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Chymopapain

Chromatographic purification and immunological characterization

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Chymopapain (EC 3.4.22.6) was purified from commercially available spray-dried latex of papaya (*Carica papaya*) fruit by $(NH_4)_2SO_4$ fractionation and fast protein chromatography on the Mono S cation-exchange column. Multiple forms of chymopapain separated chromatographically were shown to be immunologically identical. A major form was isolated and found to be homogeneous by several criteria, and fully active, and its *N*-terminal amino acid was identified as tyrosine. Latex from fresh unripe papaya fruit contained predominantly one form of chymopapain, and it is concluded that chymopapain is a single enzyme distinct from the other cysteine proteinases of *C. papaya* latex.

The cysteine proteinase chymopapain (EC 3.4.22.6) is the most abundant protein in the latex of the unripe fruit of the papaya (*Carica papaya* L). It has recently achieved medical importance through its use in the treatment of prolapsed intervertebral discs (Javid *et al.*, 1983). Chymopapain purified from spray-dried latex is well known to be heterogeneous on cation-exchange chromatography (Baines & Brocklehurst, 1982; Clagett *et al.*, 1974; Khan & Polgar, 1983; Kunimitsu & Yasunobu, 1967; Lynn, 1979; Robinson, 1975; Schack, 1967). Normally, the material eluted early from the cation-exchange column is called 'chymopapain A' and is followed by an incompletely resolved peak of 'chymopapain B'. Brocklehurst *et al.* (1980) have concluded, from results with thiol-specific reactivity probes, that the active centre of chymopapain A is different from that of chymopapain B. Khan & Polgar (1983) recently isolated a form of chymopapain that possessed properties intermediate between those of chymomapains A and B. Brocklehurst & Salih (1983) have proposed a new nomenclature for the papaya cysteine proteinases in which four 'chymopapains' are identified on the basis of the molecular environments of their thiol groups as revealed by a two-protonic-state reactivity probe and elution position from a cation-exchange column.

The purification of chymopapain (reviewed by Brocklehurst *et al.*, 1981) is made extremely difficult by the fact that its physicochemical properties closely resemble those of other components of the latex, as well as by the fact that it occurs in multiple chromatographic forms. In the present paper, we describe a new purification procedure, subject the product to rigorous tests of purity, and suggest, on the basis of immunological and other evidence, that chymopapain is best considered as a single protein that occurs in multiple forms, some natural and others as artefacts.

Abbreviations used: Bz-Arg-$NPhNO_2$, *N*-α-benzoyl-DL-arginine *p*-nitroanilide; compound E-64, L-3-carboxy-2,3-*trans*-epoxypropyl-leucylamido (4-guanidino)-butane; f.p.l.c., fast protein liquid chromatography (Pharmacia System); SP-, sulphopropyl-; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Experimental

Commercial spray-dried papaya latex ('papain') was obtained from Powell and Scholefield, Liverpool L7 3JG, U.K. SP-Sephadex C-25 was from Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K., who also supplied the f.p.l.c. system. AR-grade $(NH_4)_2SO_4$ for protein fractionation was from Fisons, Loughborough, Leics., U.K. Hydroxyethyl disulphide (98%) was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Dansyl chloride was supplied by BDH Chemicals, Poole, Dorset, U.K. Bz-Arg-$NPhNO_2$ was from Bachem Feinchemikalian AG, Bubendorf, Switzerland. Azocasein was prepared as previously described (Barrett & Kirschke, 1981). Agarose (type II) and Brilliant Blue G were from Sigma,

Poole, Dorset, U.K. Compound E-64 was a gift from Dr K. Hanada, Taisho Pharmaceutical Co., Ohmiya, Saitama, Japan, and is now available commercially from Sigma. All other reagents used were of analytical grade.

Fresh papaya latex

Unripe papaya fruit (countries of origin: Argentina and Brazil) were purchased from a local supermarket. Papaya latex was obtained by making two or three incisions, approx. 2mm in depth, in the long axis of the fruit. About 0.3ml of latex, containing approx. 15mg of chymopapain, flowed from the cuts and was collected with a micropipette, transferred to a small centrifuge tube, and mixed with 5ml of 0.05M-sodium acetate buffer, pH5.0, containing 1mM-EDTA. The solution was centrifuged, and the supernatant retained for chromatography.

Chromatographic analysis of spray-dried and fresh papaya latex

Programmed linear gradients were used for chromatography on the Mono S column. Plots of A_{280} versus gradient composition (provided automatically) were corrected for the volume between the pumps and u.v. detector, including column volume.

A pH5.0 buffer was prepared by dissolving 40.0g of NaOH in 500ml of water, adjusting the pH to 5.5 with acetic acid, allowing the solution to cool, re-adjusting to pH 5.0 and making up to 1 litre with water. Solid disodium EDTA was added to give the stock buffer (1M-Na^+/1mM-EDTA, pH5.0).

Both spray-dried and fresh latex were applied to the cation-exchange (Mono S) column in 0.05M-NaOH/acetic acid/1mM-EDTA, pH5.0, after treatment with 3mM-dithiothreitol for 30min at 20°C. The proteins were eluted with a linear gradient (0.0215M-Na^+/ml) from 0.05M-Na^+ to 1.00M-Na^+, using the stock buffer to create the gradient, at a flow rate of 1ml/min.

Fractions were collected in tubes containing sufficient 100mM-hydroxyethyl disulphide to give a final concentration of 2mM.

Purification of chymopapain

$(NH_4)_2SO_4$ fractionation. The following steps, based on the protocol of Baines & Brocklehurst (1979), were performed at 0°C. A 10g portion of dried latex was dissolved in 100ml of water. Cysteine was added to 20mM and EDTA to 1mM, and the solution was left for 15min. Hydroxyethyl disulphide was added to 100mM with stirring, and the mixture was left for a further 15min. The pH was adjusted to 9.0 with 1M-NaOH with stirring over a period of 20min, and the solution was centrifuged at 11000*g* in a MSE 18 centrifuge at 4°C for 30min. The small pellet was discarded, and the supernatant adjusted to pH6.0 with 1M-HCl over a period of 20min. $(NH_4)_2SO_4$ (0.277g/ml) was dissolved in the supernatant, and the mixture stood at 20°C for 1h. The solution was again centrifuged at 11000*g* for 30min, after which the pellet was discarded or used as a source of papain (Baines & Brocklehurst, 1979). The concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 0.472g/ml. The solution was left at 20°C for 1h, centrifuged at 11000*g* and the supernatant discarded. The pellet was dissolved in 25ml of 0.05M-NaOH/acetic acid/1mM-EDTA/2mM-hydroxyethyl disulphide/0.01% NaN_3, pH5.0, and dialysed against three changes of 500ml of the same solution.

Cation-exchange chromatography. Approx. 10mg of the $(NH_4)_2SO_4$ fraction (above) was treated with 3mM-dithiothreitol and applied to the cation-exchange (Mono S) column of the f.p.l.c. system, which had been equilibrated with the stock buffer diluted to 0.30M-Na^+ with 1mM-disodium EDTA. The proteins were eluted with the same gradient as for the chromatographic analysis of latex, up to 0.60M-Na^+. Any proteins still bound to the column were then eluted in the stock buffer. Fractions were again collected in tubes containing hydroxyethyl disulphide. If larger amounts of material were required, several identical runs were done, with collection into a single set of tubes.

Fractions containing chymopapain were combined, diluted with an equal volume of water, treated with 3mM-dithiothreitol for 30min and re-applied to the Mono S column equilibrated with 0.025M-sodium tetraborate (0.05M-Na^+) adjusted to pH9.5 with 1M-NaOH, and containing 1mM-EDTA. The column was then re-equilibrated with the borate buffer (5ml), and a linear gradient (0.01M-Na^+/ml) of NaCl (to 0.20M-Na^+) in the same buffer was developed at a flow rate of 0.5ml/min. Fractions (0.2ml) were collected in tubes containing hydroxyethyl disulphide as described above.

N-Terminal-residue identification

The enzyme (20nmol) was inactivated by reaction with 10mM-sodium iodoacetate after mild reduction with 3mM-dithiothreitol. *N*-Terminal residues were allowed to react with dansyl chloride and identified by t.l.c. on polyamide sheets as described by Gray (1972).

Immunological methods

Antiserum to papain was kindly given by Dr. E. Shapira, The Children's Memorial Hospital, Chicago, IL60614, U.S.A. Other antisera were raised in rabbits by intramuscular injection of 0.5mg of

protein emulsified with complete Freund's adjuvant for the initial injection and with incomplete adjuvant thereafter. Antisera were raised against partially purified chymopapain from f.p.l.c. at pH 5.0 (antiserum A) and the papaya proteinase III (papaya 'peptidase'; see below) peak from an SP-Sephadex chromatogram (Robinson, 1975) (antiserum B).

Double immunodiffusion was in 1% agarose in 20mM-sodium/potassium phosphate buffer, pH 7.2, containing 0.15M-NaCl. Plates were allowed to develop for 24h before being washed overnight in 1% NaCl, dried and stained with Brilliant blue G (Barrett, 1974).

Fused rocket immunoelectrophoresis in 1% agarose/90mM-Tris/80mM-boric acid/3mM-EDTA, pH 8.3, was carried out as described by Svendsen (1973), with 2% (v/v) antiserum A in the gel, with migration towards the cathode. After electrophoresis the gel was washed, pressed and stained as described by Weeke (1973).

Enzyme assays

For assays of hydrolysis of Bz-Arg-$NPhNO_2$, each sample was added to 0.10M-sodium phosphate buffer, pH 6.8, containing 1mM-EDTA and 2mM-dithiothreitol (final volume 0.975ml). The enzyme was allowed to activate for 5min at 40°C before the reaction was started by the addition of 25μl of 100mM-Bz-Arg-$NPhNO_2$ in dimethyl sulphoxide. After 10min at 40°C the reaction was stopped by the addition of 1ml of 0.10M-sodium chloroacetate/0.20M-sodium acetate buffer, pH 4.3. Released 4-nitroaniline was determined by measurement of ΔA_{410}. One unit of activity corrresponded to the release of 1μmol of 4-nitroaniline ($\varepsilon = 8800$ M·cm^{-1})/min under these conditions.

Proteolytic activity was determined with azocasein as substrate. The enzyme was activated in 0.75ml of assay buffer as described above, and the reaction was started by the addition of 250μl of 6% (w/v) azocasein. After 20min at 40°C the reaction was stopped by the addition of 5ml of 3% trichloroacetic acid. The precipitate thus formed was filtered out and the amount of trichloroacetic acid-soluble material was measured as ΔA_{366}.

Active-site titration of cysteine proteinases

The method was adapted from Barrett *et al.* (1982) with compound E-64 as titrant. A 100μl portion of the enzyme solution (5–20μM) was added to 250μl of 0.40M-sodium phosphate buffer/8mM-dithiothreitol/4mM-EDTA, pH 6.8. From 0 to 100μl of 20μM-compound E-64 was then added (11 tubes, 10μl increments) and the mixture was preincubated for 15min at 40°C. Water (prewarmed to 40°C) was added to 0.975ml and reaction was started by the addition of 25μl of 100mM-Bz-Arg-$NPhNO_2$ in dimethyl sulphoxide, and completed as described above.

The molar concentration of active cysteine proteinase thus obtained was compared with the amount of protein present, using $M_r = 28000$ for chymopapain, obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (not shown), and $A_{280,1\%} = 18.3$ (Robinson, 1975), to give a molar absorption coefficient of 51000M·cm^{-1} for chymopapain. The amount of active enzyme was then expressed as a percentage of the total protein.

Identification of the cysteine proteinases of C. papaya latex

Papain was identified immunologically by double immunodiffusion against anti-papain serum. We use the term 'papaya proteinase III' for the proteinase, a component of EC 3.4.22.6, previously called 'papaya peptidase' (IUB Enzyme Nomenclature Committee, 1979*a*), since this enzyme, like papain and chymopapain, is capable of splitting protein substrates (Schack, 1967) and is therefore a proteinase, not a peptidase (IUB Enzyme Nomenclature Committee, 1979*b*). This protein was identified by its position of elution from an SP-Sephadex column (Robinson, 1975) and by double immunodiffusion against antiserum B. The term 'chymopapain' is used to refer to the cysteine proteinase or proteinases eluted after papain and before papaya proteinase III in cation-exchange chromatography of papaya latex at pH 5.0.

Results and discussion

Analysis of spray-dried and fresh papaya latex

Chromatography on the Mono S column of proteins from spray-dried papaya latex yielded three regions of activity against Bz-Arg-$NPhNO_2$ (see Fig. 1*a*) and azocasein (not shown). Papain was only a minor component of the total activity present and, as expected from the work of Baines & Brocklehurst (1979), was eluted quite early (0.18–0.25M-Na^+) on the programmed gradient (region I in Fig. 1*a*). The largest amount of activity was associated with the 'chymopapain region' of the profile, at 0.38–0.57M-Na^+ (region II). This was followed by papaya proteinase III, eluted as a complex peak at 0.66–0.76M-Na^+ (region III).

The fractions comprising the complex series of peaks of activity in the 'chymopapain region' were applied to a fused-rocket-immunoelectrophoresis plate containing antiserum A, and two precipitin lines appeared (see Fig. 1*b*). The lower, darker-staining, line was shown to be due to chymopapain by the fact that it increased in migration distance when chymopapain purified as described below

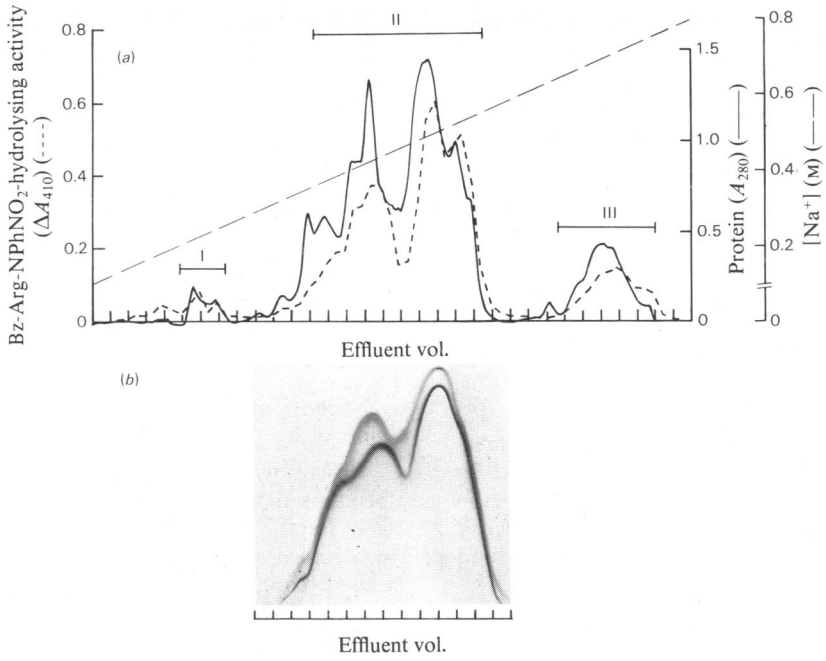

Fig. 1. *Chromatography of proteins from spray-dried latex on Mono S at pH 5.0*

(*a*) Approx. 10mg of protein was applied to the column and eluted with a sodium acetate gradient (0.05–1.0M) as described in the Experimental section. The three regions of Bz-Arg-$NPhNO_2$-hydrolysing activity were identified as follows: I, papain; II, chymopapain; and III, papaya proteinase III (see the text). (*b*) Fused rocket immunoelectrophoresis of column fractions from Fig. 1(*a*) against antiserum A. The lower, darker-staining, line was attributable to chymopapain (see the text).

was added to the wells containing column fractions. The other line represented the contaminating protein, subsequently separated from chymopapain. It can be seen in Fig. 1 that immunologically reactive chymopapain was found throughout the chymopapain region of the chromatogram and corresponded well with activity against Bz-Arg-$NPhNO_2$.

Papain, chymopapain and papaya proteinase III separated by f.p.l.c. on Mono S at pH5.0 gave reactions of non-identity in double immunodiffusion (results not shown).

The chromatographic profile for fresh papaya latex (Fig. 2*a*) was much simpler than that for spray-dried latex; there were fewer protein peaks and fewer peaks of Bz-Arg-$NPhNO_2$-hydrolysing activity. One major peak of chymopapain was eluted at a position corresponding to the latest-eluted chymopapain in spray-dried latex (0.51M-Na^+). Papaya proteinase III also was eluted as a single peak. No peak corresponding to papain activity was found, nor were we able to detect papain by testing crude fresh latex or fractions from the column in immunodiffusion plates with anti-papain serum.

Fractions were applied to a fused-rocket-immunoelectrophoresis plate as described above (Fig. 2*b*). The decrease in amount of earlier-eluted chymopapain compared with spray-dried latex was confirmed, suggesting that some of the earlier-eluted components seen in the dried latex may be artefacts produced during the drying or storage of the latex. This may also apply to papaya proteinase III, which was eluted as a complex peak with specific activity increasing towards the trailing edge of the peak when the source was spray-dried latex, but was present as a single peak in fresh latex. Furthermore, only one form of papaya proteinase III was found by use of immunological criteria, contrary to previous reports based on chromatographic separation and specific activity (Lynn, 1979; Polgar, 1981; Schack, 1967).

Purification of chymopapain from spray-dried latex

On the basis of a value for $A_{280,1\%}$ of 18.3 (see the Experimental section), 10g of spray-dried latex yielded approx. 1.8g of protein in the dialysed chymopapain-enriched $(NH_4)_2SO_4$ fraction (see the Experimental section). By active-site titration the preparation was found to be over 50% active cysteine proteinase (54%, 58% and 63% for three preparations). Activity was stable at 4°C in the presence of 2mM-hydroxyethyl disulphide, losing only 14% of its activity in 13 months.

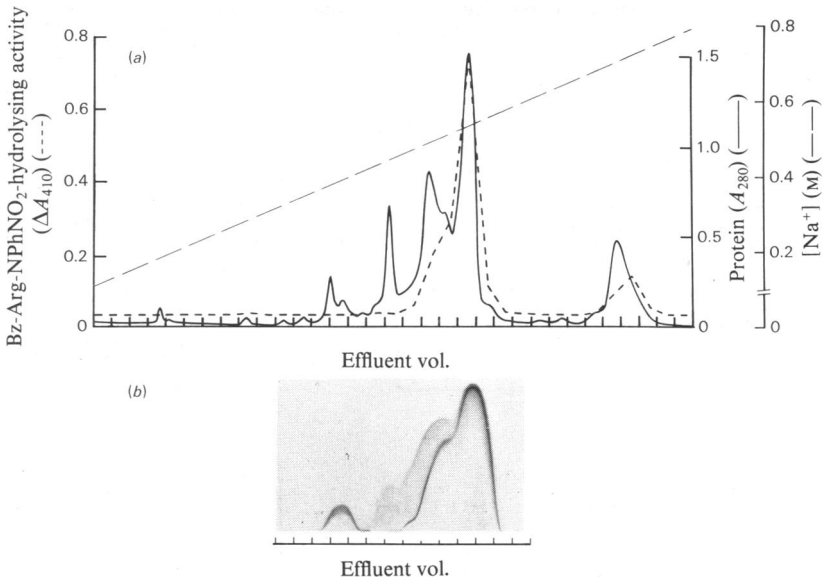

Fig. 2. *Chromatography of proteins from fresh papaya latex on Mono S at pH 5.0*
(*a*) Sufficient latex was applied to the column to give a comparable peak height for the major form of chymopapain in both this and the preceding Figure. (*b*) Fused rocket immunoelectrophoresis of column fractions from Fig. 2(*a*) against antiserum A. The lower, darker-staining, line was attributable to chymopapain.

The $(NH_4)_2SO_4$ fraction (10mg) was applied to the Mono S column and eluted at pH 5.0 as described in the Experimental section. Essentially one peak of active chymopapain, with a wide leading edge, was eluted at the position corresponding to the latest-eluted peak of active chymopapain from spray-dried latex (see Fig. 3*a*). The separation was consistently better when the preparation had been treated with dithiothreitol before chromatography. Papaya proteinase III was again eluted later.

When the fractions containing chymopapain at the highest specific activity (marked by the bar on Fig. 3*a*) were re-run on the Mono S column at pH9.5 (see the Experimental section), three major protein peaks were obtained (Fig. 3*b*); the first contained only a small amount of activity against Bz-Arg-$NPhNO_2$ and azocasein, whereas the second and third showed high activity against these substrates.

Fractions from the three peaks were compared in double immunodiffusion against antiserum A (Fig. 4). Two major antigens were detected. One was present largely in peak I, with a trace present in peak II and thus showed no correlation with enzymic activity. The other was present in all three peaks, and was the only line found in peak III, which had the highest specific activity. This line was therefore considered to be formed by reaction with chymopapain. Peak III was titrated with compound E-64 and found to be almost 100% active cysteine proteinase (98% and 99% with two preparations). This material was designated 'pure chymopapain'.

Table 1 provides a summary of the results of purification of chymopapain and properties of the various fractions. It can be seen that, owing to the abundance of chymopapain in papaya latex, purification to homogeneity involves only a 2.82-fold purification. Despite this, we obtained a very low yield of pure chymopapain, due to the small-scale chromatography employed and because the enzyme occurred in multiple molecular forms that were extremely difficult to separate completely from contaminating proteins. For our purposes, yield was considered of secondary importance when such a rich starting material was available, but the manufacture of chymopapain on a large scale would require a different approach.

Storage of pure chymopapain

Pure chymopapain gave a symmetrical peak of constant specific activity in re-chromatography on the Mono S column at pH 5.0. It was stored for 2 months at 4°C in the absence or presence of the low-M_r disulphide hydroxyethyl disulphide (2mM), which was tested on the assumption that, by reversibly blocking the active-site thiol, it would decrease the risk of autolysis. The enzyme preparations were then rechromatographed under identical conditions. Enzyme stored in the presence of hydroxyethyl disulphide showed the same activity and elution pattern as for enzyme before storage, whereas that stored in the absence of disulphide

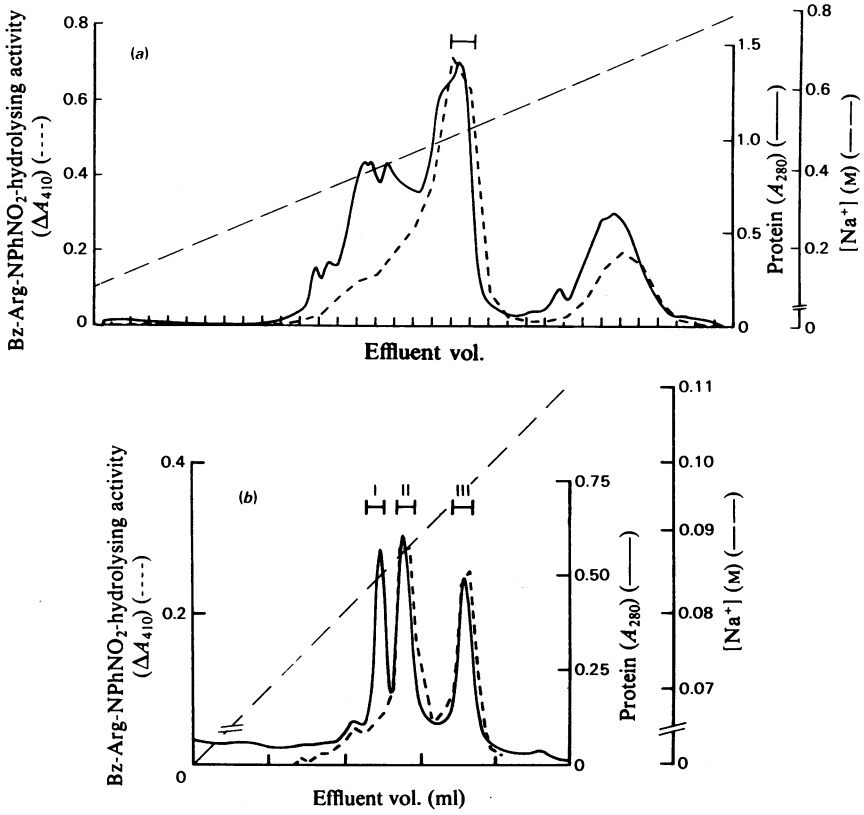

Fig. 3. *Preparative chromatography of chymopapain on Mono S*

(*a*) Chromatography of the chymopapain-enriched $(NH_4)_2SO_4$ fraction with a sodium acetate gradient at pH 5.0. Approx. 10mg of protein was applied to the column. Fractions (0.2ml) corresponding to the bar were pooled and rechromatographed as described below. (*b*) Chromatography of chymopapain from Fig. 3(*a*) with a NaCl gradient in 0.025M-sodium tetraborate, pH9.5. Fractions (0.2ml) corresponding to the bars were pooled separately and analysed further.

showed a 75% loss of activity and the appearance of a new inactive protein peak eluted earlier in the gradient than active chymopapain (not shown). These results support the argument presented above that earlier-eluted forms of chymopapain may be artefacts.

N-Terminal analyses

The three peaks obtained by chromatography at pH9.5 (Fig. 3*b*) were subjected to *N*-terminal-residue analysis as outlined in the Experimental section. The only *N*-terminal amino acid found in peak III, i.e. pure chymopapain, was tyrosine. Both tyrosine and isoleucine were identified in peaks I and II, with isoleucine being predominant in peak I. Tyrosine has been reported previously to be the *N*-terminal amino acid of chymopapain (Baines & Brocklehurst, 1982; Kunimitsu & Yasunobu, 1967; Lynn, 1979; Robinson, 1975), but so has isoleucine (Lynn, 1973). The *N*-terminal amino acid associated with preparations of chymopapain A, glutamic acid (Baines & Brocklehurst, 1982; Ebata & Yasunobu, 1962; Robinson, 1975), was not present in our purified material.

Conclusions

Chymopapain from fresh latex was eluted as chymopapain B. Therefore our results suggest that forms other than chymopapain B are artefacts produced during the commercial preparation of latex. Modifications of chymopapain could include cleavage by an aminopeptidase or proteinase, leading to the appearance of the different *N*-terminal amino acid (glutamic acid) associated with preparations of chymopapain A (see above). Such modifications could produce different elution positions in cation-exchange chromatography and alterations in the environment of active-site residues, some of which, by analogy with papain (Drenth *et al.*, 1976), may be close to the *N*-terminus.

We conclude that there are probably only three cysteine proteinases synthesized by *C. papaya* and present in the fruit latex, namely papain, chymopa-

Table 1. *Purification of chymopapain*

Monitoring the progress of the purification was made difficult by the lack of a specific substrate for chymopapain. Protein was determined by using an $A_{280,0.1\%}$ value of 1.83 to convert A_{280} units into mg of protein. Specific activity was measured by using the unit of activity for Bz-Arg-$NPhNO_2$ hydrolysis defined in the Experimental section. Active sites hydrolysing Bz-Arg-$NPhNO_2$ were quantified by titration with compound E-64 (see the Experimental section). Activity due to chymopapain was that proportion of the activity against Bz-Arg-$NPhNO_2$ eluted from f.p.l.c. at pH5.0 in the chymopapain region of the gradient, as indicated in Fig. 1(*a*). Yield was calculated as the number of units of activity attributable to chymopapain at each step. The purification factor was calculated from the specific activity corrected for the percentage of activity due to chymopapain.

	Volume (ml)	Protein (mg)	Specific activity (units/mg of protein)	Protein (mg)/ active site (μmol)	Activity due to chymopapain (%)	Yield (units)	Purification factor
Spray-dried latex extract	100	6045	0.072	61	76	331	1
$(NH_4)_2SO_4$ fraction	51	1785	0.110	48	61	120	1.23
Chymopapain after pH5.0 f.p.l.c.*	1.6	1.49	0.131	33	100	0.195	2.39
Chymopapain after pH9.5 f.p.l.c.							
Pak II	0.68	0.38	0.150	N.D.	100	0.057	2.74
Peak III	0.52	0.16	0.154	28		0.025	2.82

* Of the $(NH_4)_2SO_4$ fraction, 0.296ml (containing 10mg of protein) was applied to the Mono S column; these and subsequent values correspond to a single column run.

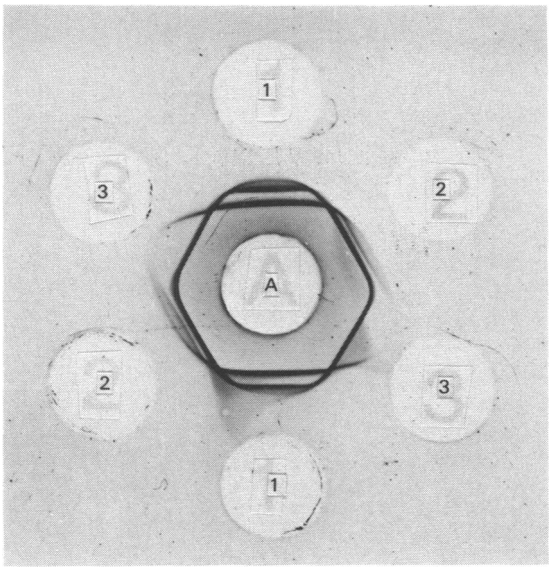

Fig. 4. *Double immunodiffusion of the three peaks separated on Mono S at pH 9.5 against antiserum A*
The wells contained: A, antiserum A; 1, peak I; 2, peak II, and 3, peak III from Fig. 3(*b*).

pain and papaya proteinase III, which are immunologically distinct and are thus the products of different genes. We suggest that future studies include work with material from the fresh fruit, so that artefacts produced during the commercial preparation of latex can be recognized.

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