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CARICA PAPAYA LATEX IS A RICH SOURCE OF A CLASS II CHITINASE

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Key Word Index—*Carica papaya*; Caricaceae; papain; chitinase; lysozyme.

Abstract—A class II chitinase is present in the latex of the tropical species *Carica papaya*. The enzyme may be readily purified by using a combination of hydrophobic interaction- and cation-exchange chromatography. This enzyme preparation is homogeneous with respect to the three physico-chemical criteria of charge, M_r (28 000) and hydrophobicity. It is also completely free of any proteolytic and bacteriolytic activities. The enzyme was classified as a class II chitinase on the basis of its N-terminal amino acid sequence up to the 30th residue. In agreement with this classification, the enzyme preparation hydrolyses chitinase substrates only very slowly and several free thiol functions are present in the polypeptide chain. These free thiol functions are buried, and to be available for titration with 2,2′-dipyridyldisulphide, the enzyme must be denatured. Unfolding of papaya chitinase requires particularly drastic conditions, not less than 4 M guanidinium hydrochloride at 25° and pH 6.8. © 1997 Elsevier Science Ltd

INTRODUCTION

Plants respond to attack by pathogenic micro-organisms by the induction of expression of a large number of genes encoding diverse proteins, among which are chitinases [EC 3.2.1.14] and β-glucanases [EC 3.2.1.19]. These glycosyl hydrolases, especially in combination, inhibit growth of several classes of phytopathogenic micro-organisms. The substrates of β-1,3-glucanases and chitinases, respectively, β-1-3-glucans and chitin (a polymer of *N*-acetyl-glucosamine) are present in the cell walls of many fungi. It is thus expected that contribution of both enzymes to plant defence may result from their ability to degrade cell walls of fungal pathogens. β-Glucan and chitin-like oligosaccharides are themselves potent inducers of several plant defence reactions such as phytoalexin synthesis, callose production and cell wall modification. Thus, a second potential function of β-1,3-glucanases and chitinases could also involve the release of defence-triggering signal molecules, the so-called elicitors [1–3]. Besides their defensive roles, chitinases exert important functions in plant metabolism, e.g. in early somatic embryo development [4] and determine the specificity of bacterium–host plant interactions [5].

Plant chitinases are a structurally diverse group with respect to their physico-chemical properties, enzymatic activities, hydrolytic mechanisms and localization in plant tissues [2, 6]. They have been divided into several classes based on amino acid sequence [7, 9]. In the classification system of glycosyl hydrolases, all chitinases are grouped into two families [9]. Class I, II, IV and V chitinases form the family 19, that is only known in plants. Family 18 includes all fungal, animal and bacterial chitinases as well as plant chitinases of class III and VI.

The presence in the latex of *Carica papaya* of a β-1,3-glucanase [10] and of a class III chitinase [3, 11–15] has been previously reported. In the course of experiments aimed at testing new chromatographic gel media for characterizing papaya proteinase isoforms, we fortuitously isolated, in an appreciable amount, a class II chitinase. Noteworthy, class II chitinases are generally produced in minute amounts, prompting us to report our discovery.

RESULTS AND DISCUSSION

Purification of class II papaya chitinase

Class II papaya chitinase was purified from the whole protein fraction of the *Carica papaya* latex. The latex of this plant is known for being a rich source

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of the rather well characterized cysteine proteinases papain, chymopapain, caricain and glycyl endopeptidase [16–19]. In the course of experiments aimed at characterizing hydrophobicity of these proteinases, measurements of the strength of their interactions with hydrophobic groups grafted on gel matrices led to the discovery of the class II papaya chitinase.

Fractogel TSK Butyl-650, one of the tested hydrophobic gel media, separated the mixture of papaya enzymes into several distinct chromatographic fractions as a function of the concentration (from 3 to 0 M) of $(NH_4)_2SO_4$ used as the eluent (Fig. 1). Four of them (peaks I to IV) contained proteinases as shown by their ability to hydrolyse the protein substrate azocoll. On the basis of several tested physico-chemical and enzymatic properties, they were identified as caricain (peak I), chymopapain (peak II), glycyl endopeptidase (peak III) and papain (peak IV). A fifth main chromatographic fraction, indicated by the solid bar in Fig. 1, contained a proteolytically inactive material which migrated on SDS-PAGE as a polypeptide chain with a M_r of 28000. This protein material was further purified by cation-exchange chromatography on S-Sepharose Fast Flow where it eluted at a position corresponding to that of glycyl endopeptidase [20] between chymopapain and caricain (Fig. 2) and identified as a class II chitinase as described in the next section.

The presence of the class III chitinase in the latex of *Carica papaya* was a serious handicap which hampered the follow up of the purification procedure by using chitinase activity tests. The actual yield of purified class II papaya chitinase is thus unknown. In any case, 75 mg of the enzyme were obtained, starting

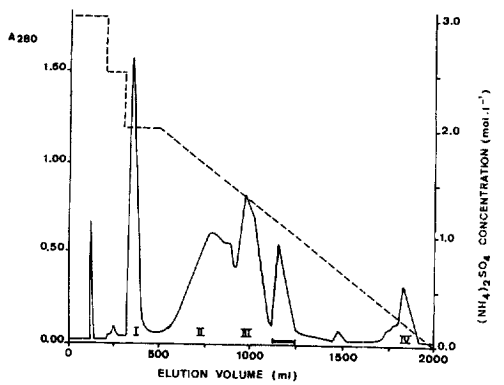

Fig. 1. Purification of the chitinase from papaya latex on Fractogel TSK Butyl-650. Column: 15 × 1.6 cm; fractions of 15 ml; flow rate: 60 ml hr^{-1}; sample: the mixture of enzymes (330 mg) present in the latex of *Carica papaya*; eluent: gradient from 3 to 0 M of $(NH_4)_2SO_4$; first eluent: 3 M $(NH_4)_2SO_4$, 200 ml; second eluent: 2.5 M $(NH_4)_2SO_4$, 100 ml; third eluent: 2 M $(NH_4)_2SO_4$, 200 ml; fourth eluent: linear gradient 2–0 M $(NH_4)_2SO_4$, 1500 ml; room temp. Each chromatographic fraction was analysed by measurement of A_{280} (continuous trace) and $(NH_4)_2SO_4$ conc. (broken lines) calculated from conductivity measurements. The chitinase pool was constituted with the chromatographic fractions noted by the solid bar.

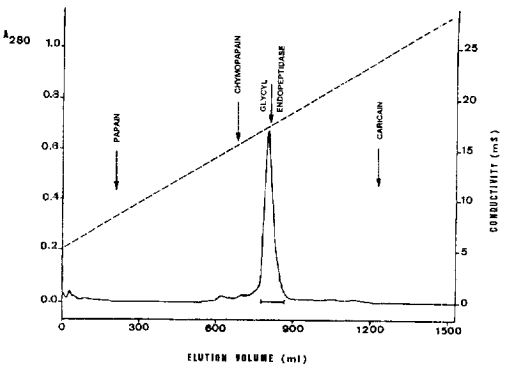

Fig. 2. Elution profile of papaya chitinase on S-Sepharose Fast Flow. Column: 15 × 3 cm; fractions of 14.15 ml; flow rate: 56.6 ml hr^{-1}; sample: the chitinase (≈ 50 mg) pool after purification on Fractogel TSK Butyl-650; eluent: gradient 100–800 mM NaOAc buffer, pH 5, 1500 ml; room temp. Each chromatographic fraction was analysed by measurements of A_{280} and conductivity (continuous and broken traces, respectively). The chitinase pool used for characterization is noted by the solid bar. The arrows indicate the elution position of the four cysteine proteinases present in the latex of *Carica papaya*.

from 1 g of the mixture of papaya enzymes. Purification yields of class II chitinases from other sources have been occasionally reported. Purified barley chitinase (CHI 26) (15 mg) was obtained from 780 mg of seed proteins [21], while extraction of 1 g of dried potato pestil led to 400 μg of purified SK-2 chitinase [22]. By reference to these values, we concluded that *Carica papaya* latex is a rich source of class II chitinase.

Before identifying class II papaya chitinase, homogeneity of the enzyme preparation was carefully checked by hydrophobic-interaction and cation-exchange chromatography as well as by electrophoresis (SDS-PAGE). Furthermore, we were also able to show that it exhibited no detectable proteolytic (azocoll as the substrate) and bacteriolytic (=lysozyme; *Micrococcus luteus* cells as the substrate) activities known to be present in the whole protein fraction of papaya latex [12]. β-1,3-Glucanase assays were however not performed.

Identification of class II papaya chitinase

Class II papaya chitinase was identified on the basis of its N-terminal amino acid sequence. This was unambiguously determined up to the 30th amino acid residue by automatic Edman degradation (Fig. 3). It was the only one which could be observed, further confirming the homogeneity of the enzyme preparation under investigation.

Searching (using the BLASTP 1.4.7 program [22]) sequence homologies to this 29 amino acids fragment through the amino acid sequence databanks (PIR, Swiss Prot and Genpept) suggested a series of candidate homologous proteins. The 88 first sequences which produced the higher-scoring segment pairs were

		Ref.	1					6				11				16				21				26					
Citrus sinensis		[23]		E	L	G	K	I	I	S	R	E	M	F	D	D	L*	L	E	Y	R	N	D	E	R	C	P	A	R	G	F	Y
Arachis hypogaea	Chi 2-1	[24]		P	I	S	S	L	I	S	K	T	L	F	D	S	I	F	L	H	K	D	D	N	A	C	P	A	R	N	F	Y
	Chi 2-2	[24]		D	A	G	T	I	I	T	Q	P	L	Y	N	E	F	L	K	H	L	T	D	S	R	C	E	A	H	G	F	Y
Gossypium hirsutum		[25]		D	I	S	S	L	I	S	Q	D	M	F	N	E	M	L	K	H	R	N	D	G	N	C	P	G	K	G	F	Y
Solanum tuberosum	SK2	[26]	Q	D	V	G	S	L	I	N	K	N	L	F	E	R	I	F	L	H	R	N	D	A	A	C	A	A	K	G	F	Y
Lycopersicon esculentum	Chi 2-1	[30]	Q	N	I	S	S	L	I	S	K	N	L	F	E	R	I	L	V	H	R	N	D	A	A	C	G	A	K	G	F	Y
Secale cereale		[27]		S	V	S	S	I	I	S	H	A	Q	F	D	R	M	L	L	H	R	N	D	G	A	C	Q	A	K	G	F	Y
Hordeum vulgare	CHI 26	[28]		S	V	S	S	I	V	S	R	A	Q	F	D	R	M	L	L	H	R	N	D	G	A	C	Q	A	K	G	F	Y
Solanum tuberosum	Cht A2	[29]	Q	N	A	G	S	I	V	T	R	E	L	F	E	Q	M	L	S	F	R	N	N	D	V	C	P	G	K	G	F	Y
Lycopersicon esculentum	Chi 3	[30]	Q	N	A	G	S	I	V	T	R	E	L	F	E	Q	M	L	S	F	R	N	N	D	A	C	P	A	K	G	F	Y
	Chi 17	[30]	Q	D	V	G	T	I	V	T	S	D	L	F	N	E	M	L	K	N	R	N	D	D	R	C	P	A	K	G	F	Y
Lycopersicon chilense	PCHT 28	[31]	Q	N	A	G	S	I	V	T	R	E	L	F	E	Q	M	L	S	F	R	N	N	D	A	C	P	A	K	G	F	Y
CARICA PAPAYA				G	I	E	K	I	I	S	R	S	M	F	D	Q	M	L	K	H	R	N	N	P	A	C	P	A	K	G	F	Y
Nicotiana tabacum	PR-P	[32]	Q	G	I	G	S	I	V	T	N	D	L	F	N	E	M	L	K	N	R	N	D	G	R	C	P	A	N	G	F	Y
	PR-Q	[32]	Q	G	I	G	S	I	V	T	S	D	L	F	N	E	M	L	K	N	R	N	D	G	R	C	P	A	N	G	F	Y
Solanum tuberosum	Cht A1	[29]	Q	N	A	G	S	I	V	T	R	E	L	F	E	Q	M	L	S	F	R	N	N	D	V	C	P	G	K	G	F	Y
Hordeum vulgare	Cht 2a	[33]	Q	G	V	G	S	V	I	T	R	S	V	Y	A	S	M	L	P	N	R	D	N	S	L	C	P	A	R	G	F	Y
	Cht 2b	[33]	Q	G	V	G	S	V	I	T	Q	S	V	Y	A	S	M	L	P	N	R	D	N	S	Q	C	P	A	R	G	F	Y
Petunia hybrida		[34]	Q	N	V	G	S	I	V	T	S	D	L	F	D	Q	M	L	K	N	R	N	D	A	R	C	F	A	V	R	F	Y

Fig. 3. *N*-terminal amino acid sequence of papaya chitinase and sequence homology with class II chitinases from other plant sources [23–34].

identified as plant chitinases. This observation prompted us to determine whether our preparation possessed any chitinase activity.

For that purpose, chitin azure and 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosaminide were used as the substrates. According to the manufacturer, chitin azure is prepared by the covalent attachment of Remazol Brilliant Violet SR dye to crab shell chitin. Upon hydrolysis of a chitin azure suspension, a fraction of the dye appears in the soluble fraction of the assay mixture. This fraction may be quantified spectrophotometrically. Using this test, our preparation exhibited a slight but quite measurable chitinase activity. Upon incubation at pH 4.6 in McIlvaine buffer, chitin azure (5 mg ml^{-1}) was hydrolysed to the extent of 0.05% per hr in the presence of the papaya enzyme (2.4 mg ml^{-1}) at 37°. Our preparation also exhibited some β-*N*-acetyl-D-glucosaminidase activity. Indeed, the presence of methylumbelliferone was detected spectro-fluorometrically (λ_{exc} nm: 390; λ_{em} nm: 450) upon incubation of 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide in the presence of the enzyme in McIlvaine buffer at pH 4.6. The observed rates of liberation of methylumbelliferone was proportional to the enzyme concentrations, at least within the range 0–15 μM tested here. As previously mentioned, the enzyme preparation under investigation did not possess any lysozyme activity. It may thus be expected that chitinase activity associated with papaya lysozyme (= class III chitinase, [3, 12, 13]) did not contribute at all to the chitinase activity measured here, confirming that the partially sequenced papaya enzyme did really possess an intrinsic chitinase activity.

A closer examination of the 88 amino acid sequences of the chitinases which exhibited strong homology to the papaya enzyme revealed that they all belong to either class I or class II chitinases. Distinction between classes I and II is still an object of debate. This is due to the fact that their primary structures are 60–65% identical while there is no more than 70% identity within the classes [17]. Originally, class I and class II chitinases were distinguished by the presence of a hevein domain and a vacuolar C-terminal targeting signal in the class I enzymes and their absence in the class II chitinases. On this basis, papaya chitinase may be classified as a class II enzyme since, its N-terminal amino acid sequence does not contain hevein (chitin binding) domain. However, since the enzyme was isolated from a particularly rich source of proteinases, the possibility that the polypeptide chain we sequenced is a truncated form of the native chitinase needs to be carefully examined. This possibility however seems unlikely. Indeed, when the sequenced fragment of papaya chitinase was aligned with the amino acid sequences of true class II chitinases [23–34], optimum identity scores were always obtained following alignment of the papaya fragment with the N-terminal amino acid sequence of these chitinases (see Fig. 3).

Preliminary studies of the denaturation of papaya chitinase by Gu-HCl

In the *Carica papaya* plant, the cysteine proteinases present in the latex are fully activated. Altogether, the proteinase concentration in this latex does exceed 1 mM, raising the question of how papaya enzymes (including the proteinases themselves) acquire resistance to proteolysis. This consideration prompted us to examine the susceptibility of class II papaya chitinase to denaturation by Gu-HCl.

The fluorescence emission spectrum of the putative native form of the enzyme was characterized by a λ_{max} at 336 nm and a quantum yield equal to 0.040 (assuming a quantum yield value of 0.13 for *N*-acetyl-L-tryptophan ethyl ester [35]). These characteristics, obtained at 25°, were independent of pH within the

range 3 to 8 which was examined here. Following incubation at 25° for 24 hr in the presence of 6 M Gu-HCl (in a 50 mM phosphate buffer at pH 6.8) the fluorescence emission spectrum of papaya chitinase (0.9 μM) exhibited a λ_{max} at 355 nm, showing unfolding of the polypeptide chain. Denaturation of papaya chitinase was also accompanied by an important (about 200%) increase of the fluorescence emission intensity.

On the other hand, accessibilities of the free thiol functions of papaya chitinase either in its native and unfolded states were compared. 2,2′-Dipyridyldisulphide was used as the titrant for this purpose. Titrations of the enzyme (0.064 nM) were performed at 25° and pH 6.8 (in a 50 mM phosphate buffer containing the titrant (0.64 mM from a 100 mM stock solution in dimethylsulphoxide)) in the absence or in the presence of Gu-HCl. The free thiol content was calculated from spectrophotometric measurements of the amount of 2-thiopyridone (λ_{max} nm: 343; $\varepsilon_{343} = 7700\ M^{-1}\ cm^{-1}$; [36]) released during the reaction. It was observed that 3.09 mol of SH per mol of enzyme were titrated when 6 M Gu-HCl was present, while no free thiol function could be detected following titrations of native papaya chitinase.

Both fluorescence intensity and free thiol content are thus valuable parameters which may be used to monitor denaturation of papaya chitinase. Both parameters were indeed used in combination to measure kinetic parameters (shown in the inset of Fig. 4) while only fluorescence intensities were used to determine the thermodynamic parameters shown in Fig. 4.

Obviously, papaya chitinase is highly resistant to denaturation. At 25° and pH 6.8, unfolding of the papaya enzyme only proceeded at Gu-HCl concentrations greater than 4 M. In 6 M denaturant, unfolding of the class II chitinase (1.4 μM) required up to 45 min to reach completion. Analysis of the preliminary results shown in Fig. 4 suggested that unfolding of papaya chitinase might be, at first sight, assimilated to a two-state transition. Indeed, identical kinetic parameters were obtained whatever the origin (fluorescence intensity or free thiol content) of the original data used to calculate these parameters. Denaturation kinetics also obeyed a first order reaction. Furthermore, the dependence of the free energy of unfolding (calculated assuming a two-state equilibrium) on the molar concentration of denaturant appeared to be linear.

It is tempting to speculate that the structure of papaya chitinase consists of a single domain, a situation reminiscent of that of barley CHI 26, the archetype of the class II chitinase family [37]. The dependence of the free energy of unfolding on denaturant concentration, which has been given the symbol m [38], provides an estimation of the surface area of protein exposed to solvent upon unfolding [39, 40]. Interestingly, the m value ($m = 3950$ cal $mol^{-1}\ M^{-1}$) calculated from the results shown in Fig. 4 is much lower than the value ($m = 5400$ cal $mol^{-1}\ M^{-1}$) expected for a polypeptide chain containing 230 amino acid residues [41]. Discovering the reason(s) why experimental m values are abnormally low may contribute to the understanding of how papaya latex

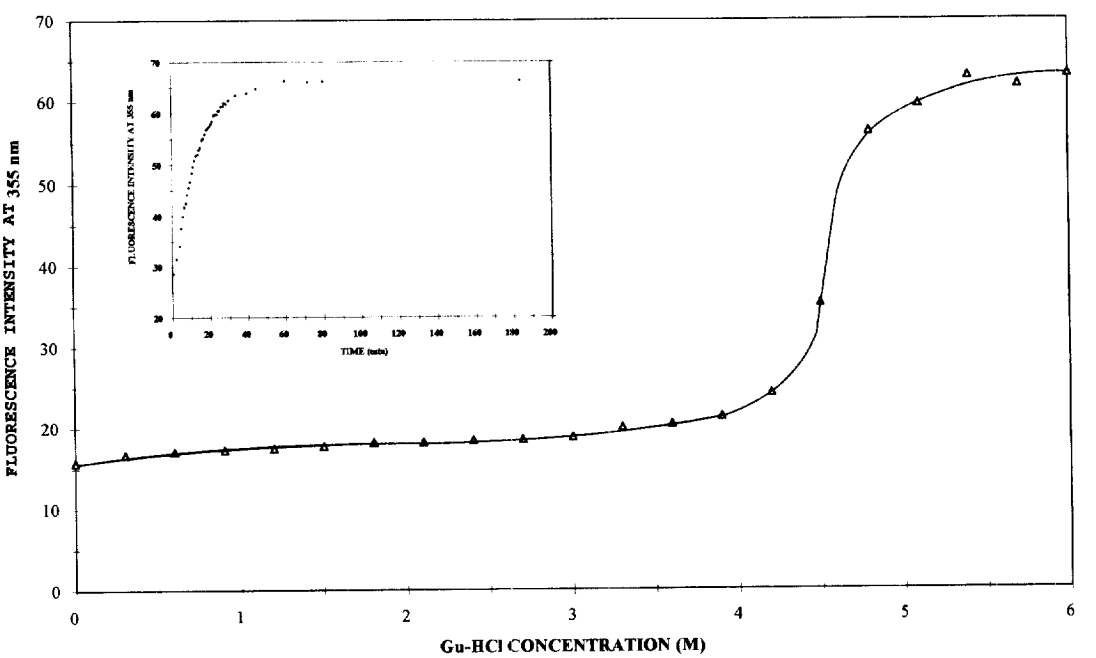

Fig. 4. Denaturation of papaya chitinase by Gu-HCl. Papaya chitinase (0.9 μM) in 50 mM phosphate buffer, pH 5 is incubated for 24 hr at 25° in the presence of Gu-HCl (various concentrations) (Δ). Inset: Kinetic of denaturation of papaya chitinase (1.4 μM) in 50 mM phosphate buffer, pH 6.8 in the presence of Gu-HCl 6 M (◆). The fluorescence emission intensity at 355 nm of each solution was then measured (λ_{exc} nm: 290) at 25° with a Shimadzu RF-5001PC spectrofluorimeter. Emission and excitation bandwidths were 5.0 and 1.5 nm each.

class II chitinase is structurally organized to retard proteolytic degradation.

EXPERIMENTAL

Materials. Aldrich-Chemie provided dithiothreitol (DTT), 2,2′-dipyridyldisulphide (2-PDS), methylmethanethiolsulphonate, 7-hydroxy-4-methylcoumarin and EDTA. Guanidinium hydrochloride (GuHCl) was from Fluka and azocoll, *N*-acetyl-L-tryptophan ethyl ester, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide and chitin azure from Sigma. All the chemicals and the M_r protein standards for SDS-PAGE were purchased from Bio-Rad. Pharmacia and Merck provided S-Sepharose Fast Flow and Fractogel TSK Butyl-650(M).

Preparation and purification of papaya chitinase. All the operations were carried out at room temp. unless otherwise stated.

Step 1: Spray-dried papain (1.65 g) was dissolved in H_2O (25 ml) and applied to a (15 × 3 cm) column of S-Sepharose Fast Flow pre-equilibrated in a 50 mM NaOAc buffer at pH 5 (in this work, the molarities of NaOAc buffers always refer to the Na^+ conc.). Most of the material contaminating the papaya enzymes was eliminated by eluting (56.6 ml hr^{-1}) with (1): 50 mM NaOAc buffer at pH 5 (start buffer; 50 ml), (2): 5 mM DTT in the start buffer (25 ml) and (3): 100 mM NaOAc buffer at pH 5 until the A_{280} reading returned to its baseline value. The papaya enzymes were then eluted, as a mixt., by pumping 2 M NaOAc buffer at pH 5 through the column, and were collected in the presence of 30 mmol of methylmethanethiolsulphonate (which converted the cysteine proteinases into their inactivated *S*-methylthioderivatives). The vol. of the enzyme (660 mg based on A_{278} (1 mg ml^{-1} = 1.85)) soln was then reduced (by rotavaporating at 30° under red. pres.) to half of its starting value.

Step 2: Hydrophobic interaction chromatography (HIC) on Fractogel TSK Butyl-650: An aliquot of the 4 M NaOAc soln from step 1 (8 ml) containing the mixt. of the papaya enzymes (330 mg) was applied on a (15 × 1.6 cm) column of Fractogel TSK Butyl-650 pre-equilibrated in 3 M $(NH_4)_2SO_4$ (start buffer). The elution was performed at 25° using a flow rate of 60 ml hr^{-1}. After loading the protein sample onto the column, the start buffer (200 ml) was pumped followed by: (1): a stepwise gradient from 3 → 2.5 (100 ml) → 2 M (200 ml) and (2): a linear gradient from 2 to 0 M $(NH_4)_2SO_4$ (1500 ml). Frs of 15 ml were collected and analysed by measurement of A_{280}, conductivity and proteolytic activity towards azocoll in the presence of 2.5 mM DTT to regenerate the active proteinases from their *S*-methylthioderivatives. Some of the chromatographic frs which did not show proteolytic activity were pooled (those indicated by the solid bar in Fig. 1), concd by ultrafiltration (Amicon system; membrane cut off: M_r 3000) and dialysed against H_2O (3 × 5 l) and then against 5 l or 100 mM NaOAc buffer at pH 5.

Step 3: Ion-exchange chromatography on S-Sepharose Fast Flow: An aliquot from the dialysed soln from step 2 was applied to a (15 × 3 cm) column of S-Sepharose Fast Flow pre-equilibrated with a 100 mM NaOAc buffer at pH 5 (start buffer). After loading the protein sample onto the column, a linear elution gradient (total vol.: 1500 ml) from 100 to 800 mM NaOAc buffer at pH 5 was then applied at a flow rate of 56.6 ml hr^{-1}. Each individual chromatographic fr (14.15 ml) was analysed by measurement of A_{280} and conductivity. The chromatographic frs indicated by the solid bar in Fig. 2 were concd by ultrafiltration, dialysed against H_2O and lyophilised.

SDS-PAGE. Experiments were carried out on slab gels using the mini protean II cell (BioRad). The resolving gels (pH 8.8; 12% acrylamide) were run at a constant voltage (200 V) and prepd according to ref [42]. The stacking gels consisted of 4% polyacrylamide (pH 6.8). The upper and lower chambers contained Tris–glycine buffer (pH 8.3) with 0.1% SDS. The sepn was towards the anode and bromophenol blue was used as the tracking dye. Before being loaded onto the gel, the protein solns were diluted with a buffer containing 25% Tris–HCl 1 M, pH 6.8; 4% SDS; 23% glycerol and 1% bromophenol blue and boiled for 4 min.

The gels were stained with 0.25% Coomassie Blue R250 dissolved in the washing soln. The latter was used also for destaining and contained MeOH, HOAc, and H_2O (9:2:9). The M_r standards were: lysozyme (14 300 M_r), soybean trypsin inhibitor (21 500 M_r), carbonic anhydrase (31 000 M_r), ovalbumin (45 000 M_r) and phosphorylase B (97 000 M_r).

Measurements of enzymatic activities. Proteinases were assayed at 37° for their ability to release soluble dye from a suspension of azocoll (1 mg ml^{-1}) in a buffer (pH 6.8) containing phosphate, citrate and borate (100 mM each) and DTT (2.5 mM). The bacteriolytic activity of papaya chitinase was measured, as described previously, using *Micrococcus luteus* cells as the substrate [14]. β-*N*-Acetyl-D-glucosaminidase activity measured at 50° using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as the substrate. Test tubes (total vol. 2 ml) contained the substrate (0.5 mM) and the enzyme (0–15 μM) in McIlvaine buffer (200 mM Na-Pi and 100 mM citric acid) adjusted at pH 4.6. The reaction was started by adding the substrate (from a 4 mM aq. stock soln) and was stopped after a 15-fold dilution of the reaction mixt. into a 200 mM glycine–NaOH buffer at pH 10.7. The amount of liberated methylumbelliferone was determined spectrofluorimetrically (λ_{exc} nm: 390 λ_{em}: 450) at 25° by reference to a standard curve (obtained by using authentic 7-hydroxy-4-methylcoumarin). The chitinase activity was measured at 37° using a suspension of chitin azure as the substrate (5 mg ml^{-1}) in the McIlvaine buffer at pH 4.6.

Determination of the N-terminal amino acid

sequence. The N-terminal sequence analysis was performed by Edman degradations of 10 mg of protein in an Applied Biosystem 447A pulsed liquid sequencer coupled to a 120A PTH-amino acid analyser.

Spectroscopic and analytical methods. The conductivities (mS) were measured with a Radiometer Conductivity meter CD M3 equipped with a Radiometer measurement cell type CDC 314. The mS values were measured at constant temp. Papaya chitinase concs were measured spectrophotometrically at 280 nm using a A (1 mg ml^{-1}) value of 2.32 [6]. A was measured with a Varian DMS 300 spectrophotometer, and fluorescence (at 25°) with a Shimadzu RF-5001PC spectrofluorimeter. For protein fluorescence measurements, excitation was at 290 nm and emission spectra were scanned from 270 to 450 nm. Emission and excitation bandwidths were 5.0 and 1.5 nm, respectively. Free thiol content was determined using 2-PDS as the titrant [7].

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