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CHROMATOGRAPHIC AND ELECTROPHORETIC ANALYSES OF PAPAYA PROTEINASES

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Key Word Index—*Carica papaya*; Caricaceae; paw-paw; proteinase; papain; electrophoresis; FPLC.

Abstract—Although it is known that unripe fruit from *Carica papaya* contains several proteinase enzymes which are used industrially, only one of these, papain, has been extensively characterized. Recently, the separate use of other enzymes of the family has been considered but information on their hydrodynamic properties is contradictory. The use of newer methods of separation has enabled us to separate a proteinase which runs slowly on acidic polyacrylamide gels (papain) from the four other proteinases. The proteinase which runs fastest on acidic polyacrylamide gels has an M_r of 25 k and a pI of 11.0. This latter pI is the same as that of a proteinase which has an M_r of 28 k and runs more quickly than papain but more slowly than chymopapain on acidic gels. We therefore have data showing that the proteinase enzymes from papaya can be classified by pI and M_r into papain (pI 8.75, M_r 23 k), chymopapains A and B (pI 10.3–10.4 or 10.6–10.7 and M_r 24 k), papaya proteinase A (Ω) (pI 11.0, M_r 24 k) and papaya proteinase β (pI 11.0, M_r 28 K).

INTRODUCTION

Latex from the unripe fruit of the papaya (*Carica papaya* L.) plant has been used as a source of proteinase enzymes for industrial use since first reported by Roy in 1872 [1]. The uses of the complex of enzymes include meat tenderizing and prevention of haze formation in beverages. However, there has recently been evidence that one of the proteinase enzymes may have medical importance in the treatment of prolapsed intervertebral discs [2]. Degradation of the proteoglycan of the nucleus pulposus is perhaps the cause of relief in this treatment. We have an interest in using these proteinases to degrade proteins partially to assess the functional properties of the fragments and the possible food implications. In particular, it is known that degradation of the caseins can produce bitter [3] or even opiod peptides [4, 5].

One of the main proteinase enzymes from papaya is papain (EC 3.4.22.2). Papain is one of the most highly studied plant enzymes; the amino acid sequence is documented [6], the crystal structure is resolved to 1.65 A [7], and the mechanistic geometry of the active site has been reviewed [8]. There have been many studies on chemical modification of the enzyme and the subsequent change in various kinetic parameters [9]. Papain is not the major proteinase in the latex of the fruit but it can be easily isolated, purified and crystallized [10]. The major latex proteinase seems to be chymopapain (EC 3.4.22.6) [11] and this general name covers a heterogeneous mixture of chymopapains A and B [12]. Cayle *et al.* [1] fractionated chymopapain into the A and B forms; the N-terminal residues of these two forms were either Glu or Tyr [13, 14]. Initially the M_r of the chymopapains was calculated as 34.5 and 36.4 k [13, 14]. Subsequent to that report the definition of chymopapains has relied on the specific activities with synthetic substrates or thiol group determination and until recently the M_r was thought to be nearer to 28 k than 35 k for chymopapain B. Dispute continued on the number of chymopapains with the identification of another form, chymopapain S [15]. This form of the enzyme had a M_r of 24 700 but the difference between chymopapain S and chymopapain A has been questioned [16]. It was acknowledged that there is difficulty in classifying the chymopapain B (perhaps three isoenzymes) and chymopapain A enzymes [17].

Schack [18] separated a very basic protein (pI 11.1), which he called papaya peptidase A (EC 3.4.22.6). It has been shown that the active site of papaya peptidase is different from that of papain [19].

Although the pIs of papain, 8.75, and chymopapain, 10.1, are sufficiently different to give reasonable separation using acidic PAGE, very few of the reports include this separation step in their work.

We report the separation of the crude latex using an FPLC method which can differentiate all the enzymes, unlike the previous methods [20, 21]. We show that one enzyme has an M_r of 28 k and can be separated on acidic gels from papain, two forms of chymopapain and papaya proteinase A. This new information is coupled with sedimentation data which indicate that the M_r of the chymopapain is 23–25 k and not 23–35 k. The newly described enzyme is similar to papaya proteinase A in its pI but with the highest M_r of the papaya proteinases.

RESULTS

Analysis of spray-dried latex

Chromatography on the Mono Q column using the concentration gradient of sodium chloride shown in Fig. 1 gave a reproducable pattern of protein elution. Care had to be taken to ensure that all the non-protein material had been dialysed away before chromatography. Incubation with dithiothreitol did not alter the pattern obtained. All the peaks shown in Fig. 1 had activity towards HPA and

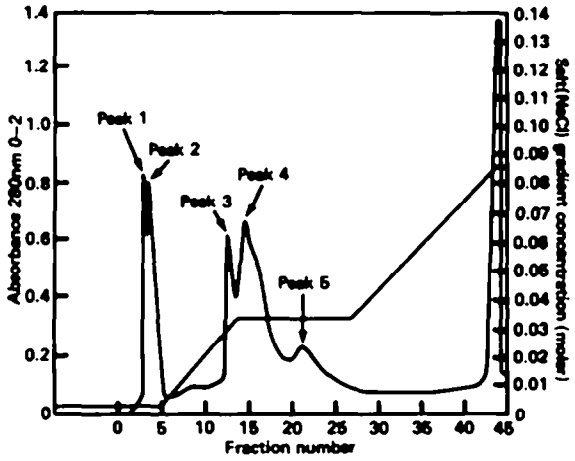

Fig. 1. Separation of proteinases using a Mono Q anion exchange column (HR 515, capacity 1 ml) attached to the Pharmacia Fast Protein Liquid Chromatographic apparatus. A buffer of 1,3-diaminopropane at pH 10.8 was used to elute the proteins with a stepped gradient of sodium chloride. The sodium chloride increased at 6 mM/min to a final concentration of 200 mM. Dried latex (1 g) was dissolved in 5 ml of buffer and after dialysis 100 μl was added to the column. The spray-dried latex varied in the amount of protein in the powder but *ca* 40% was usually protein.

β-casein. Relatively, the more protein eluted, the more the activity. Separation of a papain preparation from Sigma showed two main peaks; these peaks corresponded to peaks 4 and 5 of the spray-dried latex separation. The apex fractions from each peak were electrophoresed on acid gels and these are shown in Fig. 2. Peak 5 travelled the least far on acidic electrophoresis and this was the same as the major peak from the Sigma papain. A M_r of about 23 k was indicated from SDS electrophoresis and this peak contained the papain which is known to have an M_r of 23 406. Papain was always contaminated by other material, and as the concentration of salt increased subsequent to peak 5, a large amount of the eluted material was papain accompanied by protein from peak 4.

Analysis of peak 1 in Fig. 1 by SDS electrophoresis showed that this material had an M_r of 28 k and was not bound to the column. This peak was homogeneous and on an acidic gel ran slower than any other protein except papain (Fig. 2).

The second peak excluded from the Mono Q column contained a heterogeneous mixture of all the peaks; this could be reduced by extensive dialysis before injection but could never be totally removed. The third peak was also homogeneous, like peak 1, and ran fastest on acidic gels (Fig. 2). The M_r determined by SDS electrophoresis was 24–25 k. The large composite peak 4 contained a doublet of protein bands on acidic gels (Fig. 2) and gave an M_r of 25 k.

If the chromatographic separation was heavily overloaded a papain peak was produced which was homogeneous on acidic gels but a large proportion of papain was also associated with other material.

The best separation was achieved by using a shallower linear gradient of sodium chloride in 1,3-diaminopropane (Fig. 3). The first peak, fraction 28, was the second slowest protein on acidic gels and was homogeneous with an M_r of 28 k by SDS electrophoresis. While the majority of the second peak (fraction 30) travelled fastest of all the proteins from this separation on acidic gels, it was slightly contaminated by material from fraction 28. Fraction 45 was the faster of the two peak 4 proteins shown in Fig. 2, and fractions 62–64 contained the slower of these bands. The M_rs were, as mentioned above, between 24 and 25 k. Thus it is possible to use a sequential combination of these two methods if it is required to separate and remove completely all contamination from the proteins. Peak 3 could be run on the shallowest gradient so that fraction 30 consisted of two very similar isoenzymes which were just separable by acidic gel electrophoresis.

We repeated the separation made by Buttle and Barrett [20] (their Fig. 1) and obtained the same fractions. The papain peak was rerun on the Mono Q column and gave a single peak in the area of peak 3 shown in Fig. 1 above. Obviously the majority of papain does not appear

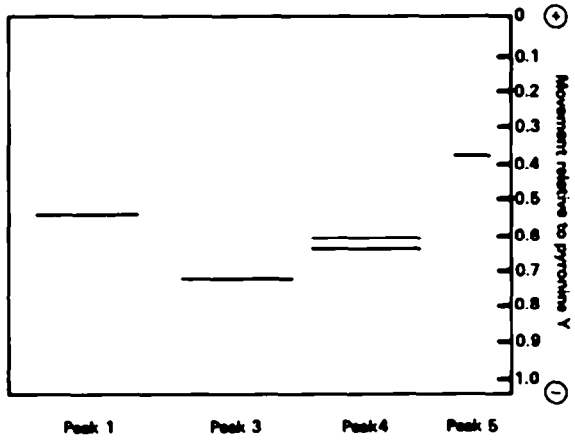

Fig. 2. Separation of fractions from Fig. 1, using 15% acrylamide gel, pH 4.3, towards the anode using pyronine Y as maker. The buffer was β-alanine acetic acid at pH 4.3. Current was 10 mA/track. Peak 2 contained a mixture of proteins when run on the gels.

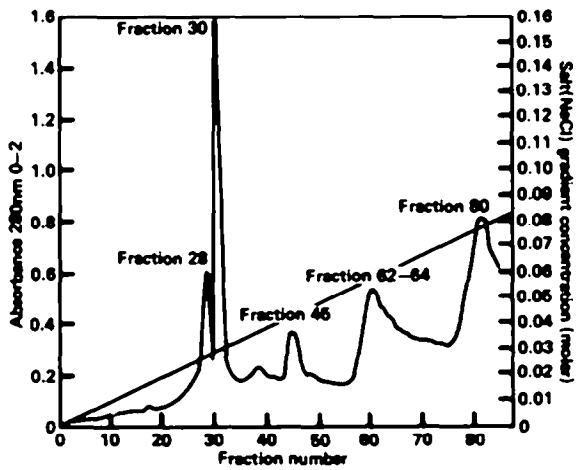

Fig. 3. Separation of proteinases using the Mono Q column mentioned in Fig. 1 but using a gradient of 2.35 mM/min and 500 μl of a 50 mg/ml sample size.

when spray-dried latex is separated on the Mono S system using acetate buffers; it is possible that it either binds to the column and is not eluted under the conditions employed, or it is present in the main peaks. Certainly we found that the main peaks from the Mono S separation were heterogeneous on SDS polyacrylamide gels. In further comparisons between the acidic separation on Mono S and the alkaline separation on Mono Q we found that the small peak eluted just before papaya proteinase on Mono S and had an M_r of 28 k; however, there was also some of this material in the major peaks of the Mono S separation. The main peaks from the Mono S separation corresponded to fractions 30, 45 and 62–64 shown in Fig. 3. We conclude that separation on Mono S does not elute papain from the ion exchange material or completely separate the other proteins.

Isoelectric focussing of the separated proteins

The data displayed in Fig. 4 show that the range of pI values obtainable using the pH 9–11 ampholytes is 7.8–11.4. The numbers on the graph indicate fractions from Fig. 3. The protein from fraction 28 had a pI of 11–11.1, as did the proteins from fraction 30. Fraction 45 had a pI of 10.7 and fractions 62–64 had pIs of 10.3–10.4, two clear bands being visible. Fraction 80 had a pI of 8.7–8.9. As well as measurements by a flat pH electrode, small samples of the gel were extracted with water and the pH was measured. Cytochrome *c* and lentil lectin were used as standard calibration proteins.

Sedimentation analysis of the proteins

Protein from fractions 45 and 62 was centrifuged in a Beckman model E centrifuge and the M_r calculated. $\bar{v}$ was calculated as 0.728 using a Pharr density meter and the concentration was determined by a synthetic boundary centrifugation. The M_r of the protein was 23 k and was the average of three different concentrations of protein.

N-Terminal analysis of the proteins from Mono Q separation

N-Terminal analysis of the dansylated protein from peak 5 in Fig. 1 or fraction 80 in Fig. 3 indicated that the amino acid was isoleucine. Peak 3 in Fig. 1 or fraction 30 in Fig. 3 had leucine as the N-terminal amino acid. The fourth protein peak and fraction 45 had glutamate as the N-terminal residue.

Digestion of β-casein by the protein fractions

The data in Fig. 5 show that the digestion of β-casein by the separate protein fractions is similar. There appeared to be ten separate fragments produced by each of the four proteinases.

DISCUSSION

The use of FPLC has greatly assisted in the general separation of enzyme and protein mixtures which are difficult to resolve. In the case of the commercially important papaya proteinases, recent papers [20, 21] have raised some important questions about the hydrodynamic properties of the enzymes. When we repeated the published separations, the content of papain appeared to be very low and the main peaks of separation, although easily reproducible, were heterogeneous on both 'native' and SDS polyacrylamide gels. Theoretically, the use of an alkaline buffer system rather than the acid system normally used [20] should lead to the highest pI proteinase not being bound to the column and eluting before the

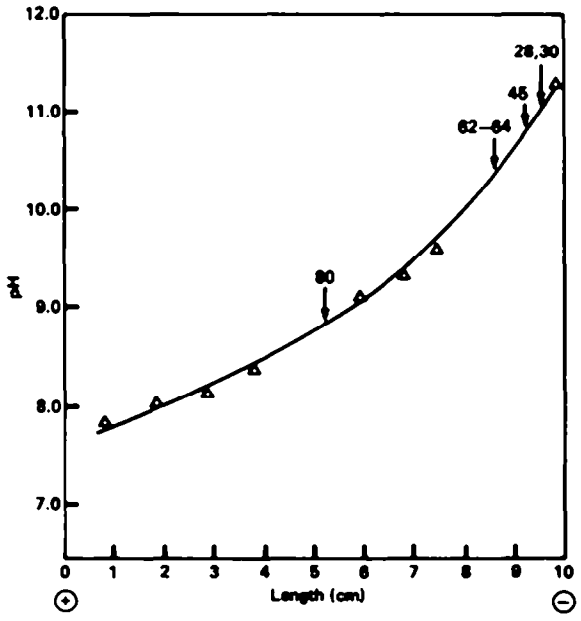

Fig. 4. Isoelectric focussing of the proteinase fractions over the pH range 7.8–11.4 100 μl of sample was placed on paper wicks just 2 cm from the anode. Bands of protein were visualized with Coomassie Blue R250.

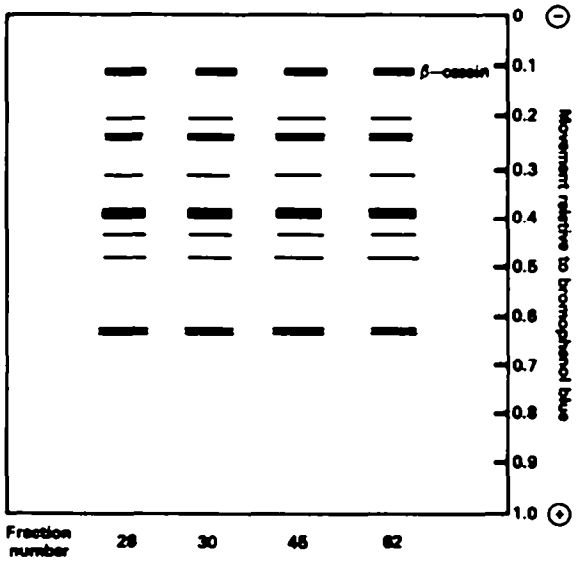

Fig. 5. Separation of fragments of β-casein after 1 min of digestion by the proteinase fractions from Fig. 3. Separation was towards the anode, the gel was 15%, pH 8.3. Total protein was 100 μg/track and the current was 3 mA for 2 hr. Staining was by Coomassie Blue R250.

gradient begins. Subsequently the chymopapains should elute early in the gradient followed later by papain. Papain will be positively charged in this system and the chymopapains will either be positively charged or just at their isoelectric point. The high pI proteinase will be negatively charged.

The original name for the proteinase with a high pI was papaya peptidase A and there seems to be general agreement, because of the proven proteinase activity, that the name should now be papaya proteinase 3, A [22] or papaya proteinase Ω. The opinion of Barrett and Buttle [22] is that the chymopapain group should be considered as one enzyme with multiple chromatographic forms, but this is opposed by Brocklehurst *et al.* [23], who identify two distinct families of chymopapain.

There is a large and fundamental difference between the work based on cation ion exchange FPLC separations [21] and the results reported here. The data of Zucker *et al.* [21] indicated three proteinases all with M_rs of 25 k. It would seem that very few other reports on these enzymes combine ion exchange chromatography, acidic gel electrophoresis and pI determination with M_r studies. We have combined these measurements and have clearly been able to identify the fractions which correspond to papain and papaya proteinase 3 or A (Ω). These two enzymes have also been characterized both by pI and N-terminal analyses. It seems clear that in all previous work these two enzymes have been easily separated and there is no dispute over their status. In addition, we have identified three proteinase molecules which are separable by acid gel electrophoresis and also by denaturing gel electrophoresis into high- and low-M_r forms. The data displayed in Fig. 4 show that the higher-M_r protein (28 k) has a pI of 11, whereas the other, lower-M_r forms have pIs of 10.3–10.7 and an N-terminal of glutamic acid.

The main historical difficulties which have arisen in the nomenclature may be due to the poor separation of the three enzymes which are not papain or papaya proteinase A or 3 (Ω). Brocklehurst and Salih [24] claim that there are four components in this group of proteinases. The first to be eluted from a sulphopropyl-Sephadex column was chymopapain A followed by three similar forms of chymopapain B [24]. In a study in 1981, Polgar indicated that a slowly moving protein in acidic gels should be given a different name than chymopapain [25]. This was generally rejected [16]. However, confusion over the M_r and relative mobility in both ion exchange and acidic polyacrylamide gels is still apparent concerning the chymopapains.

We have clearly been able to determine that there are three proteinase molecules other than papaya proteinase A or 3 (Ω) (pI 11 and 24 k) and papain (pI 8.75 and 23 k). One of these is peak 1 in Fig. 1 and fraction 28 in Fig. 3. This protein has a pI of 11.0, an M_r of 28 k and travels above chymopapains A and B and papaya proteinase Ω but below papain on acidic polyacrylamide gels. This enzyme is homogeneous and does not appear as multiple forms. This protein may well be the cause of the confusion over the M_r [20, 21] varying between 25 and 28 k. It is the only enzyme over 25 k in mass and with its high pI should not be called chymopapain. It is almost certainly the papaya proteinase B mentioned by Polgar [25], and we have shown for the first time that this enzyme has a greater M_r and pI than the chymopapains.

In our results, peak 4 (Fig. 1) and fraction 45 (Fig. 3) have a pI of 10.6–10.7 and an M_r of 24 k. This corresponds to chymopapain B whilst fractions 62–64 (Fig. 3) have a pI of 10.1–10.4 and an M_r of 24 k and correspond to chymopapain A. In acidic gels these fractions are clearly separated. When ion exchange chromatography is overloaded, these fractions exhibit closely separated multiple peaks but the same bands as previously described appear on acidic gel electrophoresis.

These data assign hydrodynamic properties to the three distinct families of proteinase molecules from papaya in a way which has not been done before. One of the high pI enzymes does not fit into the chymopapain family and should be seen as a fourth papaya proteinase.

We did not use the two protonic state probes reported by Brocklehurst as the data have obviously been difficult to reproduce in different laboratories [22] but we are preparing a monoclonal antibody to the 28 k proteinase, in the anticipation that an antibody to the region not found in the other molecules can be prepared. It is further intended to use the antibody to purify unactivated 28 k proteinase in sufficient quantities for future detailed mechanistic studies.

EXPERIMENTAL

Materials. Commercial spray-dried papaya latex was obtained from Powell and Scholefield (Liverpool, U.K.). The FPLC was supplied by Pharmacia. The chemicals were supplied by Sigma Ltd.

Chromatographic analysis of spray-dried papaya latex. A series of programmed linear gradients was used for chromatography on the Mono Q column. Plots of $A_{280\,nm}$ vs. gradient composition were corrected for the vol. between the pumps and UV detector, including column vol.

A pH 10.8, 20 mM buffer was prepared by adding 1.91 ml 1,3-diaminopropane to distilled H_2O and adjusting the pH with HCl. Spray-dried latex was applied to the column after dissolving in a small vol. (10 ml) of 1,3-diaminopropane, pH 10.8, and extensive dialysis against 5 l. of the same buffer overnight. There was no indication of loss of total activity during dialysis. The proteins were eluted by using a linear gradient of (a) 0–0.35 M NaCl followed by a period of 0.35 M NaCl and finally a linear gradient from 0.035–0.2 M NaCl, and (b) a linear gradient from 0.02 M NaCl, increasing at 0.00235 M NaCl/min. These systems should elute papaya peptidase A first (pI of 11.0) followed by chymopapain at pI 10.5 and finally papain at pI 8.5.

PAGE. Slabs of polyacrylamide (14 × 16 cm × 1.5 mm) were prepared according to the method of Maurer [26]. The separation gels were usually 15% acrylamide at pH 4.3, the spacer gel was 3% at pH 6.7, and the number of slots for samples was adjusted to be either 5, 10 or 20. Buffer in both the upper and lower electrode chambers was β-alanine–HOAc at pH 4.3. The separation was towards the cathode and pyronine Y was used as the tracking dye. Gels were stained with 0.005% Coomassie Blue R250 dissolved in 25% *iso*-PrOH and 10% HOAc; gels were destained using hot 10% HOAc. In some cases, the gels were restained with an Ag stain [27].

Separation gels at 15% were used without a spacer but with 0.1% SDS in the gel for determining relative M_r. The pH of the gels was 8.3 and the upper and lower buffer chambers contained Tris–glycine buffer, pH 8.3, with SDS at 0.1%. Separation was towards the anode and the standards were lysozyme (14.3 k), β-lactoglobulin (18.4), trypsinogen (24.0 k) and pepsin (34.7 k).

Isoelectric focusing in polyacrylamide gels. Slabs of polyacrylamide (12 × 25 cm × 3 mm) were prepared using 5% acrylamide and pH 9–11 ampholytes in the mixture. Standards in the gel were cytochrome *c* (pI 10.25), trypsinogen (pI 9.3) and lentil lectin

(pI 8.65). The pH was determined in the gel after electrofocusing for 2 hr either by using an electrode with a flat base or by dividing the gels into small slices, macerating them in distilled H_2O and determining the pH. Gels were stained with Coomassie Blue R250 after the pH profile had been determined.

N-Terminal analysis. The identity of the N-terminal residues was determined by the dansylation technique as described by Woods and Wang [28].

Protein determination and assay proteinase activity. Protein was determined by the method of Bradford [29]. Proteinase activity was assayed by the release of blue dye from Hide Powder Azure. Incubations were at 25° for 1 min at pH 6.0. Incubations of β-casein at 1 mg/ml with 1 μg of enzyme were for 1–2 min. The pH in both systems was 6.0 using maleate buffer and the enzyme preparations were activated by L-cysteine before use.

Sedimentation analysis of the protein. The protein from some FPLC peaks were each used for sedimentation analysis, using the model 'E' Beckman ultracentrifuge. The temp. was 17° (290 k) and the rotor speed was 40 000. The formula of Yphrantis [30] was used to calculate the molecular mass: $M = (RT(1 - \bar{V}\rho)\omega^2)\sigma$, where R is the gas constant = 8.314×10^7, T is the absolute temperature, ω is the angular velocity, $\bar{V}$ is the partial specific volume of the solute, ρ is the density of the solution and σ is the effective reduced molecular weight.

Protein density measurements. A Pharr density measuring cell [31] was used to obtain the density of protein solns which were then used for ultracentrifugation.

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