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PURIFICATION OF PROTEINASES FROM *CARICA PAPAYA*

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Key Word Index—*Carica papaya*; Caricaceae; pawpaw; chromatofocusing; cysteine proteinases; dried fruit latex; fresh non-fruit latex.

Abstract—A method is detailed for the fractionation and purification of cysteine proteinases from the latex of *Carica papaya*. The main advantages of this method are its ability to resolve complex mixtures based on small differences in the isoelectric points of the individual components, the amount of soluble material (*ca* 500 mg) which can be loaded, with quantitative recovery, and, unlike other high resolution analytical techniques, the lack of need for expensive equipment. The profile of proteinases is compared between unprocessed dried latex and fresh non-fruit latex, and possible reasons for the observed differences are discussed.

INTRODUCTION

The dried latex obtained from the fully grown unripe fruit of *Carica papaya* (pawpaw) contains a number of cysteine (thiol) proteinases. In order of increasing basicity they are: papain (EC 3.4.27.2); a mixture of enzymes known collectively as the chymopapains (EC 3.4.27.6); and two very basic proteins, papaya proteinase A (also called peptidase A, peptidase II, proteinase III and proteinase Ω) and papaya proteinase B [1–4].

Papain has been extensively studied in terms of its enzymatic mechanism [5] and was the first plant protein to have its three-dimensional crystal structure elucidated [6]. The remaining papaya proteinases have received less attention. Various methods for the fractionation and purification of the proteinase components of papaya latex have been described. These range from the use of ion exchange chromatography [7], to affinity methods using agarose mercurial columns [8] and activated thiol Sepharose 4B [9]. Recently FPLC methods have also been applied to the papaya proteinases [3, 4]. These techniques exhibit shortcomings associated with low yields, non-reproducibility and poor fractionation of the enzyme components of papaya latex. In particular there is widespread disagreement as to the exact number of chymopapains present in papaya latex. In order to attempt to resolve these, and other problems concerning the biology of the papaya latex proteinases, we have embarked on a programme to produce cDNA and genomic clones coding for the individual papaya proteinases [McKee *et al.*, submitted for publication]. In parallel with these studies we have used an alternative technique to fractionate and purify, in appreciable amounts and reasonable cost, the papaya proteinases. The method is based on the technique of chromatofocusing, which allows separation of proteins in terms of their isoelectric points in a column chromatographic system characterised by self-generated pH gradients giving focusing effects producing sharp, well-separated bands and high resolution. The proteins are further purified following ammonium sulphate fractionation and ion-exchange chromatography.

Using these methods we have isolated the papaya cysteine proteinases from processed and unprocessed dried fruit latex, and compared these with the components of fresh non-fruit latex.

RESULTS AND DISCUSSION

The soluble component (up to 500 mg protein) of dried papaya latex (Sigma, unprocessed latex from fruit) was chromatographed on a chromatofocusing column as described in the Experimental (Fig. 1). The most basic enzymes are eluted first, papaya proteinases A and B, and these are followed by a heterogeneous mixture of less basic proteins, the chymopapains, representing from three to five separate components. Papain, the least basic papaya cysteine proteinase, is eluted last and is essentially pure at this stage.

The activity profile for Fig. 1 reveals that there are several fractions containing relatively small amounts of protein which have a high activity towards L-BAPNA, especially towards the lower pH values. Examination of fractions showing high activity on acid, non-denaturing PAGE (not shown) indicates that these fractions contain proteins with the characteristics of papain. It is possible that the differences in pI are due to processing of these proteinases during drying and storage.

The profile of processed dry fruit latex (Sigma chymopapain) run under the same conditions, shows several qualitative differences compared with whole dried latex (Fig. 2). There is virtually no papain present in processed latex; also a reduction occurs in the overall amount of the first peak proteins, proteinases A and B, as well as a reduction in the protein components occurring between the first peak and the chymopapain fractions. Prior to further purification on a CM-Sephadex C-25 column, proteinases A and B are first separated by bringing to 60% saturation with ammonium sulphate. After dialysis, proteinases A and B, as well as chymopapains and papain, are eluted from the ion-exchange column with a 0–1.2 M sodium chloride gradient (Fig. 3).

There are three main chymopapain peaks from the chromatofocusing columns of treated and untreated latex

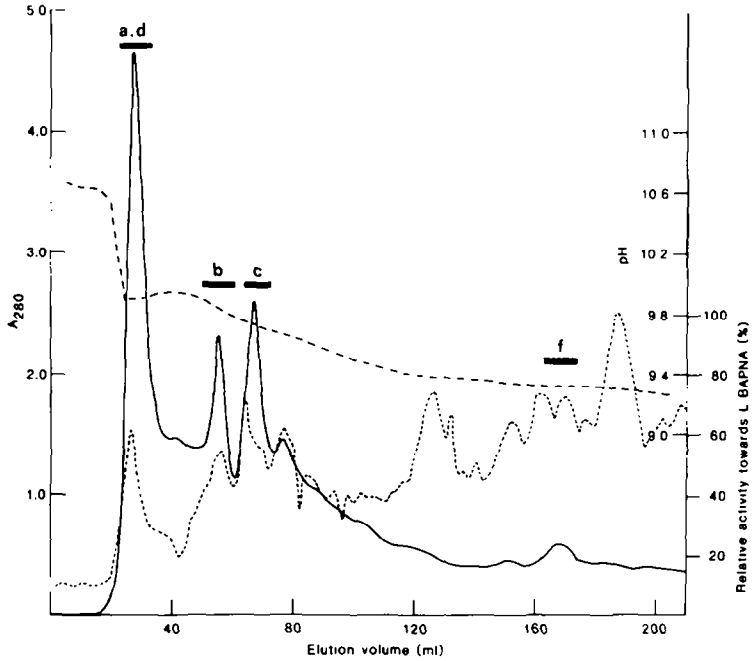

Fig. 1. Elution profile of Polybuffer exchanger PBE 118 chromatography of soluble component of dried latex from *Carica papaya* fruit (10 ml in H_2O). (— — —) pH gradient; (——) A_{280}; (- - - - - -) relative activity towards L-BAPNA, the highest activity being taken as 100%. Two ml fractions were collected. Fractions indicated by horizontal bars were processed further.

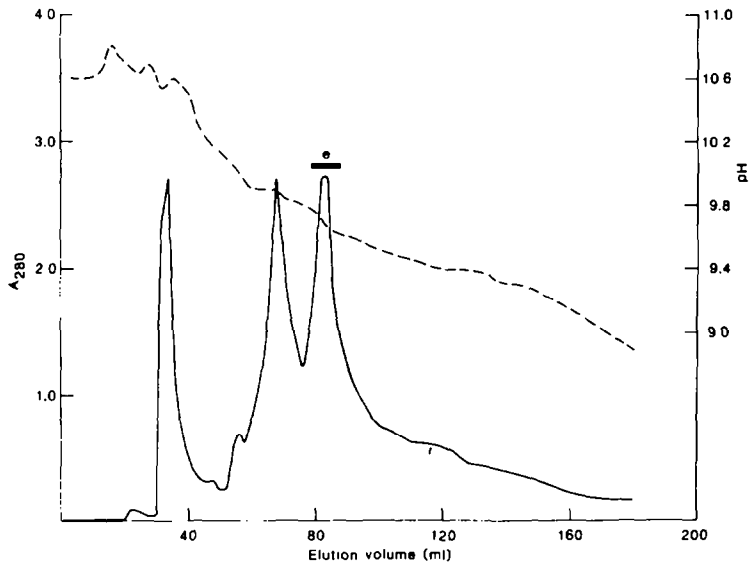

Fig. 2. Elution profile of PBE 118 chromatography of soluble component of processed dried latex from *Carica papaya* fruit (10 ml in H_2O) (Sigma chymopapain). Symbols as Fig. 1. One ml fractions were collected. Fractions indicated by horizontal bars were processed further.

and these are shown in Fig. 3b, c and e. Peaks from (b) and (c) are homogeneous as judged by electrophoresis on continuous acid, non-denaturing acrylamide gels; (e) however, appears to be a mixture of at least two proteins (Fig. 4). The other peaks in Fig. 3, proteinases A and B (d and a), as well as papain (f), run as single bands on the above gels. Determination of the specific activity of the enzymes in Fig. 3 towards L-BAPNA, shows papain to be the highest at 427 nkat/mg; proteinase A 27 nkat/mg; chymopapain (Fig. 3b) 9.4 nkat/mg and proteinase B 3.3 nkat/mg.

Two of the proteins, papaya proteinase A and chymo-

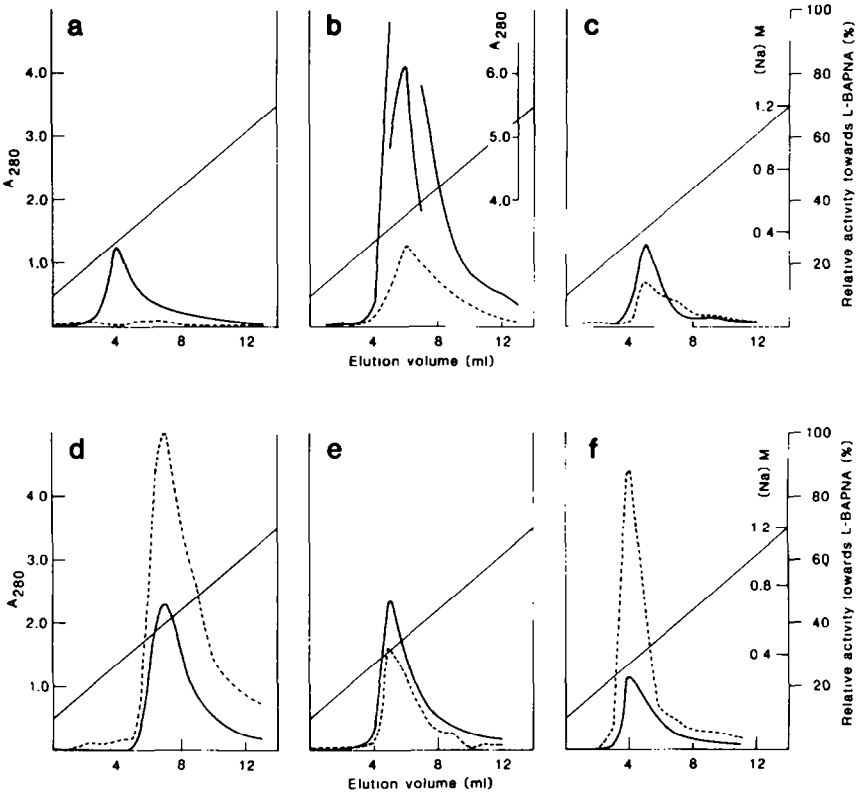

Fig. 3. Elution profiles of CM-Sephadex C-25 of fractions indicated in Figs 1 and 2. (——) NaCl gradient; (——) A_{280}; (- - - - - -) relative activity towards L-BAPNA, the highest overall activity being taken as 100%. One ml fractions were collected.

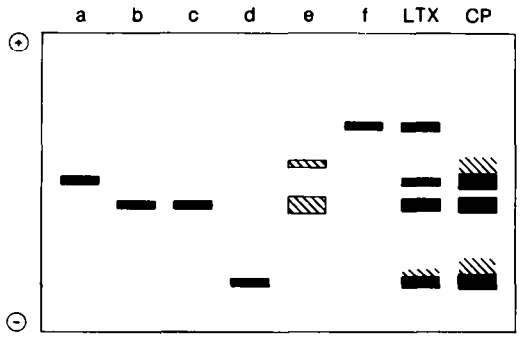

Fig. 4. Electrophoretic pattern of proteins obtained from CM-Sephadex C-25 fractions a–f, and soluble components of processed (chymopapain, CP) and unprocessed (latex LTX) dried latex from *Carica papaya* fruit; ▬ darkly staining; ▨ diffuse staining.

A.

[a] L P E N V D W R K K G A V T P V R = Q G

[b] L P E N V D W R K K G A V T P V R

B.

[a] Y P Q (E) A I D W R A K G A V T P V K N Q G A = E

[b] Y P E S I D W R A K G A V T P F K R V P D S G E C Y

Fig. 5. A, Comparison of the known *N*-terminal amino-acids of papaya proteinase A. [a] From this study; [b] from Lynn and Yaguchi [10]. B, Comparison of the known *N*-terminal amino-acids of chymopapain. [a] From this study; [b] from Lynn and Yaguchi [10]. = represents unidentified amino acids. The sequence data presented in this study were determined, using automatic methods, at the SERC facility at Leeds University.

papain (Fig. 3 b and d), were amino acid sequenced at the *N*-terminus (Fig. 5). The results show that the sequence obtained for proteinase A matches exactly that obtained by Lynn and Yaguchi [10]. Chymopapain however, differs in a number of residues from the published partial sequence of chymopapain [10], neither does it match any other known papaya proteinase sequence, including a chymopapain sequenced by Yaguchi [Lynn, Watson and Yaguchi, personal communication]. This is not surprising as there are several proteins present in the 'chymopapain' component of papaya latex, and now confirmed by partial amino acid sequencing. Furthermore a derived amino acid sequence from a partial cDNA clone constructed in this laboratory may represent another chymopapain from papaya [McKee *et al.*, submitted for publication]. The above results are at odds with those of Buttle and Barret [3], who propose, on the basis of immunological evidence, that there is only one chymopapain (chymopapain B), and

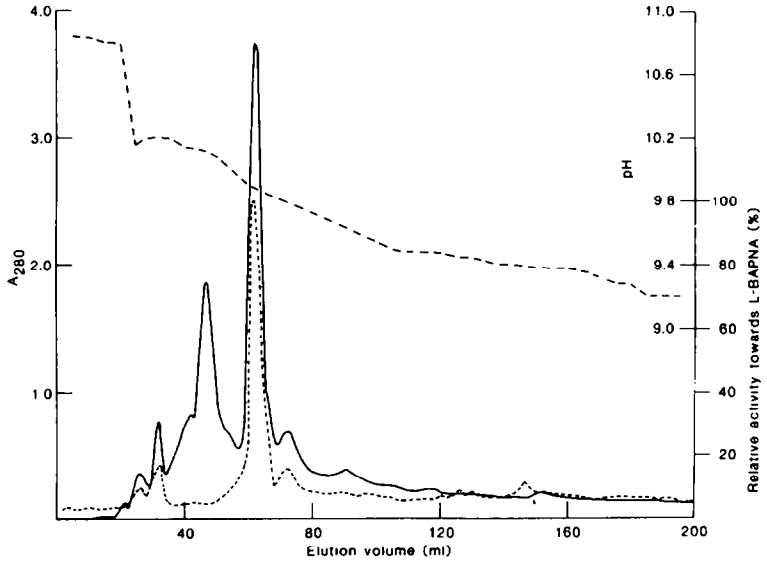

Fig. 6. Elution profile of PBE 118 chromatography of soluble component of fresh non-fruit latex from growing *Carica papaya* plants. Symbols as in Fig. 1. Ten ml of sample in H_2O was loaded into the column, and 1 ml fractions were collected.

that the other observed proteins are artefacts induced either by post-translational modification, or processing of commercial latex preparations. The present work, and also that of Brocklehurst *et al.* [4], argues strongly against there being only a single chymopapain present in papaya latex.

The chromatofocused profile of fresh non-fruit latex collected from growing plants is shown in Fig. 6, and comparison with the profile for dried fruit latex clearly shows a number of differences. The major component in non-fruit latex is the chymopapain fraction, and again several chymopapains are observed, one of which is active towards L-BAPNA. The amounts of proteinases A and B, which are a major component of dried fruit latex, are greatly reduced in non-fruit latex, as is the proportion of papain.

These observed differences in proteinase components may be due to one or a number of factors. There may be inherent differences between fruit and non-fruit latex, although this seems unlikely as similar profiles have been observed comparing fresh latex from fruit and other parts of the plant (data not shown). There may also be differences between fresh and dried latex brought about by the drying and subsequent processing. The dried fruit latex used in these studies has been untreated, although the exact drying procedure is unknown. It should be feasible to investigate the effect of different drying procedures on fresh latex, from whatever source, and determine the subsequent chromatographic profile. Finally, differences between *Carica papaya* varieties in terms of the individual latex proteinases may occur. To our knowledge this aspect has not been investigated, although it may be reasonable to assume that such differences will exist. We have found large differences in the overall proteinase patterns comparing non-fruit latex from *Carica papaya* and *Carica cauliflora* (data not shown), and it has been shown that different forms of the related cysteine proteinase, ficin, occur in different *Ficus* species.

It is our contention that the only realistic way to determine the number and diversity of the papaya latex cysteine proteinases is to investigate the phenomenon at the DNA level, and work in our laboratory is continuing towards this goal.

EXPERIMENTAL

Materials. Enzymes and reagents were purchased from Sigma. Papain, Type I; crude dried papaya latex; chymopapain; partially purified; L-BAPNA, $N\alpha$-benzoyl-L-arginine-4-nitroanilide.

Growth of plants. Seeds were obtained from shop-bought papaya fruit (paw-paw) (countries of origin Brazil and Kenya), or from Chiltern Seeds, Bortree Stile, Ulverston, Cumbria, U.K. The outer gelatinous coat was removed and the seeds soaked in numerous changes of distilled H_2O for 2–3 days. Seeds were further washed under running H_2O for 2–3 days, sown in potting compost and germinated at 25° in the dark. After germination the seeds were potted up and transferred to a controlled environment growth cabinet. Growth conditions were as follows: temp. 25° day and night; 12 hr day/night cycle; 100% relative humidity; irradiance, 200–250 μmol/m^2/sec. After 10–12 weeks the plants were moved to a greenhouse, on a 16 hr day/8 hr night cycle, with supplementary lighting at constant 20°. Plants were harvested (latex collected) at 24–30 weeks old.

Sample preparation. Latex was collected from repeated cuts along the leaf base, petioles and main stem using a sterile razor blade. The latex was collected into tubes at 0°. Fresh non fruit latex and spray dried latex was diluted 1:1 with sterile H_2O, and homogenized in a ground glass homogeniser. After centrifugation for 10 min at 10000 rpm in a Sorvall SS-34 rotor the supernatent was dialysed extensively against H_2O. The resulting dialysate was stored at −20° until use.

Chromatofocusing. Polybuffer exchanger (PBE) 118 was equilibrated with start buffer, 0.025 M triethylamine–HCl, pH 11.0, and degassed before loading onto a 1 × 35 cm column (bed vol. 28–30 ml). The gel was packed at a flow rate of 95 ml/hr

and pre-run overnight at 28 ml/hr. Immediatley prior to sample loading, 5 ml of degassed elution buffer (7.5 μmol/pH unit/ml Pharmalyte pH 8–10.5) equilibrated to pH 8.0, was run onto the column. The degassed sample (10 ml in H_2O) was eluted with the same buffer. Each 1 ml or 2 ml fraction was monitored at 280 nm. The pH of every fifth fraction was determined. Every other fraction was assayed for enzyme activity.

Ion-exchange chromatography. Samples equilibrated with 0.05 M glycine pH 8.6 were chromatographed on a 0.5 cm × 9 cm CM-Sephadex C-25 column with a linear gradient of 0–1.2 M NaCl in the same glycine buffer. Fractions (1 ml) were monitored at 280 nm and assayed for enzyme activity using L-BAPNA.

Enzyme assays. Each sample (0.2 ml) was added to 0.97 ml of 0.05 M Tris, pH 7.5, containing 2 mM EDTA and 5 mM cysteine. The enzyme was allowed to activate for 5 min at 35°. The reaction was started by the addition of 30 μl of 0.1 M L-BAPNA in DMSO. After 15 min at 35° the reaction was stopped by the addition of 0.2 ml of 30% HOAc. Released *p*-nitroaniline was determined by *A* measurement at 410 nm.

Gel electrophoresis. Protein samples were analysed on polyacrylamide gels using the methods detailed in ref. [11].

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