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Research Article

Structural characterization of two papaya chitinases, a family GH19 of glycosyl hydrolases

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Abstract. Two chitinases, able to use tetra-*N*-acetylglucosamine, chitin and chitosan as substrates, were characterized after purification from *Carica papaya* latex. The complete amino acid sequence of the major form and about 40% of the minor one were determined through proteolytic digestions and mass spectroscopy analysis. Sequencing demonstrated that both papaya chitinases are members of the family 19 of glycosyl hydrolases (GH19). Based on the known 3-D structures of other members of family GH19, it was expected that papaya chitinases would adopt all-alpha structures. However, circular dichroism and infrared spectroscopy indicated, for the papaya chitinases, a content of 15–20% of extended structures besides the expected 40% of alpha helices. Since the fully sequenced papaya chitinase contains a large number of proline residues the possibility that papaya chitinase contains polyproline II stretches was examined in the context of their resistance against proteolytic degradation.

Keywords. Amino acid sequence, secondary structure, limited proteolysis, proline concept.

Introduction

Chitinases, which belong to the large family of *O*-glycosyl hydrolases, catalyze the hydrolysis of β-1,4-*N*-acetyl-D-glucosamine linkages in chitin, one of the most abundant natural polymers. Chitin is an important structural component of many organisms, and represents the main constituent of the cuticles of insects, shells of crustaceans and cell walls of many fungi. Chitinases genes have been isolated from many different organisms including bacteria, viruses, higher plants and animals. Based on amino acid sequence homology, chitinases have been classified into two different families: GH18 and GH19 [1]. Members of these families further differ in 3-D structures and hydrolytic mechanisms. The catalytic domains of the members of family GH18 have an (α/β) eight-barrel fold as first demonstrated by structural analysis of hevamine [2]. On the other hand, the catalytic domains of the members of family GH19 have a high α-helical content [3, 4]. For glycosyl hydrolases, two distinct reaction mechanisms leads to either retention or inversion of the anomeric configuration. Family 18 chitinases use the retaining mechanism, while family 19 chitinases catalyze the hydrolysis of chito-oligosaccharides with overall inversion of anomeric configuration [5]. Family 18 and family 19 chitinases are therefore thought to have different functions and different evolutionary origins.

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Family GH18 contains chitinases able to degrade chitin but also proteins without chitinolytic activity. A recent analysis of approximately 150 plant members of glycosidase family GH18 has provided compelling evidence that xylanase inhibitors are largely represented in this family [6]. Family GH19 chitinases, on the other hand, have been found in a few bacteria and a few nematodes, but are mainly known for being present in higher plants. Papaya latex, from which two chitinases were extracted, is a thixotropic fluid with a milky appearance that contains about 6% of proteins, mainly (more than 85%) papain-like endopeptidases: papain (stricto sensu), chymopapain, caricain and glycyl endopeptidase [7]. Papaya latex may thus be grossly compared and assimilated to a 1 mM solution of cysteine endopeptidases. Although these proteases are synthesized as inactive pre-pro-enzymes, their activation requires less than 2 min after that the latex has been expelled [8]. Catalytic activity of papain-like endopeptidases requires the generation of a catalytic site imidazolium-thiolate ion pair and the protonic dissociation of a carboxylic acid function with a pKa value close to 4 that controls the ion pair geometry [9]. Three elements help to sustain full proteolytic activity in expelled papaya latex: (a) the absence of cysteine proteinase inhibitors, even though a cDNA cystatin encoding clone had been isolated as a result of screening a papaya leaf cDNA library [10], but it has never been isolated from latex despite intensive research, (b) the presence of a high concentration of low molecular weight thiol-containing compounds, and (c) a buffering capacity that maintains the latex pH around 5.2. Furthermore, with the exception of glycyl endopeptidase, papaya cysteine proteinases are unspecific endopeptidases [7].

After its collection, papaya latex refinement process require 1 week, during which time the papaya chitinase(s) cohabit(s) with the catalytically competent cysteine endopeptidases. Half-live of proteins in such an environment may be expected to be short. Despite this unfavorable prognosis, several reports have mentioned the capacity of commercial papain to degrade chitosan, a co-polymer of glucosamine and *N*-acetylglucosamine [11–13] as well as chitin (using chitin azure as substrate) [14, 15].

One of the main objective of the present work was to examine the structure of the papaya chitinase(s) to identify determinant(s) responsible for its (their) remarkable resistance to proteolytic attack by the papaya endopeptidases.

Materials and methods

Materials. Aldrich-Chemie (Steinheim, Germany) provided methylmethanethiolsulfonate (MMTS), dithiothreitol (DTT), acrylamide, succinimide, 8-anilinonaphtalene-1-sulfonic acid (ANS), chitosan (low molecular weight) and *N*-acetyltryptophanamide. SP-Sepharose Fast Flow was from Amersham Biosciences GE Healthcare (Uppsala, Sweden). Fractogel EMD Propyl (S) and 3,5-dinitrosalicylic acid were from Merck (Darmstadt, Germany). Tetra-*N*-acetylglucosamine was provided by Chemos Gmbh (Regenstauf, Germany). *N*-Benzoyl-D,L-arginine paranitroanilide (BAPA), D-glucosamine, lysozyme and albumin from chicken egg white, subtilisin Carlsberg and thermolysin from *Bacillus thermoproteolyticus* rokko were from Sigma Chemical Co (St Louis, MO), Boc-Ala-Ala-Gly paranitroanilide (Boc-AAG-pNA) was from Bachem AG (Bubendorf, Switzerland) and *N*-acetyl-D-glucosamine from Fluka (Buchs, Switzerland). Spray-dried papaya latex was from E.N.R.A. (Kivu, Democratic Republic of Congo, ex-Zaire).

Stabilization of the papaya extract. Spray-dried papaya latex (4 g) was dissolved at room temperature in H_2O (80 ml) containing 10 mM EDTA, 50 mM sodium acetate (referring to Na^+ concentration) and 2.5 mM DTT at pH 5.0. After activation of the proteinases for 30 min, MMTS (25 mM) was added and incubated for a further 30 min. The reaction mixture was then submitted to an exhaustive dialysis against 50 mM sodium acetate at pH 5.0 before being centrifuged (30 000 *g*, 30 min, 4 °C). This provided an aqueous solution containing a total of 1600 mg of the various papaya proteins.

Purification of the papaya chitinases. The papaya proteins sample in 100 ml 50 mM sodium acetate buffer at pH 5.0 (starting buffer) was first applied on a [31 × 2.6 cm inner diameter (ID)] column of SP-Sepharose pre-equilibrated in the starting buffer. Elution was performed at a flow rate of 45 ml/h at room temperature by applying a linear gradient from 50 to 1000 mM sodium acetate (total volume: 2000 ml) at pH 5.0. Protein fractions from this cation-exchange support were regrouped into three pools denoted I, II and III (as shown in Fig. 1). Further purification of the chitinases present only in pools II and III, was performed using hydrophobic interaction chromatography on Fractogel EMD Propyl 650(S). Before being submitted to the hydrophobic support, the proteins contained in pools II and III were concentrated by ultrafiltration (molecular weight cut-off: 10 kDa). Solid ammonium sulfate was then added up to 1800 mM for pool II, and 2500 mM for pool III. The resulting solutions (pH 5.0) were then applied on (18 × 2.6 cm ID) columns of Fractogel EMD Propyl pre-equilibrated in 1800 and 2500 mM ammonium sulfate, respectively. Elution was performed in both cases at a flow rate of 45 ml/h and at room temperature by applying a decreasing linear gradient from 1800 (or 2500) to 0 mM ammonium sulfate (total volumes of 1600 ml). Purification of the chitinase activity-containing chromatographic fractions (indicated by solid bars in Fig. 2a and b) was finally achieved through ion-exchange chromatography on a (15 × 1.6 cm ID) column of SP-Sepharose after

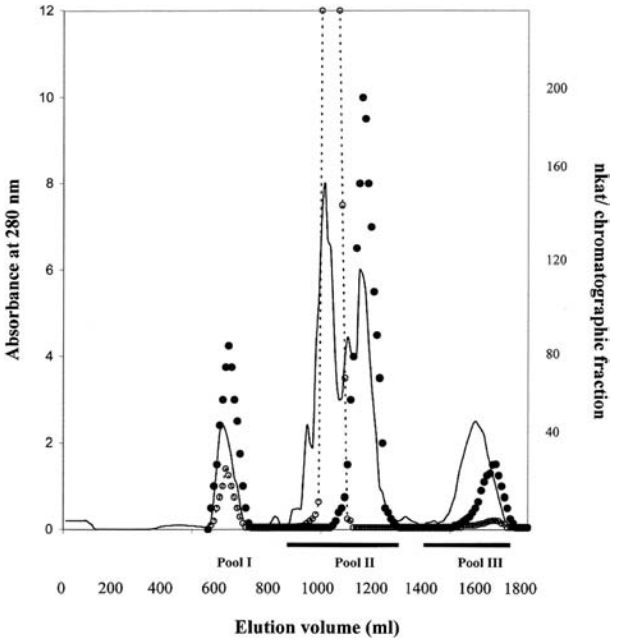


Figure 1. Ion-exchange chromatography of the papaya latex proteome on SP-Sepharose. Sample: 1600 mg papaya proteins in 100 ml 50 mM sodium acetate buffer at pH 5.0; column: 31 × 2.6 cm inner diameter (ID); fractions of 11.25 ml; flow rate: 45 ml/h; room temperature; eluting buffer: 50 mM sodium acetate at pH 5.0 followed by a linear gradient 50–1000 mM sodium acetate (total volume: 2000 ml). Each chromatographic fraction was analyzed by measurements of $A_{280\,nm}$ (continuous trace), amidase activity against BAPA (filled circles) and Boc-AAG-pNA (open circles) and of chitinolytic activity (solid bars).

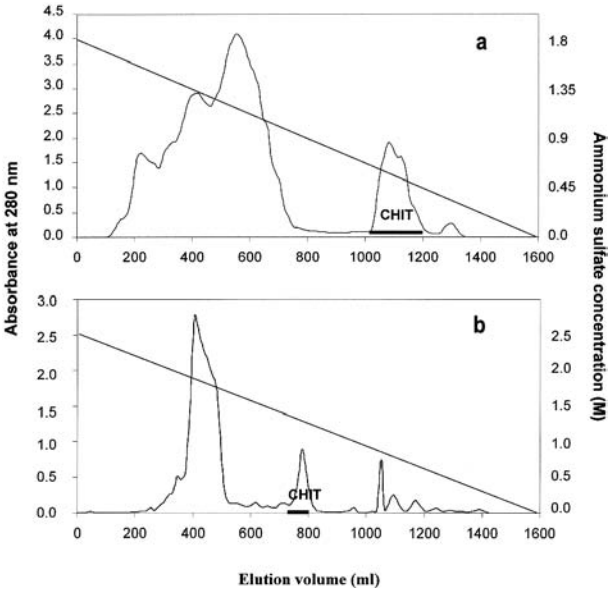


Figure 2. Hydrophobic chromatography of pools II (*a*) and III (*b*) on EMD-propyl (S): Samples: pools II and III from the SP-Sepharose after concentration by ultrafiltration (membrane cut-off: 10 kDa) and addition of solid ammonium sulfate up to 1800 mM (*a*) or 2500 mM (*b*); column: 18 × 2.6 cm ID; fractions of 11.25 ml; flow rate: 45 ml/h; eluting solutions: linear decreasing ammonium sulfate concentrations from 1800 or 2500 mM up to 0 mM (total volume: 1600 ml). Each chromatographic fraction was analyzed by measurements of $A_{280\,nm}$ (continuous trace) and chitinolytic activity. The chromatographic fractions that contained chitinolytic activity are indicated by solid bars.

being dialyzed against 200 mM sodium acetate at pH 5.0 (starting buffer). In both cases, elution was performed at a flow rate of 45 ml/h and at room temperature by applying a linear gradient from 200 to 800 mM sodium acetate buffer (total volume: 1200 ml).

Enzymes assays. Chitinase activity was measured using chitosan as the substrate as previously described [16], with only slight modifications, by measuring the release of reducing sugars [17] from a 1% chitosan solution (w/v) prepared in 100 mM sodium acetate at pH 3.5. *N*-Acetyl-D-glucosamine and D-glucosamine were used as standards. Enzyme and chitosan (1 : 100 w/w ratio, total volume: 2 ml) were incubated at 37 °C for 1 h. The reaction was stopped by adding 2 ml 2 M NaOH and the resulting suspension submitted to a centrifugation at 90 000 *g* for 2 min. Of the supernatant, 500 µl was then mixed with 500 µl of a solution containing 40 mM dinitrosalicylic acid, 400 mM NaOH and 1 M potassium sodium tartrate and the mixture was boiled for 15 min. After cooling to room temperature, the release of reducing sugars was measured spectrophotometrically at 540 nm. One chitosanase unit was defined as the number of µmoles of reducing group equivalents released per min and per mg enzyme under the above-cited conditions. Chitinase activity was measured quite similarly using tetra-*N*-acetylglucosamine (2 mM) as the substrate. The amidase activity of the papaya cysteine proteinases was measured at 37 °C and pH 6.8 in a buffer containing citrate, borate and phosphate (100 mM each) using BAPA and Boc-AAG-pNA as the substrates. Each test tube (total volume: 2 ml) contained 5% (w/v) DMSO, 1 mM BAPA (or 0.3 mM Boc-AAG-pNA), 1 mM EDTA and 2.5 mM DTT. Proteinases were preincubated at 37 °C in the buffer containing DTT and EDTA for 15 min and the reaction was started by addition of the substrates. The reaction proceeded at 37 °C and was stopped by addition of 500 µl of 50% (w/v) acetic acid. The release of 4-nitroaniline was determined spectrophotometrically using $\varepsilon_{410} = 8800/M/cm$ [18]. One unit of activity (nkat) was defined as the number of nanomoles of paranitroaniline released per second and per mg enzyme under the above-cited conditions.

SDS-PAGE. The SDS-PAGE experiments were carried out on precast gels (ExcelGel, 245 × 110 × 0.5 mm, gradient 8–18%) using the Multiphore II kit from GE Healthcare. The running conditions were 600 V, 50 mA and 35 W at constant temperature 15.0 ± 0.1 °C. Molecular weight

standards were hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and rabbit muscle phosphorylase b (97.4 kDa). Protein detection was performed using the silver staining procedure according to the instructions of the manufacturer.

Mass spectrometry. For mass determination, the major and the minor chitinases were solubilized in 50% acetonitrile/1% formic acid (v/v). The proteins were loaded into gold-palladium-coated borosilicate nanoelectrospray capillaries (Proxeon, Odense, Denmark). The mass spectra were acquired on a Q-TOF Ultima mass spectrometer (Waters/Micromass, Milford, USA), equipped with a Z-spray nanoelectrospray source and operating in the positive ion mode. The time-of-flight analyzer was operated in the W mode. Data acquisition was performed using a MassLynx 4.0 system. The molecular mass of the proteins was determined after MaxEnt1 deconvolution (Waters/Micromass, Milford, USA) of the *m/z* spectra.

For protein sequencing, after reduction of the disulfide bonds and carboxymethylation of the cysteines, the major chitinase was solubilized in 0.2% *Rapi*Gest™ SF (Waters) at a concentration of 4 mg/ml. The protein was digested overnight at 37 °C with sequencing grade trypsin (Promega, Madison, USA), sequencing grade chymotrypsin (Roche Diagnostics GmbH, Penzberg, Germany), sequencing grade endoproteinase Glu-C (Roche Diagnostics) or glycyl endopeptidase (purified from the papaya latex) at an enzyme:protein mass ratio of 1 : 20. After enzymatic digestion, *Rapi*Gest™ SF was destroyed by addition of 50 mM HCl. The water immiscible degradation product was removed by centrifugation prior to mass spectrometry. In addition, hydrolysis of the protein was also performed by microwave-assisted acid hydrolysis [19]. The major chitinase was solubilized in 25% TFA (v/v), 20 mM DTT at a concentration of 1 mg/ml. Ten microliters of the protein solution were irradiated for 10 min in a domestic 750-W microwave oven. The minor chitinase was digested overnight at 37 °C with sequencing grade trypsin (Promega, Madison, USA) after reduction of the disulfide bonds and carboxyamidomethylation of the cysteines.

For both proteins, the peptides generated by the enzymatic or chemical digestions were solubilized in 0.5% TFA (v/v), desalted on $ZipTip_{C18}$ (Millipore, Billerica, USA) and eluted in 50% acetonitrile/1% formic acid (v/v). After processing of the tandem mass spectrometry data using the maximum entropy data enhancement software MaxEnt 3, the peptide amino acid sequences were semi-automatically deduced using the Peptide Sequencing program (Waters/Micromass).

Limited proteolysis. Papaya chitinases (10 mg) were incubated separately with thermolysin (2 mg) or subtilisin Carlsberg (2 mg) in 3 ml 50 mM Tris-HCl buffer at pH 8.0 containing 1 mM KCl at 25 °C for 24 h. The reaction mixtures were then applied onto a (15 × 1.6 cm ID) column of SP-Sepharose pre-equilibrated and eluted with H_2O at pH 5.0. After elution of the proteinases, linear gradients (total volume: 600 ml) from 0 up to 1 M sodium acetate at pH 5.0, were applied. Papaya chitinases (10 mg) were also incubated with glycyl endopeptidase (2 mg) in 3 ml 50 mM phosphate buffer at pH 6.8 containing 10 mM EDTA and 2.5 mM DTT at 25 °C for 24 h. The reaction mixtures were then applied on a (15 × 1.6 cm ID) column of Fractogel EMD Propyl pre-equilibrated with 1800 mM ammonium sulfate. Elution was performed at a flow rate of 45 ml/h and at room temperature by applying a decreasing linear gradient from 1800 to 0 mM ammonium sulfate (total volumes of 600 ml). Chromatographic fractions that eluted like the native chitinases were pooled together and submitted to dialysis against H_2O before to be concentrated by ultrafiltration (membrane cut-off: 10 kDa).

Fluorescence measurements. Fluorescence spectra were recorded at 25.0 ± 0.1 °C using a Perkin Elmer LS 55 fluorimeter, and measurements were made in the concentration range where emission was linear with respect to fluorophore concentrations. In all cases, emission and excitation bandwidths were 5.0 nm each. Emission spectra were the mean of five consecutive scans.

For intrinsic fluorescence emission measurements, excitation was at 295 nm and emission spectra were collected in the 300–520 nm range. Relative fluorescence quantum yields were determined using: $Q_x = Q_s \cdot A_s \cdot S_x / A_x \cdot S_s$, where Q is the emission quantum yield, A is the absorbance at the excitation wavelength and S is the area under the emission curve. The subscripts s and x refer to the standard and to the sample, respectively. The standard used was *N*-acetyl-L-tryptophanamide with a published fluorescence quantum yield of 0.14 [20].

Acrylamide and succinimide quenching experiments were carried out at pH 5.3 in a buffer containing 50 mM phosphate and 157 mM KCl. Solutions were prepared from the buffer, a 120 mM solution of quencher in the buffer and a stock solution of chitinase in water. Fluorescence intensities were measured at 340 nm (excitation at 295 nm). Quenching data were analyzed according to the Stern-Volmer equation for collisional quenching: $F_0 / F = 1 + K [Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher and K is the Stern-Volmer quenching constant [21]. Data were subjected to a linear fit up to 100 mM acrylamide ($r^2 = 0.9969$) or succinimide ($r^2 = 0.9961$), and assuming that static quenching did not interfere under these conditions.

Emission spectra of ANS (excitation at 350 nm) were collected in the 400–550 nm range.

Circular dichroism measurements. Circular dichroism (CD) measurements were made at 25 °C on a Jasco J-710 polarimeter using desalted and filtered protein solutions in water (pH 5.25). Far-UV spectra were collected in the range 185–260 nm in a 0.2-mm circular cell. Near-UV spectra were collected at 240–350 nm in a 1-mm cell. All CD spectra were an accumulation of eight scans recorded at 50 nm/min with a 1-nm slit width and a time constant of 0.5 s for a nominal resolution of 1.7 nm. The far-UV spectra were scaled to molar ellipticities [θ] ($deg \cdot cm^2 \cdot dmol^{-1}$).

Attenuated total reflection Fourier transform infrared spectroscopy. ATR-FTIR spectra were recorded at room temperature on a Bruker IFS-55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector at a nominal resolution of 2 cm^{-1}, and encoded every 1 cm^{-1}. The spectrophotometer was purged continuously with air dried on a FTIR purge gas generator 75-62 Balston at a flow rate of 5.8 L/min. The internal reflection element was a germanium plate (50 × 20 × 2 mm) with an aperture angle of 45°, yielding 25 internal reflections. Samples were prepared by gently evaporating the solvent from 20 or 40 µl of aqueous solutions (pH 5.3) of chitinases using a stream of nitrogen gas [22]. The thin films prepared in this manner were transparent, indicating that no substantial aggregation of the chitinases occurred during sample preparation [23]. For each spectrum, 124 scans were accumulated. The signal from a water-vapor spectrum collected in the same manner was subtracted based on the area of the peak at 1717 cm^{-1}.

Homology modeling of papaya chitinase. A 3-D model of papaya chitinase was built with the automated comparative modeling program Modeller v6.2 [24], using three X-ray structures of barley chitinase as homologous protein templates (protein codes: 1cns, 1dxj and 2baa). The stereochemical quality of the final model was further evaluated with Procheck-NMR [25].

Prediction of the proteolytic sites. Based on the method of Novotny and Bruccoleri [26], the localization of the proteolytic sites was predicted by profiles of accessible contact surface and of backbone flexibility. The X-ray structure of barley chitinase (protein code 1CNS) was used to compute residue backbone flexibility, which was defined as the average crystallographic B values of the backbone atoms N, C, Cα and O. The accessible contact surface was calculated on papaya chitinase 3-D model, using NACCESS program (Hubbard, S. J., 1992, University College, London), by the contact areas on the van der Waals surface of atoms that come into direct contact with a spherical probe of 1-nm radius [27]. Residue contact areas were obtained as the sums of computed atomic contact areas. Contact areas and B factor values for each residue were smoothed by a seven-point moving window algorithm according to the formula:

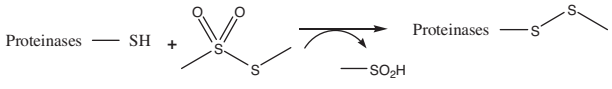
$$N_i = \{7N_i + 6[N_{(i-1)} + N_{(i+1)}] + 3[N_{(i-2)} + N_{(i+2)}] + [N_{(i-3)} + N_{(i+3)}]\}/27$$

where N_i is the considered value at position i and plotted against the residue numbers.

Other spectroscopic and analytic methods. Absorbances were measured with a Perkin Elmer Lambda 45 spectrophotometer. Concentrations of the papaya chitinases were measured spectrophotometrically using, for ε at 280 nm, a value of 51 140 $M^{-1} \cdot cm^{-1}$. This value was calculated from the knowledge of the amino acid sequence of the major chitinase form as previously described [28]. ANS concentrations were also determined using the value of 4950 $M^{-1} \cdot cm^{-1}$ at 350 nm [29]. The phenol sulfuric acid method was used to determine the carbohydrate content of the chitinases using glucose as the standard and lysozyme and albumin (both from chicken egg white) as negative and positive control proteins, respectively [30].

Results

As previously mentioned, papaya latex has a high content of active cysteine endopeptidases. The catalytic competence of these enzymes, *i.e.* papain, chymopapain, caricain and glycyl endopeptidase, is due to the presence of an imidazolium-thiolate ion-pair in their active site pockets. To protect the papaya chitinases from degradation during their purification, papaya cysteine proteinases were first converted into their inactive S-thiomethyl derivatives (Scheme 1).



Scheme 1. Conversion of the papaya proteinases into their S-thiomethyl derivatives.

This chemical modification does not affect the papaya chitinase structures and/or activities, and is reversible since addition of DTT allowed the quantitative regeneration of the essential thiol functions of the proteinases when required.

Purification of the papaya chitinases. Three protein pools denoted I, II and III were separated on a SP-Sepharose Fast Flow column. Pool I, the first to be eluted from the column (Fig. 1), contained papain as the main constituent [31]. Fractions from this pool exhibited no chitosanase activity but they hydrolyzed BAPA and Boc-AAG-pNA.

Fractions from pool II contained glycyl endopeptidase (as demonstrated by its ability to hydrolyze Boc-AAG-pNA but not BAPA) and chymopapain variants (recognizable by their ability to hydrolyze BAPA but much less efficiently Boc-AAG-pNA), while fractions from pool III contained caricain as the major constituent [31]. Two chitinases were isolated from, respectively, pools II and III by sub-fractionation using hydrophobic interaction chromatography (Fig. 2a and b) as previously observed [14, 15]. Their purification was achieved by ion-exchange chromatography as shown in Figure 3a and b. These papaya chitinases exhibited distinct chromatographic elution patterns. The major chitinase fraction, isolated from pool II, appeared more hydrophobic (Fig. 2) but less basic (Fig. 3) than its minor fraction counterpart. The major chitinase represented 5% of the total protein material (80 mg out of 1600 mg). The minor chitinase, isolated from pool III, contained 16 mg of protein material, representing 16.5% of the total chitinase content. The exact proportion of the two papaya chitinases in the latex is not known. To determine this proportion, latex collected in the presence of a thiol-specific reagent (like MMTS) should be used as the starting material.

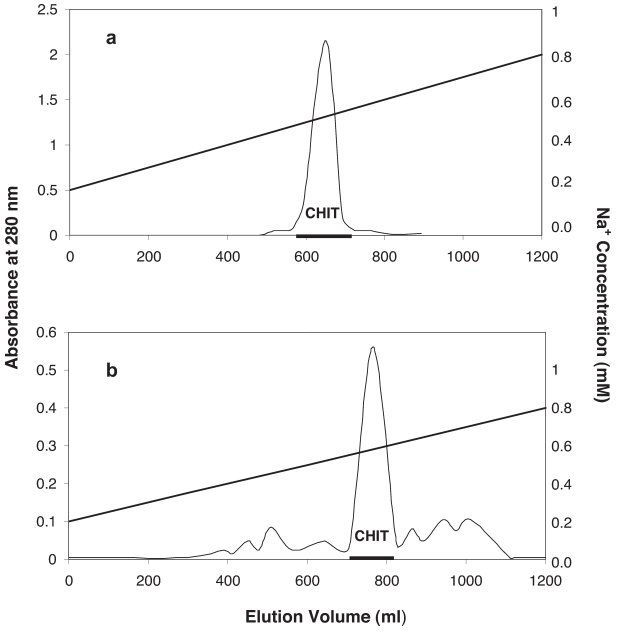


Figure 3. Ion-exchange chromatography of the papaya chitinases on SP-Sepharose: Samples: the pools of the chitinases after the hydrophobic chromatography, concentration by ultrafiltration (membrane cut-off: 10 kDa) and dialysis against 200 mM sodium acetate at pH 5.0; column: 15 × 1.6 cm ID; fractions of 11.25 ml; flow rate: 45 ml/h; eluting buffer: 200 mM sodium acetate buffer at pH 5.0 followed by a linear gradient from 200 to 800 mM sodium acetate buffer (total volume: 1200 ml). Each chromatographic fraction was analyzed by measurements of $A_{280\ nm}$ (continuous trace) and chitinolytic activity. The chromatographic fractions that contained chitinolytic activity are indicated by solid bars.

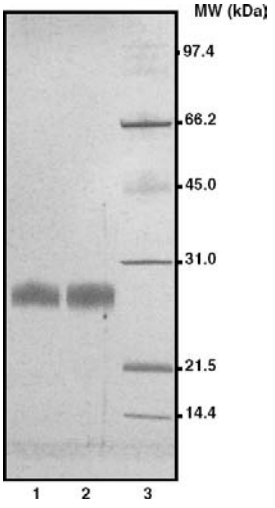


Figure 4. Analysis by SDS-PAGE of the papaya chitinases. Lanes 1 and 2, the major and minor fractions of the papaya chitinases from step 3; lane 3, molecular weight standards.

Several criteria were used to evaluate the homogeneity of the chitinase preparations. Both preparations were devoid of cysteine proteinases as no detectable amidase activities (less than 0.2%, using BAPA and Boc-AAG-pNA as substrates) were observed. SDS-PAGE experiments (Fig. 4) did not reveal any contaminants, even though gels were overloaded and silver stained. The purified papaya chitinases contained virtually no glycans, as shown by the phenol sulfuric acid test.

Determination of molecular masses by mass spectrometry provided values of 26 534 Da and 26 733 Da for the major and the minor chitinases, respectively. A less intense peak with a 114-Da mass increase was observed in the spectra of both proteins, suggesting the presence of a subpopulation containing an additional asparagine residue.

Chitosanase activity. It was not the purpose in the present study to fully characterize the enzymatic activity of the papaya chitinases. These enzymes, however, were found to act on chitin (using chitin azure as substrate) and tetra-*N*-acetylglucosamine (a 2 mM solution of this substrate was completely hydrolyzed by 1 mg enzyme at pH 3.5 and 37 °C within 5 h) showing that these enzymes are true chitinases. These enzymes also used chitosan as a substrate. The hydrolysis of chitosan was studied here at pH 3.5, a pH previously described as being optimum for the hydrolysis of this substrate by spray-dried papaya latex [16]. Using chitosan (75–85% deacetylated) as substrate, the papaya chitinases were characterized by a capacity to liberate 0.082 µmol (major constituent) and 0.087 µmol (minor constituent) of reducing sugars per min and per mg of enzyme.

Sequencing of the papaya chitinases. The major chitinase constituent was cleaved by trypsin, chymotrypsin, endoproteinase Glu-C, glycyl endopeptidase and acid hydrolysis. The digestion products were analyzed and sequenced using tandem mass spectrometry. A nearly complete amino acid sequence of the major papaya chitinase was reconstructed using the overlapping sequences (Fig. 5a). Two amino acids located at positions 155 and 156 in the papaya sequence were not identified as no peptide covering these positions has been detected. This gap was filled using alignment of the papaya sequence on those of other plant chitinases. The signature 2 of members of family GH19 encompasses the region from residues 149 to 159. Position 156 in this region is strictly conserved and occupied by a tryptophan residue in the amino acid sequence of all chitinases belonging to family GH19. A tryptophan residue was thus assigned to position 156 in the amino acid sequence of the major papaya chitinase. Position 155 is occupied in this chitinase family by either leucine, isoleucine, valine, methionine or phenylalanine. A leucine (or isoleucine) residue was thus assigned to this position in the papaya enzyme sequence to fit with the determined molecular mass. Taking into account the formation of three disulfide bonds as expected for all the members of this chitinase family, the calculated molecular mass (26 535 Da) from this amino acid sequence is in perfect agreement with the measured molecular mass (26 534 Da). Analysis of the glycyl endopeptidase digestion products allowed us to identify C-terminal peptides containing an additional asparagine. This additional residue is represented in parentheses in Figure 5a, and is probably associated to the above-mentioned subpopulation at 26 648 Da.

Sequence information was also obtained by tandem mass spectrometry for several tryptic peptides from the minor chitinase constituent. These peptides could be easily aligned on the sequence of the major constituent, as shown in Figure 5b. Noteworthy, out of these 106 positions (more than 40% of the whole sequence) that were examined, 96 residues were found to be strictly identical. Examination of the results in Figure 5 definitively shows that the two papaya chitinases are true chitinases belonging to family GH19 and not chitosanases (regrouped in family GH46) [1].

Limited proteolysis. Proteolysis experiments on papaya chitinases were conducted under experimental conditions (24 h, 37 °C, pH 8, protease/protein substrate ratio 1 : 5) that favored proteolysis by thermolysin, subtilisin or glycyl endopeptidase. Theses proteases were selected because they are known for being considerably stable in the pH range 5–9.5 and because the low cleavage specificity of thermolysin and subtilisin generates numerous short fragments. Both proteinases have thus often been used in limited proteolysis experiments. Despite the optimal conditions that were used to favor proteolysis, the materials eluting as the native chitinases on the SP-Sepharose or the Fractogel EMD Propyl columns, accounted for 70–85% of the starting chitinase materials. Furthermore, their chitosanase activities were comparable to those of the native enzymes. This (preliminary) result strongly suggests that papaya chitinases offer a strong resistance to proteolysis by thermolysin and subtilisin Carlsberg

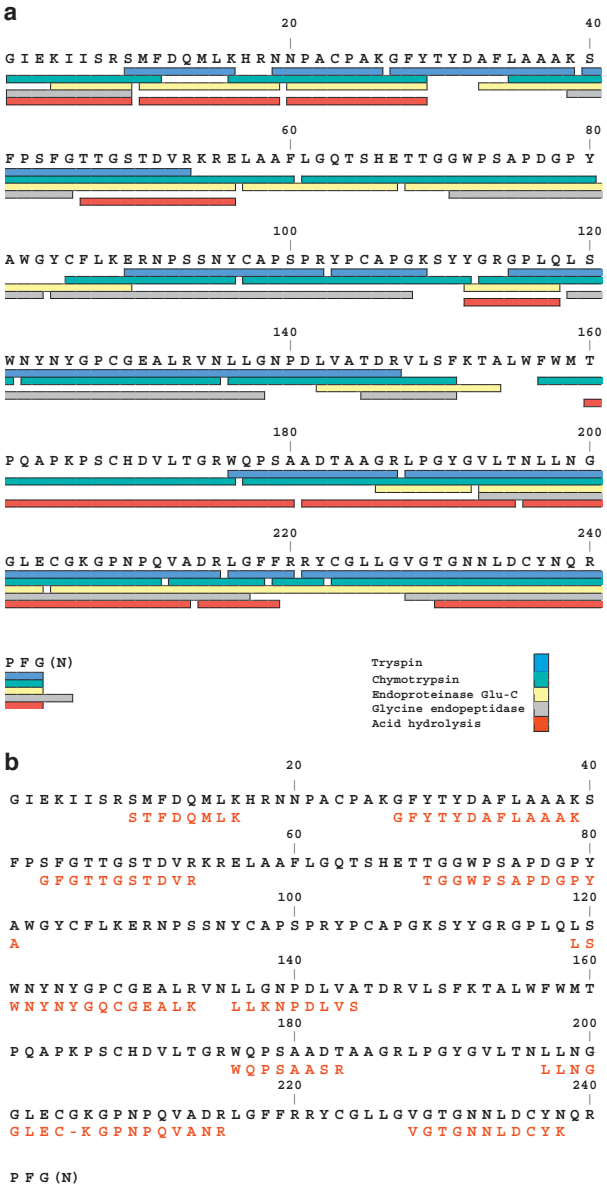


Figure 5. (*a*) Amino acid sequence of the major chitinase deduced from the alignment of the peptides obtained after digestion with trypsin, chymotrypsin, endoproteinase Glu-C, glycyl endopeptidase and acid hydrolysis. Due to the approach used in this study, the leucine and the isoleucine residues were indistinguishable and are represented here by the symbol L except in the domain covering the 30 first N-terminal residues previously identified by Edman degradation [8]. (*b*) Alignment of the tryptic peptides of the minor chitinase (in red) with the major chitinase sequence.

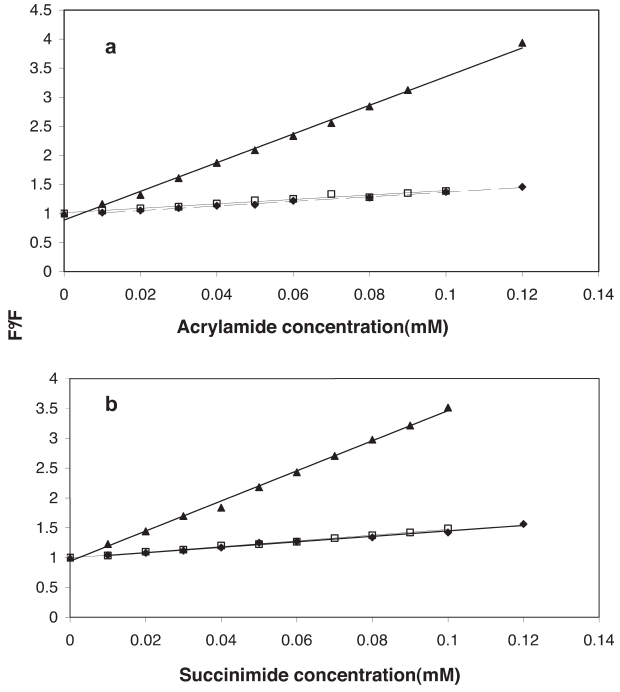


Figure 6. Stern-Volmer plots illustrating the dynamic quenching of the tryptophan residues of the major (diamonds) and the minor (squares) papaya chitinases by acrylamide (*a*) and succinimide (*b*). F and F_0 are the measured tryptophan fluorescence intensities in the presence and absence of quenchers. The quenching of a solution of *N*-acetyltryptophanamide (triangles) is shown for comparison.

(85% of recovery of fully active material). Unexpectedly, both chitinases were more susceptible to proteolysis by glycyl endopeptidase (with only 70% of recovery of fully active material).

Fluorescence studies. The fluorescence emission spectra ($\lambda_{exc.}$ = 295 nm) of both papaya chitinases were characterized by a maximum at 340 nm and quantum yields of 0.019 for the major form and 0.021 for the minor form assuming that the quantum yield of *N*-acetyl-L-tryptophanamide is 0.14 [20].

These characteristics are unchanged within a broad pH interval of 2–10, a pH range in which the papaya enzymes do not bind ANS at all. Below pH 2 and above pH 10, the enzymes become less structured, a conclusion that is essentially based on the observation that the enzymes do bind ANS somewhat more strongly. ANS, indeed, is well known to only binds to partially denatured but not to native or totally unfolded proteins.

Tryptophan exposure to the external solvent was determined, at pH 5.3, by monitoring fluorescence intensity (at 340 nm) in the presence of increasing concentrations (0–100 mM) of the aqueous quenchers acrylamide and succinimide. These neutral quenchers differ in their average molecular radii and are able to diffuse into the interior of the protein and to quench the fluorescence of even partially buried tryptophans [21]. The data were analyzed using Stern-Volmer equation as described in the Materials and methods and reported in Figure 6. Assuming that both chitinases contain six tryptophan residues, the results shown in Figure 6 indicated that, on the average, one out of the six tryptophan residues is accessible to both acrylamide and succinimide. Such a conclusion was based on the observation that the Stern-Volmer constant associated to these chitinases (K_{SV} = 0.004 mM^{-1}) represents only one sixth of the value of the Stern-Volmer constant (K_{SV} = 0.024 mM^{-1}) associated to the model (*N*-acetyl-tryptophanamide) used here. It has been shown that quenching of partially buried indole chains is less efficient than quenching of totally accessible indole rings. This has been observed with both acrylamide and succinimide. The loss of quenching efficiency, however, is more pronounced in the case of succimimide [21]. Taking into account that the efficiency of the quenching by succinimide was comparable to the quenching by acrylamide, as shown in Figure 6, it is suggested that, for both chitinases,

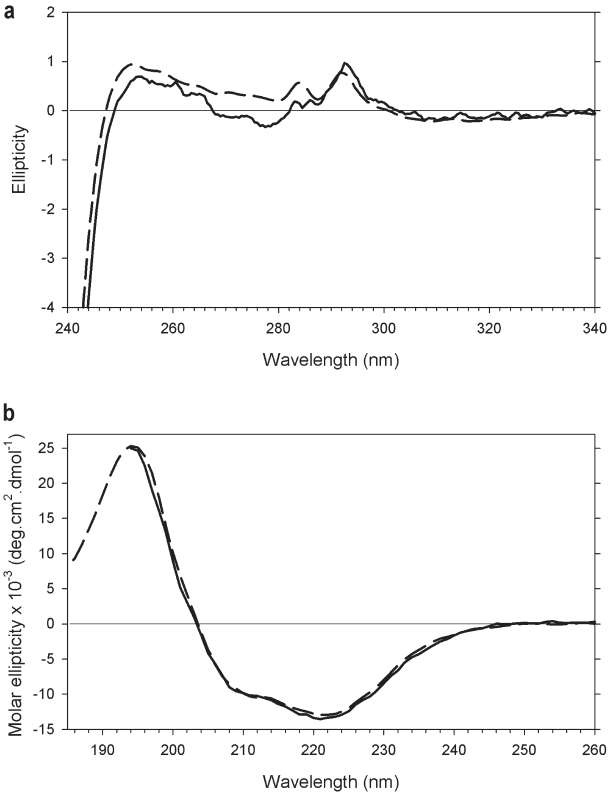


Figure 7. Circular dichroism (CD) spectra of the major (continuous line) and the minor (dotted line) in the aromatic (*a*) and the far-UV regions (*b*). Far-UV spectra were collected in the range 185–260-nm in a 0.2-mm circular cell. Near-UV spectra were collected at 240–350 nm in a 1-mm cell. All CD spectra were an accumulation of eight scans recorded at 50 nm/min with a 1-nm slit width and a time constant of 0.5 s for a nominal resolution of 1.7 nm. The spectra in the far-UV region were scaled to molar ellipticities [θ] ($deg \cdot cm^2 \cdot dmol^{-1}$).

a small number of tryptophan would be highly accessible to the solvent, while the majority would be deeply buried. Such a conclusion however is questionable since it implies that the six tryptophan residues equally contribute to the total fluorescence emission.

CD and infrared spectroscopic studies. Figure 7 shows the CD spectra of the papaya chitinases in the aromatic (Fig. 7a) and the far-UV (Fig. 7b) regions, both recorded at pH 5.3. The spectra in the aromatic region (250–350 nm) exhibit several positive and negative signals that can be assigned to tryptophan and tyrosine side chains as well as to disulfide bonds. In the far-UV region, the spectra of the major and of the minor forms are quite superimposable. These far-UV spectra were analyzed using the CONTIN, the SELCON and the CDSSTR methods with 48 reference proteins [32]. The results of this analysis indicated that α helices (40%), β sheets (15%) and turns (18%) contribute to the secondary structure of the two papaya chitinases. Analysis [22, 23] of the ATR-FTIR spectra (shown in Fig. 8) provided very similar results for the content α helices (37–43%) and turns (15%) and a slightly higher content of β sheets (23%).

Homology modeling of papaya chitinase. The major papaya chitinase was modeled using X-ray barley chitinase structures as homologous templates. The equivalent resolution according to H-bond energy criteria was 1.6 Å, with only one residue in the disallowed regions of Ramachandran map. The tertiary structure of papaya chitinase model was very similar to that of family GH19 chitinases, consisting of three disulfide bonds, one short β sheet and ten α helices [3, 4].

Discussion

Two similar but distinct chitinases have been separated and purified to homogeneity from the latex of the tropical species *Carica papaya*. Both belong to chitinase family GH19 as shown by their chitinase and chitosanase activities as well as by their amino acid sequences.

On the basis of the completely determined amino acid sequence of the major form and the partial sequence of the minor species (40% of the full-length sequence), the degree of homology between the two amino acid sequences may be expected to be high. According to sequence homology, it can be predicted that both papaya chitinases would adopt the same folding pattern [33].

The two isoenzymes are indeed structurally related and share several physicochemical characteristics. Both have fluorescence emission spectra characterized by a maximum at 340 nm. Their indole rings are quite similarly quenched by acrylamide and succinimide. Also, the two chitinases could not be distinguished on the basis of their CD spectra in the far-UV region nor on the basis of their ATR-FTIR spectra. Both enzymes are stable over a wide pH range from 2 to 10 and hydrolyze chitosan at a comparable rate.

Yet, they adopt different chromatographic behaviors. In this regard, the major form appears somewhat less basic but more hydrophobic than its minor counterpart. The minor form is also characterized by a higher molecular mass and a higher (10%) quantum yield of fluorescence emission than that of the major constituent. Despite the fact that the degree of homology between the two amino acid sequences is expected to be high, as previously mentioned, several peptides derived from the major and minor constituents were found to clearly differ. Altogether these observations show that the two papaya isoenzymes are distinct enzymes.

Several observations suggest that the papaya chitinase(s) described here had been previously isolated and char-

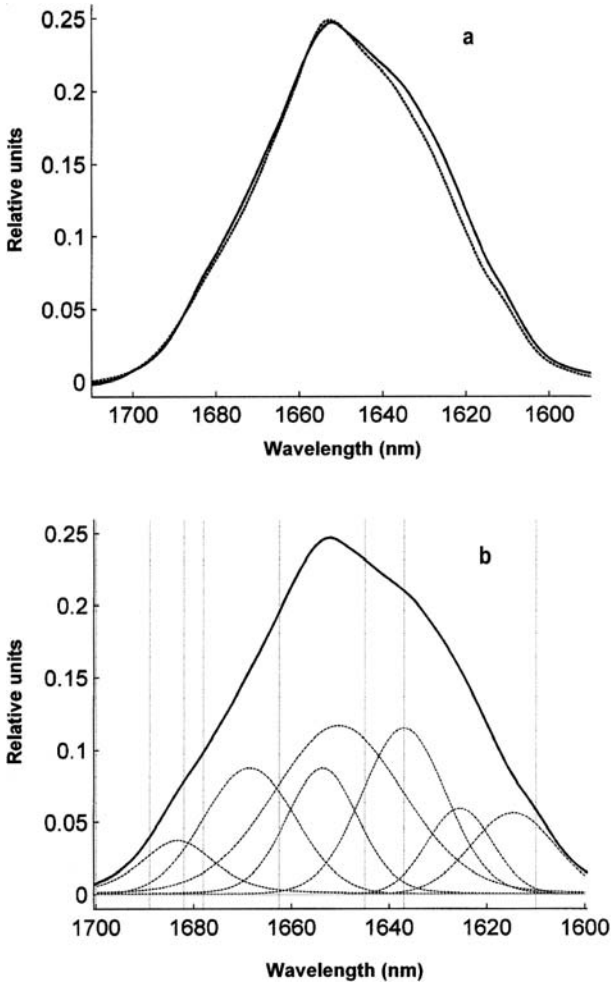


Figure 8. (*a*) Infrared spectra in the amide I region of major (solid line) and minor (dotted line) chitinases. (*b*) Results of the curve fitting process on the major chitinase spectrum; vertical bars correspond to the limits of the different secondary structures.

Table 1. Amino acid composition of papaya lysozyme and chitinase (major constituent) *.

Amino acid	Lysozyme	Chitinase	Amino acid	Lysozyme	Chitinase
K	11	10	G	28	29
H	3	3	A	22	21
R	14	15	V	8–9	8
D + N	23–24	24–25	M	4	3
T	14	14	I + L	24–25	26
S	17	15	Y	14	13
E + Q	12	13	F	13	12
C	8–9	9	W	7–8	6
P	19–20	22	Total	241–247	244

* The values provided by Howard and Glazer [30, 31] were recalculated to account for a total of approximately 244 amino acid residues.

acterized [as plant lysozyme(s)] by Howard and Glazer several decades ago [34, 35], and later on by Beintema and coworkers [36]. The papaya lysozyme and the papaya (major) chitinase, indeed, exhibit bacteriolytic activity against *Micrococcus lysodeikticus* [34, 36] and chitinase/chitosanase activity ([34], this work). The N-terminal amino acid sequence, GISKI, that had been determined for papaya lysozyme [35] does not greatly differ from that, GIEKI, found here for the major chitinase constituent (Fig. 5). Furthermore, it has been reported that papaya lysozyme, like the papaya chitinases which belong to family GH19, also catalyzes the hydrolysis of chitooligosaccharides with overall inversion of anomeric configuration [5, 37]. We finally noted that the amino acid composition of papaya chitinase (major constituent) that can be calculated from its amino acid sequence (Fig. 5) is very close (see Table 1) to that previously established for papaya lysozyme [34].

Alignment of the sequence of papaya chitinase (major form) on that from barley seeds [38] does not require any gap and shows that both the percentage of identity (73%) and the percentage of homology (84%) between the two plant chitinases are quite high. Such a high sequence homology leads obviously to the adoption of similar folds [33], a prediction that is fully supported by our papaya chitinase model, which shows that the tertiary structure of papaya chitinase model was very similar to that of family GH19 chitinases, consisting of three disulfide bonds, one short β sheet and ten α helices [3, 4].

Applying the program NACCESS on this model, it was shown that, out of the six tryptophan residues of the papaya chitinase, four (tryptophan residues 82, 156, 158 and 176) would be completely buried, while tryptophan residues 72 and 121 would be partly (around 50% each) accessible to the solvent, an observation that is supported by our fluorescence emission quenching analysis and which, in turn, provided a good validation of our 3-D model of the protein.

To explore how the papaya chitinases resist proteolysis by thermolysin, subtilisin and glycyl endopeptidase, a nicking-site profile was computed for this enzyme (Fig. 9). Typically, proteolytic sites in proteins are found within flexible loop regions that are exposed to the protein surface and are outwards regions of regular secondary structures, especially outwards of β-sheet stretches [39]. Regions identified as highly susceptible to be cleaved by proteases were located in nine loops, encompassing residues 16–28, 40–46, 70–80, 88–98, 98–110, 134–148, 160–170, 174–180 and 206–210. Our analysis reveals that proline residues (22 out of a total of 243–244 amino acid residues) present in papaya chitinase (major form) are all positioned in these regions. In addition to the proline residues that are conserved between barley and papaya chitinases (P42, P76, P99, P104, P107, P140, P164, P166, P178), papaya chitinase contains nine additional prolines (P21, P24, P73, P79, P92, P101, 161, P208, P210) perfectly located so that the nine potential cleavable loops contain at least one proline residue (Fig. 9). Substitution of an amino acid residues by a proline is known, from protein engineering studies, to increase the proteolytic resistance [40, 41]. This strategy called the 'proline-concept' was probably used by evolution to offer a better resistance of papaya chitinase to the extreme conditions found in papaya latex in the presence of fully active cysteine proteases.

Chitinases, generally speaking, seem to be resistant to proteolysis. Several commercial crude sources of proteases have been shown to be able to depolymerize chitosan because they contain chitinases. This is not only the case for the already mentioned papain, but also for, for instance, ficin [42], bromelain [43] and pepsin [44].

Another indication was provided by the rye seed chitinase-a (RSC-a) constituted by a N-terminal cysteine-rich chitin-binding domain, called the hevein domain, and a C-terminal catalytic domain; these two domains are linked by a flexible segment known as the hinge region. It has been shown that, unlike the catalytic domain, the hevein domain could be easily reduced (by 2-mercaptoethanol) in the absence of denaturing agent and that the hinge region was the sole domain susceptible to cleavage by thermolysin [45].

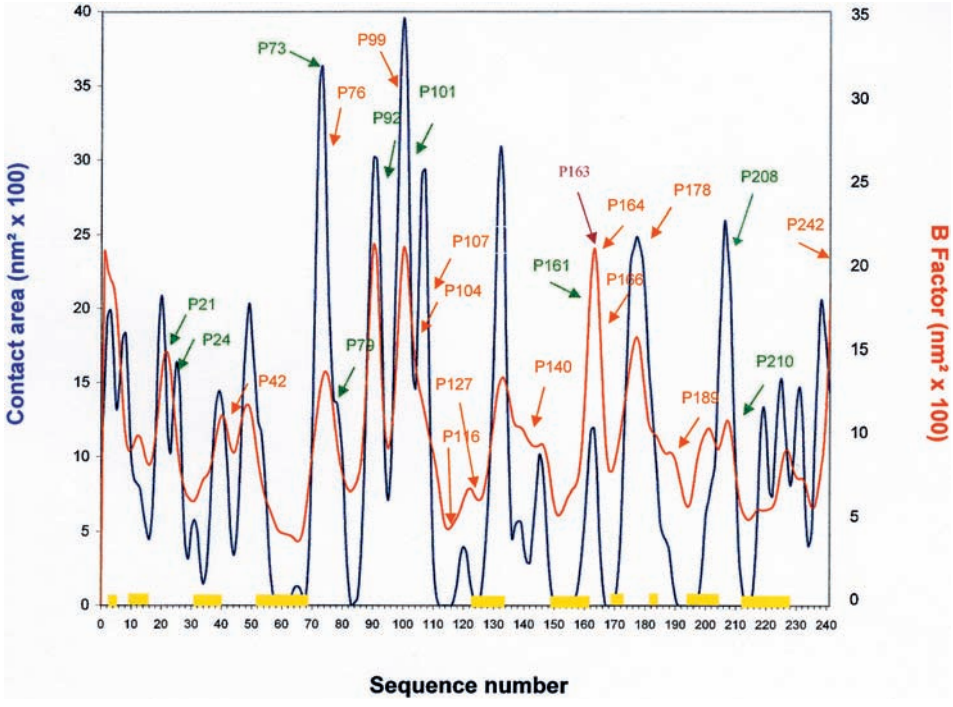


Figure 9. Large probe accessibility profile of papaya chitinase 3-D model and average backbone temperature factors of the X-ray 3-D structure of barley chitinase. Blue line: the smoothed contact area profile computed with a 1-nm radius probe, red line: smoothed average backbone crystallographic B value profile of the backbone atoms. The α helices are shown above the sequence number by yellow bars. Proline positions are indicated by arrows, in red for proline residues of barley chitinase that are conserved in the papaya chitinase sequence and in green for the additional proline residues that are present in papaya chitinase.

Finally, it was also expected that both papaya chitinases should contain, like barley chitinase, a pronounced α-helical secondary structure, a prediction that is fully supported by the CD and ATR-FTIR spectra. The α-helix content that is predicted by both methods is in excellent agreement with that deduced from the X-ray structures of the barley and jack bean chitinases [3, 4]. In marked contrast to barley and jack bean chitinases that do not contain β-sheet segments, the CD and ATR-FTIR spectra predicted a high β-sheet content (15–23%) for the papaya chitinases. Such a prediction is unexpected taking into account the high degree of homology between the papaya and the barley chitinases amino acid sequences. This prompted us to re-examine our CD and ATR-FTIR spectra to see whether there was another possible interpretation.

A careful examination of the barley chitinase structure (PDB code: 2BAA) showed that several loop regions adopted long extended conformations. In fact, a rough anti-parallel β-sheet comprising three β strands (residues 87–89, 112–114 and 118–120) was seen. Two of these (residues 112–114 and 118–120) have been observed previously [3]. Three similar β strands have also been identified in the structurally related chitinase isolated from *Streptomyces griseus* that also belongs to family 19 chitinases [46], leading to a β-sheet content of around 7% in both chitinases, and thus far less than the 15–23% predicted here. The lack of regular hydrogen-bonding patterns in these short β-sheet segments prevents their detection by conventional secondary structure assignment programs, like DSSP [47].

Furthermore, an analysis of the backbone φ-ψ torsion angles revealed the presence of short polyproline II structures in two proline-rich regions of the barley X-ray structure (residues 104–107 and 163–167). The larger number of proline residues present in the papaya chitinase sequence is expected to increase the propensity of proline-rich loop regions to adopt polyproline II conformations [48].

The CD spectra in the far-UV region of all β-sheet proteins and of polyproline II-rich proteins are characterized by negative peaks around 215 nm and 207 nm, respectively [49, 50]. Such subtle differences are expected to be largely masked in the case of papaya chitinases in view of their high content in α-helix. ATR-FTIR is generally recognized as the most appropriate spectroscopic technique for detection of β-sheet structures [51]. The deconvoluted spectra of proteins rich in such structures exhibit a strong absorbing peak centered between 1613 and 1637 cm^{-1}. The imino group of proline is known to strongly absorb near 1630 cm^{-1} [52]. Therefore, β-sheet fragments from polyproline II structures cannot be really distinguished

by any of these spectroscopic techniques. Polyproline II structures are known to contribute to the stability of proteins [48]. This point thus deserves further attention and this has prompted us to attempt crystallization of the papaya chitinases.

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