initial active enzyme concentration and a_t is the remaining active enzyme concentration at time t (Laidler, 1958).

Chicken cystatin

The inhibition of papaya proteinase IV by chicken cystatin was investigated with Boc-Gly-OPhNO₂ as substrate. Papaya proteinase IV (0.15 µM final concentration by active-site titration) was preincubated with or without chicken cystatin (1 µM final concentration) in the pH 6.0 assay buffer (see above) (975 µl final volume) for 20 min at 40 °C. Then 25 µl of 10 mM-Boc-Gly-OPhNO₂ was added and substrate hydrolysis was allowed to continue for 5 min, after which the reaction was stopped and $A_{347.5}$ determined.

The interaction of papaya proteinase IV and chicken cystatin was also studied with azocasein as substrate in 0.10 M-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 2 mM-dithiothreitol. The enzyme (30 nM final active enzyme concentration) was preincubated with chicken cystatin (up to 1.13 µM final concentration) for 20 min at 40 °C. Substrate was then added. After 30 min the reaction was stopped by the addition of trichloroacetic acid (10%, w/v). The precipitate was removed and A_{366} of the supernatant was measured.

RESULTS AND DISCUSSION

Inhibition of the papaya proteinases by Boc-Gly-Phe-NHCH₂CN

$K_{i(\text{app.})}$ values for the inhibition of papain, chymopapain and papaya proteinase III by Boc-Gly-Phe-NHCH₂CN determined by the method of Nicklin & Barrett (1984) were 0.42 µM, 6.6 µM and 7.9 µM respectively.

The inhibition of papain by a nitrile derivative was previously reported by Sluyterman & Wijdenes (1973) (benzoylamidoacetonitrile; $K_i = 0.14$ mM) and Lewis & Wolfenden (1977) (acetyl-Phe-NHCH₂CN; $K_i = 0.73$ µM). More recently, Moon *et al.* (1986) and Liang & Abeles (1987) demonstrated by ¹³C n.m.r. the binding of the nitrile carbon atom of peptide nitriles to Cys-25 of papain.

Purification

Two peaks of Bz-Arg-NHPhNO₂-hydrolysing activity were eluted from the Sepharose-Ahx-Gly-Phe-NHCH₂CN column: one that passed straight through (peak 1), and one that was bound and eluted the next day in hydroxyethyl disulphide (peak 2) (see Fig. 1a). Of the activity applied to the column 70% was recovered in these two peaks, 52% in peak 1 and 18% in peak 2. The capacity of the affinity column was 1–2 mg of protein/ml bed volume.

The two peaks from the affinity column were loaded separately on to the Mono S HR 5/5 column of the Pharmacia f.p.l.c. system. When the dithiothreitol or hydroxyethyl disulphide had washed through, and the A_{280} had returned to baseline, the proteins were eluted with a gradient of sodium acetate/acetic acid buffer, pH 5.0.

Peak 1 gave protein and amidase activity in the positions characteristic of chymopapain and papaya proteinase III (results not shown, but see Buttle & Barrett, 1984). In addition, a small protein peak with associated lysozyme-like activity was eluted between the

Fig. 1. Chromatographic purification of papain and a new cysteine proteinase

(a) Spray-dried papaya latex proteins were applied to the Sepharose-Ahx-Gly-Phe-NHCH₂CN column. A proportion of the Bz-Arg-NHPhNO₂-hydrolysing activity was bound by the column and eluted in 50 mM-hydroxyethyl disulphide at pH 4.5. This material (peak 2) was collected and chromatographed as described in (b). ●—●, Protein (mg/ml); □—□, Bz-Arg-NHPhNO₂-hydrolysing activity (ΔA_{410}). (b) Cation-exchange chromatography of peak 2 (above) on the Mono S column at pH 5.0. Fractions (1 ml) corresponding to the bars were pooled separately and analysed further. —, Protein (A_{280}); ·····, azocasein-hydrolysing activity (ΔA_{366}); ———, Bz-Arg-NHPhNO₂-hydrolysing activity (ΔA_{410}); ———, [Na⁺] (M).

chymopapain and papaya proteinase III regions (Buttle *et al.*, 1986).

The material that had bound to the affinity column produced two major protein peaks on cation-exchange chromatography (Fig. 1b). Peak a, eluted at about 0.17 M-Na⁺, displayed high activity against both Bz-Arg-NHPhNO₂ and azocasein. This protein was subsequently shown to react with a monospecific antiserum to papain (Fig. 3). The larger peak b, eluted at approx. 0.38 M-Na⁺ (close to the position expected for early-eluted forms of chymopapain), exhibited no detectable activity against Bz-Arg-NHPhNO₂. It was, however, associated with azocasein-digesting activity, although the specific activity was much lower than that of the papain peak. Peaks a and b were collected, dialysed separately into 1 mM-EDTA, freeze-dried and stored at -20 °C awaiting further analysis.

Our finding that Boc-Gly-Phe-NHCN₂CN inhibited papain with a $K_{i(\text{app.})}$ over an order of magnitude less

than those for chymopapain and papaya proteinase III presumably explains the preferential binding of papain by the deprotected and immobilized ligand. Since the second enzyme adsorbed by the column, and separated from papain in cation-exchange chromatography, seems not to have been described previously (see below), we named it papaya proteinase IV. This is in line with our proposal of a chronological scheme for the naming of new proteinases from *C. papaya* (Barrett & Buttle, 1985).

Gel electrophoresis

The four papaya proteinases, including papain and papaya proteinase IV isolated by our new method, were examined by SDS/polyacrylamide-gel electrophoresis (Fig. 2a). The apparent M_r of papaya proteinase IV was slightly greater than the M_r of papain (23406; Mitchel *et al.*, 1970) and very similar to that of chymopapain. Semi-logarithmic M_r calibration curves were plotted, and apparent M_r values of 26000, 25000 and 24500 were

Fig. 2. Gel electrophoreses of the four papaya proteinases

(a) SDS/polyacrylamide-gel electrophoresis (with reduction) of: lane 1, M_r standards; lane 2, peak a in Fig. 1(b); lane 3, peak b in Fig. 1(b); lane 4, chymopapain; lane 5, papaya proteinase III. (b) Multizonal cathodal gel electrophoresis of: lane 1, peak a in Fig. 1(b); lane 2, peak b in Fig. 1(b); lane 3, chymopapain; lane 4, papaya proteinase III.

found for papaya proteinase III, chymopapain and papaya proteinase IV respectively. The complete amino acid sequence of papaya proteinase III has recently been determined (Dubois *et al.*, 1988), from which we found its M_r to be 23262. Papaya proteinase III therefore behaves anomalously on this gel system, possibly as a result of its extreme basicity (Kaarsholm & Schack, 1983).

Migration of the four native proteinases in a cathodal electrophoresis system (Fig. 2b) confirmed the similarity in overall charge between papaya proteinase IV and chymopapain first suggested by their similar elution positions from the Mono S column (compare Fig. 1b with Fig. 1 in Buttle & Barrett, 1984). Papaya proteinase IV thus resembles chymopapain in both molecular size and charge. Papain from the Mono S column ran as a single band.

Active-site titration

The molarity of cysteine-proteinase active sites was found by using compound E-64 as titrant. The value thus determined was compared with the molar concentration of protein on the basis of the molar absorption coefficient for papaya proteinase IV [from the $A_{280}^{1\%}$ of 16.5 and the M_r of 24500 (see above)] of $4.04 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The molar absorption coefficient for papain of $5.85 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Glazer & Smith, 1971) was used. Papain and papaya proteinase IV purified by the method

described above were 84% and 95% active cysteine proteinase respectively.

Double immunodiffusion

The antiserum raised against papaya proteinase IV gave a single precipitin line in double immunodiffusion with this protein (Fig. 3). Papaya proteinase IV failed to react with monospecific antisera directed against papaya proteinase III and papain. It did form a precipitin reaction with our anti-chymopapain IgG, but it is clear in Fig. 3 that this is a reaction of non-identity with chymopapain. We conclude that the chymopapain used for raising the antibody was contaminated with this distinct antigen, and that papaya proteinase IV is immunologically distinct from the three previously described proteinases from papaya latex.

Immunological quantification of papaya proteinase IV in spray-dried latex and a chymopapain preparation

The specific IgG preparation directed against papaya proteinase IV was used in single radial immunodiffusion to estimate the amount of protein that was papaya proteinase IV in the batch of spray-dried latex from which the enzyme had been purified. The value obtained, $28.2 \pm 0.5\%$ (mean $\pm$ S.E.M., $n = 12$) demonstrated that this previously unrecognized proteinase was in fact a major component of the latex.

The same method was used to find the amount of contamination by papaya proteinase IV in a preparation of chymopapain obtained by our published method (Buttle & Barrett, 1984). Papaya proteinase IV contributed $13.6 \pm 0.4\%$ (mean $\pm$ S.E.M., $n = 5$) of total protein.

Fig. 3. Double immunodiffusion of the four papaya proteinases and IgG preparations directed against them

The wells contained: A, anti-papain IgG; B, an IgG preparation raised against chymopapain; C, anti-(papaya proteinase III) IgG; D, an IgG preparation raised against peak b in Fig. 1(b); 1, peak a in Fig. 1(b); 2, chymopapain; 3, papaya proteinase III; 4, peak b in Fig. 1(b).

It seems likely that the presence of papaya proteinase IV in other chymopapain preparations, particularly in the early-eluted forms of that enzyme, has previously gone undetected.

Catalytic activities of papaya proteinase IV

Papaya proteinase IV was inactive until a thiol compound (dithiothreitol or cysteine) was added to the incubation mixture. The enzyme then solubilized Hide Powder Azure and produced trichloroacetic acid-soluble peptides from haemoglobin, casein and azocasein, with a broad pH optimum around 7.0 with the last-mentioned substrate. The assays with casein and azocasein proved the most sensitive, but, even with these, papaya proteinase IV appeared to have only about 10% of the activity of papain.

The time course of production of small peptides from azocasein by papaya proteinase IV and papain was investigated. It can be seen (Fig. 4) that the assay of papaya proteinase IV, unlike that of papain, was not linear. Above a ΔA_{366} value of 0.1, the rate of soluble peptide formation by papaya proteinase IV was very low. This suggested substrate depletion, which could occur if only a limited number of peptide bonds in azocasein were susceptible to cleavage by this enzyme.

The degradation of casein by papain and papaya proteinase IV was investigated by subjecting the incubation mixture to SDS/polyacrylamide-gel electrophoresis at various times. Fig. 5 demonstrates that papain was capable of degrading casein to peptides too small to detect by this method, via a number of distinct intermediates. Papaya proteinase IV initially cleaved casein to produce, by 60 min, a number of discrete digestion products. After this time, no further digestion was detected. This was not due to loss of enzymic activity; samples removed at 180 min showed over 80% of the expected activity on azocasein for both papain and papaya proteinase IV. It was therefore concluded that

Fig. 4. Time course of formation of trichloroacetic acid-soluble peptides from azocasein by papain and papaya proteinase IV

Azocasein was incubated with papain (60 nM) (●—●) or papaya proteinase IV (300 nM) (○—○) for the specified times, in 0.10 M-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 2 mM-dithiothreitol at 40 °C. Trichloroacetic acid-soluble digestion products were measured as A_{366} .

the peptide products formed after a 60 min incubation with papaya proteinase IV represented the limit of activity of the enzyme on this substrate.

At earlier time points it is clear that papain and papaya proteinase IV produced different peptides, highlighting differences in specificity between these two cysteine proteinases.

Papaya proteinase IV failed to hydrolyse the synthetic substrate, Bz-Arg-NHPhNO₂, normally used for assaying the papaya proteinases. We therefore screened a series of synthetic substrates for susceptibility to cleavage by the new enzyme. No activity was detected against the *p*-nitroanilide ester derivatives of leucine, Suc-Ala-Ala-Pro-Leu or Suc-Ala-Ala-Pro-Phe. In addition, papaya proteinase IV, up to an active enzyme concentration of 0.94 μM (24 μg/ml), failed to release detectable amounts of aminomethylcoumarin from a series of 15 peptide-NHMe substrates: glycine, Boc-Gly, Z-Phe-Ala, Z-Phe-Met, Z-Arg-Arg, Z-Phe-Arg, Z-Gly-Gly-Arg, Bz-Phe-Val-Arg, Boc-Val-Pro-Arg, Boc-Ile-Glu-Gly-Arg, Z-Phe-Cit, Z-Gly-Phe-Cit, Z-Leu-Tyr, Ala-Ala-Phe and Suc-Ala-Ala-Phe.

We examined the esterase activity of papaya proteinase IV with a small series of *p*-nitrophenyl ester substrates: Boc-Phe-OPhNO₂, Boc-Leu-OPhNO₂, Boc-Ala-OPhNO₂, Boc-Tyr-OPhNO₂ and Boc-Gly-OPhNO₂. Of these, Boc-Gly-OPhNO₂ was hydrolysed most rapidly. The specific activity of papaya proteinase IV against this substrate was 81 μmol/min per μmol, under our standard assay conditions ([S] = 250 μM). Unfortunately, Boc-Gly-OPhNO₂ was not a specific substrate for papaya proteinase IV, being cleaved rapidly by papain, chymopapain and papaya proteinase III (results not shown).

Inhibitors

Papaya proteinase IV was rapidly inhibited by compound E-64, with $k_{2(app.)} = 15300 \pm 1400 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ S.E.M., $n = 4$). No inhibition by chicken cystatin was observed, however, even up to 1.13 μM-cystatin.

CONCLUSIONS

Our characterization of the enzymic activity of papaya proteinase IV shows it to be very different from the three previously known proteinases from papaya latex. Papain, chymopapain and papaya proteinase III have been shown to share many characteristics with regard to substrate and inhibitor specificity. For instance, they favour the same peptide bonds in the B-chain of oxidized insulin (Johansen & Ottesen, 1968), and produce very similar initial cleavage patterns from other proteins. They are then capable of continued hydrolysis, leading to the formation of low- M_r products (Zucker *et al.*, 1985). In addition, the previously described papaya proteinases have very similar $k_{cat.}/K_m$ values with two synthetic substrates, Bz-Arg-NHPhNO₂ (Buttle, 1985) and Z-Phe-Arg-NHMe (Zucker *et al.*, 1985). Chicken cystatin is a tight-binding inhibitor of papain (Nicklin & Barrett, 1984), chymopapain ($K_{i(app.)} = 3.3 \times 10^{-10} \text{ M}$; Buttle, 1985) and papaya proteinase III (A. J. Barrett & D. J. Buttle, unpublished work).

In all these respects papaya proteinase IV is dissimilar. Its lack of inhibition by chicken cystatin is reminiscent of one other plant cysteine proteinase, stem bromelain (Rowan *et al.*, 1988). With regard to substrate specificity,

Fig. 5. SDS/polyacrylamide-gel electrophoresis of casein cleavage products

Casein (1.0 mg/ml final concentration) in 0.10 M-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 2 mM-dithiothreitol was incubated with pre-activated papain (4.6 nM active) (lanes 3, 6, 9, 12 and 15) or papaya proteinase IV (46.0 nM active) (lanes 2, 5, 8, 11 and 14), or without added proteinase (lanes 1, 4, 7, 10 and 13) at 40 °C. At various times up to 180 min, 20 μ l samples were removed and mixed with 20 μ l of 0.10 M-sodium chloroacetate/0.20 M-sodium acetate buffer, pH 4.3. The samples were run in SDS/polyacrylamide-gel electrophoresis (with reduction).

papaya proteinase IV appears to be unique among the plant cysteine proteinases. We have yet to find a synthetic amide substrate for it, and its limited cleavage of a protein substrate suggests a stringency of subsite specificity unique among the known plant cysteine proteinases.

Papaya proteinase IV has previously escaped detection because of its lack of activity against Bz-Arg-NHPhNO₂ and other common synthetic substrates, as well as its limited action on proteins. Even so, we were surprised to find that the new enzyme is abundant in spray-dried papaya latex. Owing to their similar physicochemical properties, papaya proteinase IV tends to contaminate chymopapain preparations. This may help to explain some formerly puzzling data for differences in specific activity between chymopapain preparations. For instance, Kóródi *et al.* (1986) were surprised that 'two similar enzyme forms, not easily separable even by f.p.l.c., exhibit considerable differences in their rate constants. This questions the reliability of all studies to date pertinent to the catalytic properties of chymopapain'. Chymopapain preparations have previously been shown also to vary in their reactivity with 2,2'-dipyridyl disulphide (Brocklehurst *et al.*, 1980, 1984, 1985; Khan & Polgár, 1983; Polgár, 1984). Papaya proteinase IV differs from chymopapain in its reactivity with substrates and inhibitors, and it may also differ in rates of reaction with the disulphide.

A convenient method for detecting papaya proteinase IV contamination of chymopapain preparations takes advantage of the different behaviour of these two proteinases towards chicken cystatin. Activity against Boc-Gly-OPhNO₂ or azocasein that is resistant to inhibition by micromolar chicken cystatin can be attributed to papaya proteinase IV.

The immobilized ligand that led to the discovery of papaya proteinase IV is the acetonitrile of a dipeptide coupled to Sepharose, and represents a new class of affinity ligand that may have further applications in the purification of cysteine proteinases. The ligand also bound papain, and, like previously described methods for the affinity purification of this enzyme (Blumberg *et al.*, 1970; Sluyterman & Wijdenes, 1970; Brocklehurst *et al.*, 1973), ours discriminated between the active and

inactive forms of papain. Unlike the methods cited above, however, the acetonitrile ligand also differentiated between the active forms of papain, chymopapain and papaya proteinase III, binding only the former. We were therefore able to purify both papain and papaya proteinase IV by a method involving just two chromatographic steps without the need for the salt precipitations used previously for separating papain from the other proteolytic components of papaya latex before its application to the affinity matrix (e.g. Stuchbury *et al.*, 1975).

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REFERENCES

- Anastasi, A., Brown, M. A., Kembhavi, A. A., Nicklin, M. J. H., Sayers, C. A., Sunter, D. C. & Barrett, A. J. (1983) *Biochem. J.* **211**, 129–138
- Barrett, A. J. (1970) *Biochem. J.* **117**, 601–607
- Barrett, A. J. & Buttle, D. J. (1985) *Biochem. J.* **228**, 527
- Blumberg, S., Schechter, I. & Berger, A. (1970) *Eur. J. Biochem.* **15**, 97–102
- Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1973) *Biochem. J.* **133**, 573–584
- Brocklehurst, K., Baines, B. S. & Mushiri, M. S. (1980) *Biochem. J.* **189**, 189–192
- Brocklehurst, K., Baines, B. S., Salih, E. & Hatzoulis, C. (1984) *Biochem. J.* **221**, 553–554
- Brocklehurst, K., Salih, E., McKee, R. & Smith, H. (1985) *Biochem. J.* **228**, 525–527
- Bury, A. F. (1981) *J. Chromatogr.* **213**, 491–500
- Buttle, D. J. (1985) Ph.D. Thesis, The British Library, London
- Buttle, D. J. & Barrett, A. J. (1984) *Biochem. J.* **223**, 81–88
- Buttle, D. J., Abrahamson, M. & Barrett, A. J. (1986) *Spine* **11**, 688–694
- Clagett, J. A., Tokuda, S. & Engelhard, W. E. (1974) *Proc. Soc. Exp. Biol. Med.* **145**, 1250–1257
- Dubois, T., Kleinschmidt, T., Schnek, A. G., Looze, Y. & Braunitzer, G. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 741–754
- Glazer, A. N. & Smith, E. L. (1971) *Enzymes* 3rd Ed. **3**, 501–546
- Heide, K. & Schwick, H. G. (1978) *Handb. Exp. Immunol.* 3rd Ed. **1**, 7.1–7.11

- Johansen, J. T. & Ottesen, M. (1968) *C. R. Trav. Lab. Carlsberg* **36**, 265–283
- Kaarsholm, N. C. & Schack, P. (1983) *Acta Chem. Scand. Ser. B* **37**, 607–611
- Khan, I. U. & Polgár, L. (1983) *Biochim. Biophys. Acta* **760**, 350–356
- Kóródi, I., Asbóth, B. & Polgár, L. (1986) *Biochemistry* **25**, 6895–6900
- Kunimitsu, D. K. & Yasunobu, K. T. (1967) *Biochim. Biophys. Acta* **139**, 405–417
- Laidler, K. J. (1958) *The Chemical Kinetics of Enzyme Action*, pp. 19–54, Oxford University Press, Oxford
- Lawson, W. B. & Schramm, H.-J. (1965) *Biochemistry* **4**, 377–386
- Lewis, C. A. & Wolfenden, R. (1977) *Biochemistry* **16**, 4890–4895
- Liang, T.-C. & Abeles, R. H. (1987) *Arch. Biochem. Biophys.* **252**, 626–634
- Lynn, K. R. (1979) *Biochim. Biophys. Acta* **569**, 193–201
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254
- Mitchel, R. E. J., Chaiken, I. M. & Smith, E. L. (1970) *J. Biol. Chem.* **245**, 3485–3492
- Moon, J. B., Coleman, R. S. & Hanzlik, R. P. (1986) *J. Am. Chem. Soc.* **108**, 1350–1351
- Nicklin, M. J. H. & Barrett, A. J. (1984) *Biochem. J.* **223**, 245–253
- Polgár, L. (1984) *Biochem. J.* **221**, 555–556
- Rowan, A. D., Buttle, D. J. & Barrett, A. J. (1988) *Arch. Biochem. Biophys.* **267**, 262–270
- Schack, P. (1967) *C. R. Trav. Lab. Carlsberg* **36**, 67–83
- Sluyterman, L. A. E. & Wijdenes, J. (1970) *Biochim. Biophys. Acta* **200**, 593–595
- Sluyterman, L. A. E. & Wijdenes, J. (1973) *Biochim. Biophys. Acta* **302**, 95–101
- Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L. & Suschitzky, H. (1975) *Biochem. J.* **151**, 417–432
- Thomas, J. M. & Hodes, M. E. (1981) *Anal. Biochem.* **118**, 194–196
- Zucker, S., Buttle, D. J., Nicklin, M. J. H. & Barrett, A. J. (1985) *Biochim. Biophys. Acta* **828**, 196–204

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