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ORIGINAL ARTICLE

Papaya latex enzymes capable of detoxification of gliadin

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Abstract Assay of fractions obtained from ion exchange chromatography of papaya latex on CM Sephadex-C50, size exclusion chromatography on Sephacryl S-300 and size exclusion HPLC have provided an insight into the relative contributions of the gluten-detoxifying enzymes present. This outcome has been achieved by the use of the above chromatographic techniques, coupled with assays of lysosomal activity, protease activity using benzylarginine ethyl ester (BAEE) as substrate, prolyl endopeptidase (PEP) using glycylprolylnitroanilide and a prolidase assay using acetylprolylglycine. These procedures have shown that the activity in papaya latex is due largely to caricain and to a lesser extent, chymopapain and glutamine cyclotransferase. The presence of caricain and these other enzymes was confirmed by mass spectrometry of trypsin digests of the most active fraction obtained by CM Sephadex-C50 chromatography and size exclusion HPLC. Fractions rich in caricain would be suitable for enzyme therapy in gluten intolerance and appear to have synergistic action with porcine intestinal extracts.

Keywords Gluten intolerance · Enzyme therapy · Gliadins · Papaya · Caricain

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Introduction

The broad aim of previous work was to characterise the enzyme present in the normal population but which is missing in individuals with gluten intolerance such as in coeliac disease. This enzyme is present in the intestinal mucosa and is able to detoxify wheat gliadin in vitro (Cornell 2005). A pig intestinal extract has been found to be of value in ameliorating the symptoms of coeliac disease in a clinical trial, thus providing further evidence for this deficiency (Cornell et al. 2005). Cornell and Stelmasiak (2007) proposed a "Unified Theory of Coeliac Disease" in which all of the mechanisms of tissue damage may be explained in terms of a missing or defective enzyme in the small intestine which is required for digestion of gluten proteins to the point where they are no longer toxic, as is the case in those who do not suffer from this disease.

These authors also showed that extracts of intestinal mucosa from the cow and sheep achieved high levels of detoxification of gliadin in vitro (Cornell and Stelmasiak 2004). The findings are consistent with the observations that other mammals do not suffer from coeliac disease and that the only useful animal intestinal mucosa for studies of toxic proteins in this disease is from foetal animals (Auricchio et al. 1984).

The work of Cornell and Rivett (1995) and Cornell (1998) on comparisons of coeliac and normal mucosal digestion indicated that there was defective digestion of toxic synthetic peptides, based on A-gliadin structures, in the case of remission coeliac mucosa. Amino acid analysis of the undigested residues, coupled with the evaluation of toxicity in vitro (Cornell and Mothes 1993, 1995) suggested the necessity of a prolidase type of enzyme for cleaving peptide bonds on the N-terminal side of proline as shown:

$X_{\uparrow}PY$, requires an oligoiminopeptidase (not a prolinase or iminodipeptidase). This is in contrast to enzymes that attack peptides containing proline on the C-terminal side, such as prolyloligopeptidase (Szeltner et al. 2000). An example is prolyl endopeptisdase (PEP) that can be isolated from microbial sources (Szwajcer-Dey et al. 1992) as well as from various animal tissues.

To be useful in the detoxification of gluten, an enzyme must be able to attack the key motifs associated with the toxicity of the three main cereals known to be toxic to individuals with coeliac disease. The motifs, showing points of attack, are:

$QQQ_{\uparrow}PF_{\uparrow}P$, $QQQ_{\uparrow}PY$ and $PQQ_{\uparrow}PY$ (McLachlan et al. 2002). This would define the enzyme as a prolidase. These key motifs are only associated with toxicity when they form part of larger peptides such as peptide 77–84 of A-gliadin (QQPYPQPQ) and peptide 11–19 of A-gliadin (QNPSQQQPQ), the latter sequence being found only in wheat. The residues which remain after digestion of related peptides with remission coeliac intestinal mucosa are mainly octapeptides with the motifs intact. They are present in larger amounts than the smaller peptides which remain after digestion by normal mucosa, suggesting there is a deficiency of a peptidase in coeliac disease that targets the toxic peptides.

For the present studies, it was decided to examine the possibility of using enzymes from other than animal sources as palliative agents. The one which sprang immediately to mind was crude papain from carica papaya. Messer et al. (1964) postulated that this crude preparation contained an enzyme capable of detoxifying N-terminal glutaminyl peptides by converting them to N-pyrrolidone carboxylyl peptides. There was limited evidence that crude papain was useful in a preparation which could be administered orally to patients with coeliac disease to lessen the severity of the symptoms (Messer and Baume 1976). It was postulated that the active enzyme in crude papain was glutamine cyclotransferase (QCT). Hence, it was decided to evaluate the various enzymes present in papaya latex using methods based on chromatographic techniques and designed to yield fractions for evaluation of in vitro activity and ultimately, confirmation of their identity by mass spectrometry.

Crude papain (as dry papaya latex) contains many types of proteolytic enzymes and other impurities. Several other thiolhydrolases are present, such as chymopapain and caricain, as well as QCT (Dubey et al. 2007). The possibility of endopeptidases and prolidases being present was also investigated. Molecular weight, pH optimum, selective enzyme assays and mass spectrometric fragmentation analysis of enzymic digests have all been used to provide a clearer picture of the most active fractions suitable for enzyme therapy.

Materials and methods

Lysosomal activity of plant enzymes

It was important to this project to determine if there were enzymes, other than those in pig, cow and sheep, which had potential for enzyme therapy in coeliac disease. The major emphasis was the assessment of plant enzymes using the rat liver lysosomal assay. Samples of each enzyme (2 mg in 0.2 mL PBS taken from a solution of 0.10 g enzyme/10 mL PBS) were pre-incubated with 0.10 mL of a solution of a peptic-tryptic-pancreatinic (PTC) digest of wheat gliadin (50 mg/mL in 0.15 M NaCl) for 2 h at 37°C. A suspension of rat-liver lysosomes (0.10 mL) was then added and the mixtures allowed to further incubate at 37°C for 1.5 h. After this time PBS (3 mL) was then added and the absorbance at 410 nm measured in a spectrophotometer. Blank experiments without the incubations (zero-time controls) were also run as were controls without any pre-incubation of enzyme with PTC digest. All tests were carried out in duplicate. The protection index (P%) was then calculated as described previously (Cornell and Townley 1973). All chemicals for buffers and reagents throughout this work were of AR quality.

Synergistic action of different enzymes

Mixtures of two different enzymes were tested in the rat liver lysosomal assay to determine if their combined efficiency in detoxification of gliadin was greater than either enzyme used alone.

The enzyme under test (0.06 g) was dissolved in PBS (10 mL) and 0.2 mL aliquots tested using the rat liver lysosomal assay. Comparisons were made against mixtures of 0.1 mL of two different enzyme solutions and calculation of protection indices were made as described previously. All tests were carried out in duplicate.

Size exclusion chromatography on Sephacryl S-300

A column (88 × 2.2 cm) of Sephacryl S-300 (Pharmacia, Sweden) was equilibrated with phosphate buffer 0.05 mol/L, containing 0.12 mol/L sodium chloride at pH 5.2. Dry papaya latex MG50000 (Enzyme Solutions Pty Ltd, Melbourne, Australia) (1.2 g) was dissolved in this buffer (10 mL) and applied to the column, followed by 2 careful additions of buffer (10 mL total) before connecting the buffer to flow at 30 mL/h. Fractions of 15 mL were collected beginning at the void volume of the column up to the total volume of the column monitoring by the use of UV absorbance at 280 nm (on diluted aliquots).

Each fraction was assayed for activity in the lysosome assay (see "Lysosomal activity of plant enzymes") and the

PEP assay (see "Enzyme assays") after adjustment of the pH to 7.5 and 7.0, respectively with 0.5 mol/L sodium hydroxide. Fractions were selected for the BAEE assay (see "Enzyme assays") and dialysed against distilled water and freeze-dried.

Molecular weights of the major protein fractions were estimated from plots of Kav against molecular weight using dextran blue, human gammaglobulin, bovine serum albumin and trypsin inhibitor as standards.

Three runs were carried out on papaya latex.

Ion exchange chromatography on CM Sephadex

A column (3.2 × 20 cm) of CM Sephadex C-50 (Pharmacia, Sweden) was equilibrated with 0.02 mol/L monosodium dihydrogen phosphate adjusted to pH 4.6 in a cold room at 5°C. A sample of dry papaya latex, MG50,000 (2.5 g) in starting buffer (30 mL) was applied to the column which was run at 180 mL/h and the eluate from the column monitored for protein at 280 nm. After the unabsorbed material was obtained, the pH was increased by application of a pH 6.5 phosphate buffer (0.02 mol/L) comprised of monosodium dihydrogen phosphate and disodium monohydrogen phosphate. A linear salt gradient was then applied from 0.1 to 0.3 mol/L sodium chloride, followed by a buffer containing 0.8 mol/L NaCl. Fractions corresponding to the peaks at 280 nm were collected, dialysed against distilled water (×3 changes) and freeze-dried.

Fractions were assayed in the rat liver lysosome assay (see "Lysosomal activity of plant enzymes") and in the BAEE, PEP and prolidase assays (see "Enzyme assays"). Three runs were carried out on the papaya latex. Fractions 3 and 4 were also subjected to tests for heat resistance by heating in a water bath to 85°C over 15 min. in PBS and determining the change in lysosomal activity (see "Lysosomal activity of plant enzymes"). It has been reported that QCT has a high resistance to heat (Zerhouni et al. 1998) but so also has caricain (Ikeuchi et al. 1998).

pH optima

The pH optima for papaya latex and fractions from size exclusion chromatography were focussed on the ideal pH values for pre-incubation of the gliadin digest and the enzyme preparation. This was readily achieved using a set of phosphate buffers (pH 4.0–9.5) in which the enzyme was dissolved (0.1 g/10 mL) and used with a suspension of lysosomes in 0.154 mol/L sodium chloride. The gliadin digest was made without buffer, as normal, but aliquots were adjusted by the addition of 0.3 mol/L NaOH and 0.3 mol/L HCl as required. Normal incubation times of 2 h were employed throughout. Dry papaya latex concentrations of 0.1 g/10 mL of buffer and fractions 10 mg/mL of buffer were used. P values were calculated as described previously (see "Lysosomal activity of plant enzymes").

High performance liquid chromatography (HPLC)

Preparative HPLC on a column (300 × 7.8 mm) was carried out on an Agilent Technologies (USA) model 1100 instrument with injection of Fractions 3 and 4 from CM Sephadex C-50, using the size exclusion medium Bio Sep SEC-S2000 (Phenomenex, USA). Elution was carried out in 0.05 mol/L phosphate buffer, pH 6.8 in the isocratic mode, at 0.5 mL/min monitoring at 214 nm. These two most active fractions derived from papaya latex were chromatographed, first at a loading of 0.3 mg, then increasing to 3.0 mg for assay purposes (see "Lysosomal activity of plant enzymes").

Enzyme assays

PEP assays

PEP was assayed by the action of this enzyme on Z-Gly-Pro-4-nitroanilide (Fluka, Switzerland) from which coloured 4-nitroaniline is released and measured in a spectrophotometer at 405 nm (Kocna and Frič 1980). The enzyme was detected in the pig intestinal extracts and it was important to determine if it was a contributor to the lysosomal activity of the papaya latex fractions.

Suitable aliquots of undialysed fractions (0.4–0.8 mL), adjusted to pH 7, were mixed with a solution of Z-Gly-Pro-4-nitroanilide (0.4 mmol/mL) in PBS containing 10% v/v 1,4-dioxane (2.5 mL) and allowed to react at room temperature, measuring the absorbance at 405 nm at the start and after each min. over a period of 5 min. Activity at 30°C was calculated from the formula.

$$\text{Activity} = \frac{\text{Increase in absorbance/ min} \times 0.46}{\text{Mass of sample (mg)}}$$

Freeze dried samples (10 mg) were dissolved in pH7 phosphate buffer (0.10 mol/L) and tested in the same way allowing the specific activity to be calculated. All assays were carried out in duplicate.

BAEE assays (Arnon 1970)

Enzyme activity was carried out using benzoyl arginine ethyl ester (BAEE) as substrate (80 mg) and measuring the release of acid by titration with 0.01 mol/L sodium hydroxide, maintaining the pH at 6.3 and temperature at 20°C. Units of enzyme activity were calculated as μ mol of acid released/min/mg sample.

Prolidase assays

Prolidase assays were carried out using acetyl prolylglycine as substrate, measuring acid released at 40°C by titration with 0.01 mol/L sodium hydroxide, maintaining the pH at 6.3. Again, freeze dried fractions of 10–20 mg were used, each dissolved in distilled water (3 mL) and substrate (5 mg) dissolved in distilled water (3 mL).

Electrophoretic separations

Comparisons of electrophoretic patterns of the fractions obtained from the chromatography of porcine intestinal extract and papaya latex were made in order to work towards obtaining bands which could be identified by mass spectrometry with the highly purified enzyme in each case.

Agarose

Electrophoresis was carried out initially in 0.1 mol/L phosphate buffer (pH 7.5) for 1 h at 150 V, using agarose gel (Robyt and White 1987).

SDS-PAGE

Several electrophoretic runs were then made using polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). The method used was based on that of (Laemmli 1970) with the following modification:

The running buffer (pH 8.3) consisted of Tris (3.03 g), glycine (14.4 g) and SDS 1.0 g dissolved in Milli Q water and made up to 1 L. Gels were run in a Bio-Rad Criterion gel tank over a period of 1–1.5 h at 150 V using the dye front as indicator. Protein molecular weight markers of 10–250 kD (10) were used for reference. Staining was carried out with Coomassie Brilliant Blue. All other details were provided by the AfCS Procedure Protocol PP000000002, version 1, 24 April 2002 of Invitrogen Inc. (USA).

Mass spectrometric (MS) analysis

MS analysis of trypsin-digested fractions from chromatography of papaya latex on CM Sephadex C-50 and subsequent HPLC were carried out on an Agilent 6520 Q-TOF LC/MS (Agilent, Palo Alto, USA) coupled to an Agilent 1100 LC system and run under standard conditions in ESI positive mode. The scan range acquired was 100–3,000 m/z.

The chromatographic separation was on Zorbax Poroshell 5 μm SB300-C18 with column 1 × 75 mm, using a gradient of acetonitrile in water (20 to 60% B in 30 min) at 0.25 mL/min.

Trypsin digestion was carried out with diphenylcarbamyl chloride (DCC) treated trypsin (ICN, USA) using 0.5 mg of sample, dissolved in 0.05 mol/L ammonium bicarbonate (1 mL) containing 12 mmol/L calcium chloride, treated for 20 h at 37°C with 2% trypsin on weight of substrate.

The mass spectra of the fragments were analysed using a Mascot data base.

Nano-liquid chromatography-mass spectrometric analysis

Excised gel sections from SDS-PAGE were digested with trypsin (0.25 μg) and the resultant digests were concentrated to ~10 μL by centrifugal lyophilization (Savant, USA) in readiness for electrospray-ion trap (ESI-IT) tandem mass spectrometry (MS/MS) (LCQ-Deca, Finnigan, USA). Protein digests [~10 μL in 1% (v/v) formic acid] were transferred into 100 μL glass autosampler vials and analysed by nano-reversed-phase-HPLC (Agilent Model 1100 capillary HPLC) using a butyl-silica (C4) 150 × 0.15 mm I.D. RP-capillary column (Vydac^{TM}-C4MS, 5 μm, 300 Å Vydac, USA) developed with a linear 60 min gradient from 0 to 100% B with a flow rate of 0.8 μL/min at 45°C. Solvent A was 0.1% (v/v) aqueous formic acid and Solvent B was 0.1% aqueous formic acid/60% (v/v) acetonitrile. The capillary HPLC was coupled on-line to the ESI-IT mass spectrometer for automated MS/MS analysis of individually isolated peptide ions.

Database searching

Acquired MS/MS spectra were searched against the non-redundant protein subset database (Ludwig NR_subset) using the Mascot(tm) search algorithm (v2.1, Matrix Science, UK). Searches were conducted with carboxymethylation of cysteine as a fixed modification (+58 Da), variable oxidation of methionine (±16 Da), and the allowance for up to three missed tryptic cleavages.

Results and discussion

The use of crude papain

Dry papaya latex MG50,000 was shown to have high activity in the rat liver lysosome assay, making it a good starting material for further purification. Hence chromatography was then used as an important way of determining what types of enzymes were responsible for this activity.

Rat liver lysosomal assays on a number of commercial enzymes indicated that they had less activity than papaya extracts. Next to papaya latex, the highest activity of all the

Table 1 Protection indices for enzymes evaluated in the rat liver lysosomal assay for their ability to detoxify gliadin

Enzyme	Source	Protection index (*P* %) (means of 2 determinations)
Papaya latex	Enzyme solutions	93
Aspergillus oryzae	Enzyme solutions	29
Fungal protease	Enzyme solutions	7
Aspergillus niger	Enzyme solutions	14
Bromelain	Enzyme solutions	64
Crystalline papain	Sigma, USA (P4762) 2× crystallized	7
Chymopapain	Sigma, USA chromat Purified (C8526)	46
Prolyl Endopeptidase[a]	Seikagu, Japan (Product 26260)	35

All enzymes were tested at 2 mg/mL concentration except the PEP, which was a highly purified product and used at 0.8 mg/mL

[a] From *Chryseobacterium meningosepticum*

Table 2 Enzymes contributing to 'in vitro' activity of papaya latex in detoxifying gliadin (rat liver lysosome assay)

Enzyme	Classification (EC number)	Approximate molecular weight (kDa)
Procaricain[a]	3.4.22.30	40
Chymopapain	3.4.4.11	36
Prolyl endopeptidase	3.4.21.26	35
Glutamine cyclotransferase	2.3.2.5	30
Caricain[b]	3.4.22.30	25
Papaya proteases[c]	3.4.22.2 (papain)	23
Autolysis products	–	10–25

[a] An endopeptidase with pro region (Groves et al. 1996)

[b] Contributing the highest activity in assay

[c] The major protein components

Table 3 Results of some combinations of crude enzymes to test for synergistic activity

Enzyme 1	*P* (%)	Enzyme 2	*P* (%)	Combination *P* (%)
Pig intestinal extract	61	Papaya latex	71	92
Pig intestinal extract	48	Papaya latex	54	68
Bromelain	21	Papaya latex	62	61

The protection index (*P*) from the rat liver lysosome assay was used as the indicator. Each enzyme evaluated at 6 mg/mL in mixture of two and also at 12 mg/mL separately. Results are the means of two determinations

enzymes tested was bromelain from pineapple. Chymopapain is another peptidase in papaya latex which is measured by the BAEE assay. However, a sample of purified enzyme had less activity than papaya latex in the lysosomal assay and does not appear to be the major contributor to detoxification of gliadin.

Several other plant-derived enzymes have been tested in the lysosomal assay including crystalline papain, chymopapain, bromelain and *Aspergillus niger* and *Aspergillus oryzae*. These were all less protective than the papaya latex. The lysosomal activities of the plant-derived enzymes, which measure their ability to protect the rat liver lysosomes against damage by the gliadin digest, are shown in Table 1.

The experiments henceforth focussed upon crude papaya latex and bromelain as the most important source of enzymes comparable to the activity of the enzymes in the porcine extract. Bromelain has pharmacological properties, some of which are dependent upon its proteolytic activity (Maurer 2001).

The papaya enzymes of importance to this project are shown in Table 2, together with their molecular weights. It was important to determine their relative contributions to activity in the rat liver lysosomal assay.

Pure PEP showed much lower lysosomal activity at the level determined in papaya latex (5.3×10^{-4} U/mg) indicating that other enzymes were largely responsible for the activity of papaya latex and the porcine extract, the latter containing PEP at a level of 6.7×10^{-4} U/mg.

The activity of pure papain was quite low, in agreement with the work of Messer et al. (1964) who showed that crude papain, but not pure papain, was responsible for detoxification of gliadin. However, these authors thought that QCT was responsible for the detoxification process. The present work indicates that it is only a contributor to this process.

Synergistic action of different enzymes

Synergism was most noticeable with mixtures of the pig extract and papaya latex whereby the lysosomal activity of a mixture of equal parts by weight of each enzyme is greater than that of the same total mass of each product tested separately (refer Table 3). The reason for this may well lie in the different mechanisms of the active enzymes—the active pig enzyme selectively splitting toxic peptides on the N-terminal side of proline residues and the enzymes in papaya latex having much broader specificity, such as the cysteine proteinases.

Thus the toxic peptides from A-gliadin residues 11–19 (QNPSQQQPQ) and 75–86 (RPQQPYQPQPQP) would benefit from the action of both a proline-specific hydrolase in the pig intestinal extract and enzymes of broad specificity in the papaya latex. With each product, the reactions bring about detoxification of oligopeptides such as peptides

Table 4 Activity of fractions of papaya latex on Sephacryl S-300

Fraction no	Approximate molecular weight (kDa)	RLL assay (% *P*)	PEP assay (U/mg, 30°C)	BAEE assay (U/mg, 20°C)
1		8	–	–
2		1	Not detectable	–
3		8	Not detectable	–
4	40	58	2.8×10^{-5}	–
5	35	64	1.8×10^{-4}	0.20
6	30	99	1.8×10^{-5}	0.38
7	25	100	2.7×10^{-5}	0.58
8	10	75	0.9×10^{-5}	1.10
9		29	Not detectable	0.55
10		19	Not detectable	0.18
Papaya latex	–	81	5.3×10^{-4}	3.5

Column (88 × 2.2 cm). Elution buffer 0.05 mol/L phosphate containing 0.12 M NaCl, pH 5.2. Papaya latex applied (1.2 g) in buffer (10 mL). Fr 4 may contain procaricain, Fr 5 represents the peak of PEP activity, Fr 6 and 7 represent peak of lysosomal activity due to caricain, Fr 8 represents peak of BAEE activity

11–19 and 75–86 of A-gliadin or their products of partial digestion. When the enzymes are combined, complementary mechanisms bring about a more complete detoxification. There was some synergy with bromelain and papaya latex, but not with pig intestinal extract. Generally, there was no significant synergism detected in combinations of the plant-derived enzymes.

Size exclusion chromatography

Sephacryl S-300 size exclusion (SE) chromatography at pH 5.2 in 0.05 M phosphate buffer containing 0.12 mol/L NaCl, yielded one major peak due to protein, two peaks of high lysosomal activity and one peak of PEP activity. The higher molecular weight fraction had highest activity while the lower molecular weight fraction had less activity and contained most of the PEP activity (refer Table 4).

Pooled fractions of high molecular weight were compared against those of lower molecular weight for optimal pH of lysosomal activity. The indications were that the high molecular weight fraction exhibited two peaks of activity, one at pH 6.5 and a broader peak at pH 7.0–8.5. The lower pH optimum probably corresponds to papaya peptidases, whilst the higher pH optimum may correspond to caricain and PEP (optimal pH approx. 7) and QCT (optimal pH 8.5). The low molecular fraction showed highest lysosomal activity at lower pH values (pH 5.5–6), suggesting mainly papaya proteases were responsible for its activity. However, further experiments are required before a separation of the three main types of enzymes is able to be achieved. It was obvious that the Sephacryl S-300 was useful in separating some components, particularly the PEP. We should always allow for some components being retarded on the column. Thus, only estimates of molecular weights of the various species were able to be provided. Other columns were run in phosphate buffered saline at pH 7.6, but retardation of some component at this pH was more noticeable.

Size exclusion chromatography of papaya latex, a starting material favoured as a useful alternative to the porcine extract, has indicated the presence of at least two active enzymes in the lysosomal assay. Both of these enzymes have molecular weights less than the active enzyme in the porcine extract, ranging from, on present estimates, about 10 kDa (perhaps autolysis products) to 40 kDa. Apart from PEP, these enzymes may correspond to cysteine hydrolases such as caricain. In humans with coeliac disease, the missing enzyme is likely to be related chemically to the porcine prolidase.

Ion exchange chromatography

Chromatography on CM Sephadex C-50 showed that the major part of the lysosomal activity was contained in two fractions—one eluted at low concentrations of sodium chloride (<0.3 mol/L) at pH 6.2 and the other at higher concentrations of sodium chloride (refer Fig. 1). Table 5 shows the results of the lysosomal assay and other enzyme assays on the dialysed freeze-dried fractions obtained.

Attention has focussed on Fraction 4 because it has the highest specific activity in the lysosomal assay (168 U/mg) obtained by dividing the *P* value by the mass of sample (in mg) used in the assay. Fraction 3 contributes a significant proportion of the total activity recovered, although its specific activity is only about 70 U/mg.

The BAEE assay has only been helpful in measuring proteases which are involved in digestion of gliadin but which play a less significant role in detoxification. In both the porcine extract and papaya latex, these proteases are not enriched in the fractions of high lysosomal activity from CM Sephadex. In size exclusion chromatography, they appear at higher levels in the lower molecular weight fractions instead of those fractions with peak lysosomal activity.

Protease activity by BAEE assay of 3–4 U/mg has been determined in the papaya latex and 0.6 U/mg in the pig intestinal extract. The papaya proteases hydrolyse a number of different types of peptide bonds and are commonly assayed by the hydrolysis of BAEE. There is little doubt that they are contributing to the detoxification of gliadin, since a significant part of the BAEE activity of papaya latex is associated with fractions of high lysosomal activity obtained by ion- exchange chromatography on CM Sephadex and by Sephacryl S-300 size exclusion chromatography.

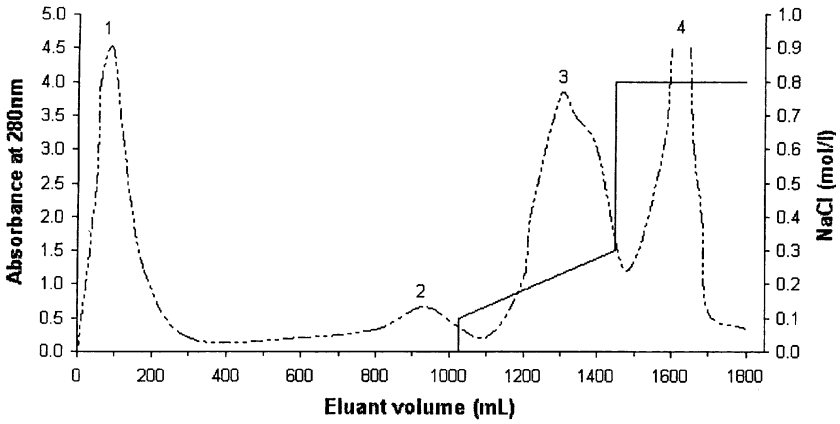

Fig. 1 Elution profile of papaya latex (2.5 g) when chromatographed on CM Sephadex. C-50 showing UV absorbance and application of phosphate buffer containing different concentrations of sodium chloride as shown. Fraction 1 was obtained in the starting buffer pH 4.6), Fraction 2 when eluate increased to pH 5.4–6.2 and Fractions 3 and 4 at pH 6.5. For details see "Ion exchange chromatography on CM Sephadex"

Table 5 Activity of fractions of papaya latex from chromatography on CM Sephadex C-50 (3.2 × 20 cm)

Fraction no.	Yield (%)	Elution conditions	Rat liver lysosome assay *P* (%)	BAEE assay (U/mg)	PEP assay (U/mg)
1	9.4	Unabsorbed	40	0.72	5.4×10^{-5}
2	2.5	Change of pH	53	0.77	5.9×10^{-5}
3	20.0	0.1–0.3 mol/L NaCl	70	0.82	7.8×10^{-5}
4	15.1	0.8 mol/L NaCl	84	1.20	7.8×10^{-5}
Papaya latex	–	–	80	3.6	5.3×10^{-4}

Standard buffer 0.02 mol/L phosphates, pH 4.6. Dry papaya latex (2.5 g) in standard buffer (30 mL)

1, rat liver lysosome assay used 2 mg in assay tube; 2, specific activity of Fraction 4 was 168 U/mg compared with 35 U/mg for papaya latex; 3, all assay results are means of duplicates

Prolidase activity of 0.01 U/mg was detected in the papaya latex and 0.025 U/mg in the pig intestinal extract. These figures are fairly low for this type of assay, but calculations of the acetic acid released from acetyl prolylglycine appear to be significant, especially for the fractions of increased lysosomal activity. For example, ion exchange chromatography of the pig intestinal extract resulted in an increase in prolidase activity to 0.06 U/mg, in the active salt-elutable fraction. This type of activity has been reported previously, but only for dipeptides. In the case of the papaya latex, Fractions 3 and 4 also were higher in prolidase activity (0.04 and 0.05 U/mg, respectively) indicating some enrichment in this enzyme.

Fraction 4 displayed a higher resistance to heat than Fraction 3, retaining 85% of lysosomal activity compared with 50% for the latter fraction. This observation is consistent with it containing caricain, chymopapain and QCT, all of which have good heat stability (Ebata and Yasunobu 1962; Ikeuchi et al. 1998). Furthermore, all are eluted at high concentrations of salt in cation exchange chromatography because of their basic nature (Dubey et al. 2007).

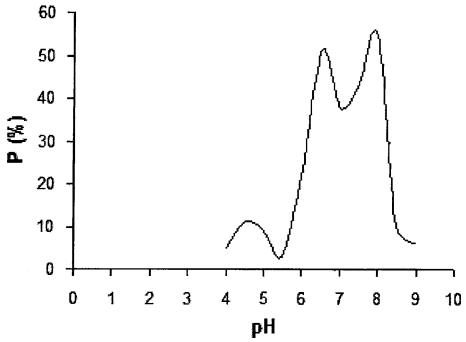

Fig. 2 Graph showing activity of papaya latex in the lysosomal assay at different pH values. Two peaks of high activity at pH 6.6 and 8 were obtained. For details see "pH optima"

pH optima

The supplier of the purified papain (Sigma, St Louis, USA) lists the optimal pH for this product as 6.0–7.0. However, with the lysosome assay, two pH maxima in the lysosome assay were observed with papaya latex—one at pH 6.6 and the other, a broader band peaking at pH 8 (refer Fig. 2). For the enzymes suspected of being present, the first peak may correspond to several enzyme including the cysteine hydrolases of papaya, like caricain and chymopapain, the pH optima of these enzymes being listed in the literature as 6.8 (Ikeuchi et al. 1998) and 7.2 (Ebata and Yasunobu 1962). The second and broader peak activity is probably due to QCT reported as pH 8.5 (Dubey et al. 2007). It must be pointed out, however, that the activity between these two peaks is still very significant.

Further experiments on Fractions 3 and 4 from ion exchange chromatography showed that Fraction 3 had the lower pH optimum of pH 6.9 and Fraction 4 had a broad peak form pH 7.0 to 8.5, thus confirming that in the fractions of highest activity, this activity was due to caricain and QCT.

Experiments with fractions from size exclusion chromatography on Sephacryl S-300 were not so convincing,

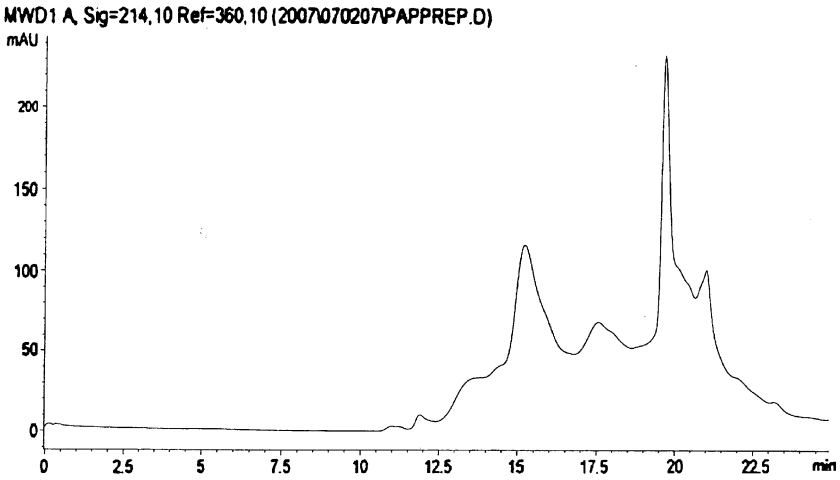

Fig. 3 HPLC profile of Fraction 4 (0.3 mg), the most active fraction from CM Sephadex C-50 chromatography of papaya latex, on Bio Sep SEC-S2000 in 0.05 mol/L phosphate buffer (pH6.8). For details see "High performance liquid chromatography (HPLC)"

but there was a tendency for the lower molecular weight fraction (20–25 kDa) to have a lower pH optimum compared with the higher molecular weight fraction (30–35 kDa), suggesting that the papain itself (23.4 kDa) and other related cysteine proteases are the main enzymes present, whereas the chymopapain, QCT and PEP are mainly in the higher molecular weight fraction with caricain present in both. Cysteine proteases all exhibit pH optima in the range 5.0–8.0 (Dubey et al. 2007).

HPLC of CM Sephadex fractions

HPLC of the two most active fractions from CM Sephadex gave the following results. Fraction 3 gave two major peaks of absorbance quite close together, followed by two other much smaller peaks. Peak activity was associated with fractions with retention times beginning at 17.5 min (30 kDa) to 19 min (25 kDa), inclusive of the two major absorbance peaks.

HPLC of Fraction 4 gave two major peaks of absorbance with again, two smaller peaks (refer Fig. 3). The most active fractions were those which eluted at about 17–17.5 and 19–19.5 min, the latter corresponding to the major peak of absorbance, indicating molecular weights of active enzymes in the range 30–35 and 23–26 kDa, respectively. Thus the active principles in both fractions 3 and 4 from CM Sephadex chromatography after HPLC have molecular weights which are consistent with caricain, chymopapain and QCT. It is suggested that Fraction 3 is higher in papain proteases/proteinases, while Fraction 4 (the most basic proteins) is rich in caricain with some chymopapain and QCT, accounting for the higher activity and somewhat higher heat stability of the latter fraction. A summary is presented in Table 6.

The use of HPLC as a method of further purifying active fractions of papaya latex appears to be very promising for sequence studies. The examination of enzyme activity

Table 6 Size exclusion HPLC of active fractions from CM Sephadex chromatography of papaya latex

Fraction	Tubes	t_R (min)	Molecular weight (kDa)	Activity P (%)[a]
3	3	17.0	35	44
	4	17.5	30	60
	7	19.5	20	42
	10	21.0	15	33
	14	23.0	10	22
4[b]	4	15.0	45	79
	7	17.0	35	100
	8	17.5	30	100
	10	18.5	28	48
	11	19.0	26	71
	12	19.5	23	71
	14	20.5	20	64
	17	22.5	12	33

Loading, 3 mg; column, Biosep SEC-S2000 (300 × 7.8 mm); buffer, 0.05 mol/L phosphate buffer, pH 6.8; collection, tubes of 0.20 mL

[a] means of duplicate determinations

[b] Range of molecular weight by MS is 12–35 kDa

relevant to detoxification has been helped considerably by studies of both animal (Cornell et al. 2005) and plant sources and it is interesting to note that there appears to be a synergistic effect brought about by the different mechanisms of action of enzymes in the pig intestinal extract and those in papaya latex. These experiments support further studies of enzyme therapy in coeliac disease.

Previous studies (Cornell and Rivett 1995) suggest that the main activity in the human intestinal mucosa is not PEP (3.4.21.26) but is an endopeptidase targeting the N-terminal side of proline residues which could be classified as a prolidase. It is not an imidodipeptidase which attacks only certain dipeptides, although these enzymes are present in pig intestine (Sjöström et al. 1973) but instead, the vital

peptidase is able to attack larger peptides containing proline on the N-terminal side of that amino acid. Support for an endopeptidase comes from work which showed that such an enzyme was present in the endosperm of germinated triticale, suggesting it plays a major role in the mobilisation of storage proteins (Prabucka and Bielawski 2004).

Electrophoretic preparations

Agarose gels

In pH 7.5 buffer, most proteins in papaya latex ran to the cathode which indicated they have high isoelectric points (pI), i.e. they are positively charged even at pH 7.5 and therefore their pIs must be greater than 7.5. This is considerably higher than most other peptide hydrolases.

Fraction 4 from CM Sephadex chromatography of papaya latex presented a changed pattern in which proteins of pI higher and lower than 7.5 were observed.

Polyacrylamide gels

The presence of sodium dodecyl sulfate (SDS) coats the proteins with a negative charge and permits the estimation of their molecular weight in polyacrylamide gel electrophoresis (PAGE). Under such conditions, papaya latex was shown to contain many bands in the range 10–65 kDa. Fraction 4, from CM Sephadex chromatography of papaya latex was shown to contain proteins in the range 10–33 kDa in good agreement with the HPLC size exclusion experiments. Most of these bands were concentrated in the 25–30 kDa range. The heat-stable enzymes present in Fraction 4 correspond to those in the 30 kDa region, as determined by size exclusion chromatography on Sephacryl S-300 and appear to be a significant portion of the proteins in this fraction. Other workers (Oberg et al. 1998) have obtained major bands on papaya latex in the same region, with highly purified PEP from the same source at 33 kDa.

Proteomics and MS analysis

The Mascot search resulted in 10 protein hits with caricain precursor (EC 3.4.22.30) or papaya proteinase omega the one with by far the highest Mowse Score, this being 230. Scores greater than 56 indicate extensive homology ($P < 0.05$) so that this score is very strong evidence for the major component in the HPLC purified Fraction 4 being the Caricain precursor, or more likely, its active enzyme form. Matches were made on 8 out of 70 fragment ions using the 17 most intense peaks.

The results with the Ludwig data base led to the same conclusion. In this case, coverage figures were presented as percentages where

$$\%\,\text{coverage} = \frac{\text{total number of amino acids sequenced}}{\text{full protein sequence}}$$

Two sample runs gave 38 and 39% coverage for the caricain precursor with lower coverages of 18 and 24% coverage for the chymopapain precursor (EC 3.4.22.6) also called papaya proteinase II (refer Table 7). It was thought that chymopapain may be contributing to the activity of papaya latex, being a member of the papaya cysteine proteinase family. It was important to note that QCT precursor (EC 2.3.2.5) gave only 7 and 5% coverage.

In other experiments carried out using bands from SDS-PAGE of Fraction 4 the results gave 41 and 39% coverage of the caricain precursor, making it clear that caricain was the major active principle. The amino acid sequence covered was from 133 to 348 in the sequence of procaricain, corresponding closely to the active enzyme (Groves et al. 1996).

Table 7 Mass spectrometric fragmentation analysis of Fraction 4 from CM Sephadex chromatography of papaya latex showing caricain precursor as the major protein identified

Sample name and run no.	Protein(s) identified	Number of peptides identified	Accession number
624-1 DO70621-01	Caricain Precursor (EC 3.4.22.30) (Papaya proteinase omega)	17 (38% coverage)	P10056
	Chymopapain precursor (EC 3.4.22.6)	6 (18% coverage)	P14080
	Glutamine cyclotransferase precursor (EC 2.3.2.5) (Carica papaya)	2 (7% coverage)	081226
624-2 DO70621-01	Caricain precursor (EC 3.4.22.30) (Papaya proteinase omega)	20 (39% coverage)	P10056
	Chymopapain precursor (EC 3.4.22.6)	6 (24% coverage)	P14080
	Keratin.Type II cytoskeletal I	4 (10% coverage)	P04264
	Endochitinase (EC 3.2.1.14) (Carica papaya)	4 (14% coverage)	P85084
	Keratin.Type I cytoskeletal 9	2 (8% coverage)	P35527
	Glutamine cyclotransferase precursor (EC 2.3.2.5) (Carica papaya)	1 (5% coverage)	081226

Further studies

There is a need for further work to be carried out on the kinetics of the reactions using highly purified enzyme preparations as well as studies of the effects of various enzyme inhibitors. Both of these studies could utilise the rat liver lysosome assay.

Conclusions

1. The rat liver lysosomal assay has been a useful mainstay of an investigative plan for elucidation of the gliadin-detoxifying enzymes in porcine intestinal extract and papaya latex.
2. The activity of a papaya latex is mainly due to caricain whereas the activity in a porcine extract is likely to be due to a prolidase, e.g. an oligoiminopeptidase.
3. Research on papaya latex indicates that it is a viable starting material for preparation of gliadin-detoxifying enzymes such as caricain (EC 3.4.22.30). It also has some activity due to impurities such as chymopapain, QCT and PEP.
4. Coupled with chromatographic techniques and assays of PEP and proteases (as measured by the BAEE assay), the rat liver lysosomal assay has shown that these enzymes do not play a significant role in the way in which pig intestinal extract and crude papain act in gliadin detoxification.
5. A combination of the porcine extract and papaya latex was more effective than either product alone, suggesting synergistic action due to the different types of mechanism of the active enzymes in each.
6. The use of the cation exchanger CM Sephadex C-50, followed by size exclusion HPLC, has resulted in greatly enriched forms of the active enzymes in Fractions 3 and 4 which were used successfully for their further characterisation and an indication of the main species of enzyme present. The behaviour of the principal enzyme indicated that it was highly basic as is reported for caricain.
7. The use of Sephacryl S-300 in size-exclusion chromatography has yielded active fractions from papaya latex in the range of 40 kDa down to 10 kDa. The highest lysosomal activity was observed in the higher molecular weight fractions (23–35 kDa) and is ascribed mainly to caricain.
8. The heat stability of enzymes in Fraction 4 up to 85°C was consistent with this fraction containing caricain and QCT, both of which have good heat stability.
9. MS results have confirmed the enrichment of caricain in the most active fraction which also contains some chymopapain and QCT, indicating that this fraction would be effective in enzyme therapy for gluten intolerance.
10. There was little detectable PEP activity in the most active fractions from CM Sephadex chromatography of papaya latex and pure commercial PEP had low activity in the rat liver lysosome assay, indicating that this enzyme would not be effective in enzyme therapy for gluten intolerance.

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