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# Detection of three wound-induced proteins in papaya latex

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**Abstract**

The effects of routine mechanical wounding for latex collection from unripe fruits of the tropical *Carica papaya* tree were investigated. For that purpose, the protein composition of three different latexes was analyzed. The first one, commercially available, was provided in the form of a spray-dried powder, the second one was harvested from fully grown but unripe papaya fruits that are regularly tapped for latex production and the last one, was obtained from similar fruits wounded for the first time. Repeated mechanical wounding was found to profoundly affect the protein content of the latex inducing, among others, activation of papain. Regularly tapped latexes also accumulated several low molecular weight proteins not yet identified, as well as three proteins identified as a trypsin inhibitor, a class-II chitinase and a glutaminyl cyclase on the basis of their enzymatic or inhibitory activities and chromatographic elution profiles. This latter was found here, for the first time, to be a wound-induced protein. The roles of these proteins in the plant defense mechanism are discussed.


*Keywords:* *Carica papaya*; Caricaceae; Plant defense; PR-proteins; Glutaminyl cyclase; Trypsin inhibitor; Chitinase; Latex; Wounding

## 1. Introduction

Plants are continuously exposed to a variety of environmental stresses. Considering the drawbacks to be anchored to the ground and to be deprived of a circulating immune system, plants have developed various sophisticated strategies to protect themselves against pathogens or predators, such as insects, herbivores as well as against abiotic stresses. The different defense mechanisms are known to be either constitutive or inducible.

Plant defense reactions are generally classified into direct or indirect ones. Direct defenses make the plant more resistant to further attacks (Hammerschmidt and Shultz, 1996). For example, hypersensitive reactions constitute an effective defense mechanism. In this case, a rapid death of the plant cells at the infection site leads to a restricted colonization of the potential pathogen. This sacrificed site helps then the plant to prevent further pathogen spread to the unaffected tissues (Grant and Mansfield, 1999). By analogy to mammalian apoptosis, in plants, the hypersensitive reaction is also a form of programmed cell death in plants (Greenberg, 1997; Lam et al., 1999; Heath, 2000). Other examples of direct defenses include production of secondary metabolites and defensive proteins (Bowles, 1990; Bohlmann and Appel, 1991; Bohlmann, 1994; Broekaert et al., 1995, 1997). Secondary metabolites, such as alkaloids and especially nicotine, could act as defensive toxins by directly acting on the herbivore's nervous system (Wink, 1998; Shoji et al., 2000). Proteins with enzymatic activity could also have a direct role in plant defense. These include antifungal enzymes, such as chitinases, glucanases (Bowles, 1990; Raikhel et al., 1993) and protease inhibitors. These latter proteins target the major proteolytic enzymes of the aggressors, therefore acting as anti-digestive or anti-nutritive bio-molecules (Koiwa et al., 1997).

In the case of plants producing latex, mechanical wounding leads to an abrupt release of latex. This latter rapidly coagulates, sealing the injured area and preventing further entry of the pathogens into the phloem. This may thus constitute a very efficient defense mechanism. For instance, *Hevea brasiliensis*' latex

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coagulation occurs as a result of lutoid bodies (vacuolar organelles) bursting followed by subsequent electrostatic interactions between the negatively charged rubber particles and the positively charged proteins released upon wounding (d'Auzac and Jacob, 1989). The presence of the so-called pathogenesis-related proteins (PR-proteins), e.g. glucanases and chitinases/lysozymes (Van Loon and Van Strien, 1999), suggests that these latter ones play an active role in the defense of the plant against wounding and/or insects and pathogens attacks. For *Carica papaya*, the mechanism leading to latex coagulation is not yet precisely known although there exists a direct relation between this process and the activation of various papaya proteinases present in the latex as inactive precursors (Silva et al., 1997; Moutim et al., 1999). This latex also contains many proteins belonging to the PR-proteins family (El Moussaoui et al., 2001). These proteins were identified as a basic class-II chitinase (Azarkan et al., 1997), a serine proteinase inhibitor (Odani et al., 1996), a cystatin (Song et al., 1995), a β-1,3-endoglucanase (Wilson, 1974) and a lysozyme (Smith et al., 1955; Howard and Glazer, 1967). In addition, four well-known cysteine proteinases are present in this latex at a concentration of about 1 mM (Oberg et al., 1998; Azarkan et al., 2003). Besides all these proteins, a glutaminyl cyclase (papaya QC or PQC), which catalyzes the cyclization of N-terminal glutamine of polypeptide chains into pyroglutamyl moieties, has been isolated from papaya latex (Messer and Ottesen, 1964, 1965; Gololobov et al., 1994, 1996; Zerhouni et al., 1997, 1998). While the role of mammalian QCs is well established (Sykes et al., 1999), that of PQC remains quite enigmatic.

On the other hand, emission of volatile molecules from plant tissues, which is considered as an indirect defense reaction (Baldwin et al., 2001), has been recognized for many years as an important feature capable to perturb plant-insect interactions. This involves both, pollinators attraction and deterrence of herbivores (Pichersky and Gershenzon, 2002). The emission of specific volatile subtances could also act as natural attractants of carnivorous enemies and parasitoids of herbivores (De Moraes et al., 1998; Dicke et al., 1999) and further as chemical messengers to alert neighbouring plants (Karban et al., 2000; Bruin and Dicke, 2001; Karban and Maron, 2002).

The enzymatic reaction catalyzed by PQC is accompanied by the release of volatile ammonia, a molecule that could adversely affect the growth of micro-organisms (Banuelos et al., 2000). It has thus been suggested that PQC could also possibly be involved in the plant defense mechanism (El Moussaoui et al., 2001).

In order to confirm this hypothesis and to gain better insights into the exact role played by this enzyme, in particular within the plant defense context, we analyzed the protein compositions of two types of papaya latexes collected from either regularly injured plants, or either from newly wounded plants.

## 2. Results and discussion

### 2.1. Electrophorosesis of the whole protein fraction

SDS-PAGE experiments, performed on the whole protein fraction and shown in Fig. 1, revealed major differences in the protein content of the latexes depending on whether they originate from untapped or regularly tapped trees. The major difference concerned the protein material associated with low molecular weights (below 14,000 Da) that is virtually absent in untapped fruits but very abundant in regularly tapped trees. Unfortunately, with the possible exception of papaya cystatin, the individual constituents of this protein fraction in papaya latex have not yet been identified (Azarkan et al., 2003). It becomes clear however that in a context of the defense mechanism of *Carica papaya*, these constituents, indubitably, deserve further attention. Another noticeable difference was associated with the protein band attributed to PQC (33,000 Da) (Zerhouni et al., 1998) also absent in the latex of untapped fruits but present in the latex of regularly tapped fruits. Finally, mention should be made that both latexes collected from regularly tapped fruits exhibited only slight differences that could possibly be attributed to the fact that one of them had been processed in a manufacture.

### 2.2. Cation-exchange chromatography

The fractionation on a cation-exchange support also revealed significant differences between the three latexes

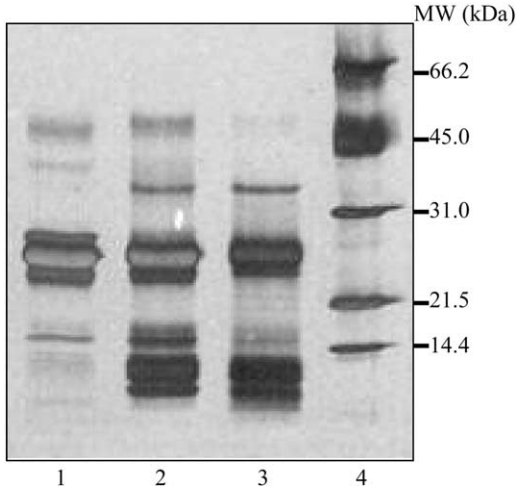



Fig. 1. SDS-PAGE experiment using the silver staining procedure. Lane 1: whole protein fraction from latex of papaya fruits tapped for the first time, lane 2: whole protein fraction of regularly tapped fruits latex, lane 3: whole protein fraction from commercially available latex and lane 4: molecular weight standards.

studied (Fig. 2). The protein fractions were usually grouped into three pools. The first pool (denoted pool I) contained the papain enzyme as the main constituent, the second pool (pool II) was essentially composed of chymopapain and glycyl endopeptidase and the third pool (pool III) contained caricain and, as minor components, a class-II chitinase, a glutaminyl cyclase and a trypsin inhibitor (Azarkan et al., 2003). Both, the protein composition and the enzymatic activities were affected depending on the origin of the latex. For instance, while the peak of the papain component (pool I) displayed very high amidase activity in the latex provided by enzymase (Fig. 2a) and in the latex prepared from repeatedly injured fruits (Fig. 2b), in the latex from newly tapped fruits, this peak was almost inactive and contained only few protein material. Examination of pools II also revealed dramatic changes in the amidase activity and in the protein profiles. Finally, observation of pools III indicated that while the amidase activity profile was different according to the latex investigated, the protein profile seemed to be only slightly affected. The significant differences affecting pools I and II will not be further investigated here. In this paper, we focused our investigation on the composition of the pools III exclusively. First because it normally contained PQC but particularly because exhaustive analysis of each difference observed between the three latexes requires many efforts and is time consuming. This work is currently under investigation, and will be published separately.

### 2.3. Electrophoresis of pools III

After cation-exchange chromatography, the three pools III isolated from the different latexes were further analyzed by means of SDS-PAGE using Coomassie Brilliant Blue as the staining technique. The results, sustained by this experiment confirmed those obtained after SDS-PAGE experiments when starting from whole latex protein fractions and using silver staining (Fig. 1). Indeed, while the PQC band was clearly visible on the gel in the case of regularly harvested latexes, in the latex harvested from newly wounded fruits, the PQC band became detectable but only after the gel was overloaded with the protein material of pool III. The bands corresponding to the two other minor proteins, ~27,000 Da for the class-II chitinase and ~24,000 Da for the proteinase inhibitor, were not directly distinguishable on the gel partly because their molecular weights were very close to that (~26,000 Da) of caricain, the most abundant constituent present in pool III.

### 2.4. Hydrophobic interaction chromatography

In order to gain more insights on pool III protein composition, this material was further fractionated according to the hydrophobic character of its components, by using hydrophobic interaction chromatography. Under the experimental conditions used here, caricain was eluted in the void volume of the column well before the gradient was applied. The results obtained after elution of pools III on the Fractogel EMD propyl 650 (S) hydrophobic gel medium (Fig. 3a, b and c) unambiguously demonstrated that the protein elution profile significantly differed according to the latex used as the starting material, reflecting the numerous differences between repeatedly injured unripe papaya fruits and those injured for the first time.

As shown on hydrophobic chromatography profiles of regularly wounded plants (Fig. 3a and b), the three minor proteins present in pool III can be separated as a function of their increasing hydrophobicity. Their molecular weights, enzymatic activities or inhibitory activity confirmed that these three proteins were the class-II chitinase, the glutaminyl cyclase and the trypsin inhibitor (see below). In contrast, the hydrophobic profile of newly wounded plants differed significantly (Fig. 3c). Neither the glutaminyl cyclase nor the trypsin inhibitor were visible on this chromatogram, although the former protein was slightly visible when overloading the electrophoresis gel with the pool III material and using the Coomassie Brilliant Blue in the staining procedure. Furthermore, the class-II chitinase, indicated by the arrow in Fig. 3c, can also be detected according to its molecular weight and enzymatic activity but in a much smaller amount comparatively to the quantity obtained with regularly wounded plants. To estimate the relative chitinase quantities in the different latexes, we performed a deconvolution of the chromatographic profiles (Fig. 3) using PeakFit program. The analysis of the surface areas of the chitinase peaks indicated that this protein represented less than 20% in the latex from newly tapped fruits compared to the latexes of regularly wounded fruits.

### 2.5. Trypsin inhibitory activity of pools III

We also assayed the inhibitory activity of the three different pools III against the proteolytic enzyme trypsin. The results obtained clearly demonstrate that while both latexes of routinely wounded fruits had nearly the same efficiency to inhibit this proteinase, no trypsin inhibitory activity could be measured in the latex obtained from newly injured fruits (results not shown).

It is important to note that the comparison between the three chromatograms is made possible by the fact that they were obtained under the same experimental conditions and with similar initial protein quantities (quantified by using the Modified Lowry Protein Assay Kit or by measuring the absorbance at 280 nm).

The hydrophobic profile of newly wounded plants (Fig. 3c) also showed that an unidentified protein

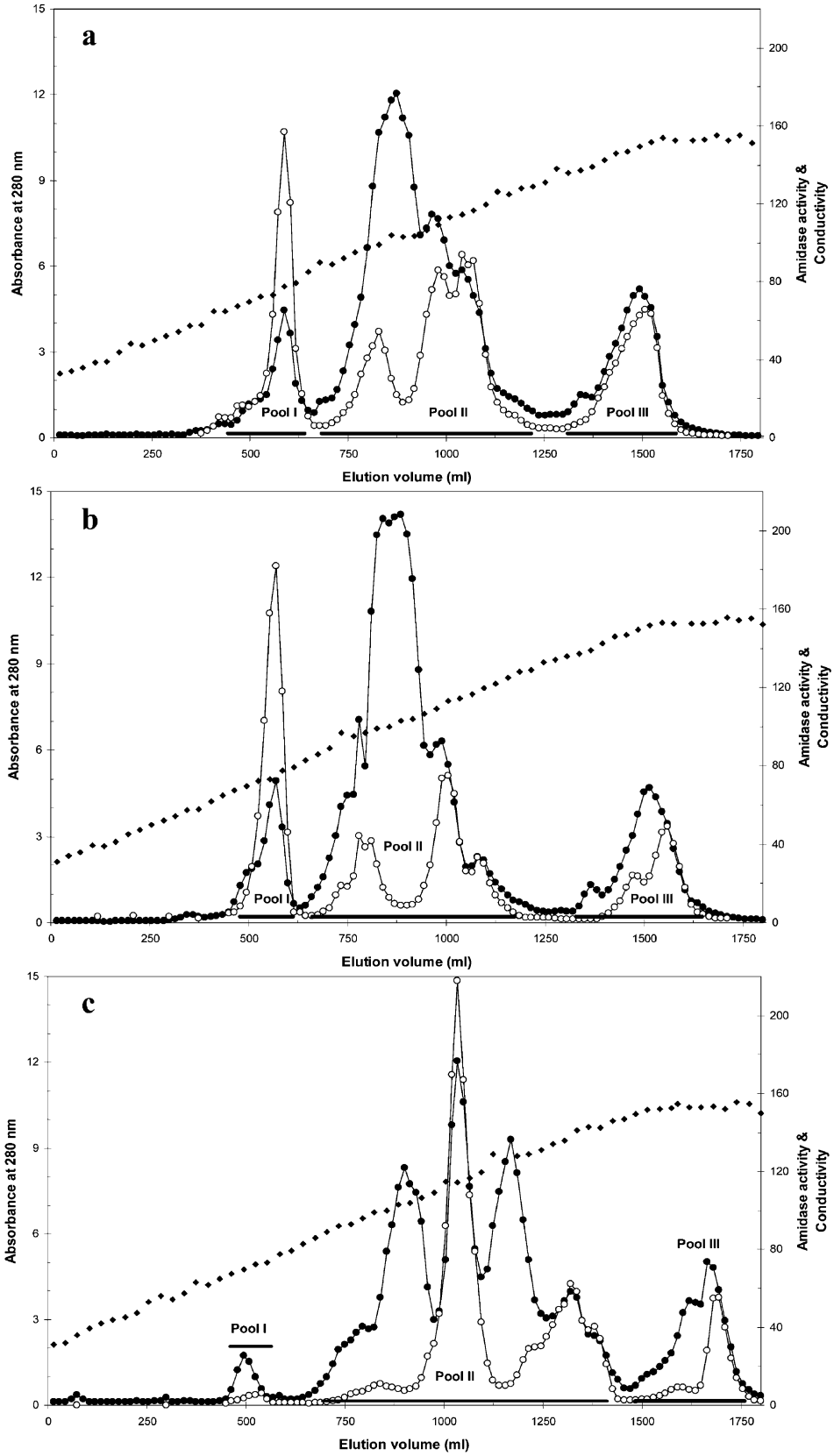



Fig. 2. Fractionation on SP-Sepharose Fast Flow column of the whole papaya latex soluble protein fraction obtained from different latexes (a) commercially available papaya latex, (b) regularly tapped fruits or (c) firstly wounded fruits. Fractions of 15 ml were collected at a flow rate of 60 ml/h and analyzed by measurement of $A_{280}$ (•), amidase activity (nkat/chromatographic fraction, ○) and conductivity (mS×1/5, dotted trace). The fractions indicated by the solid trace, denoted pool III, were selected for further characterization.

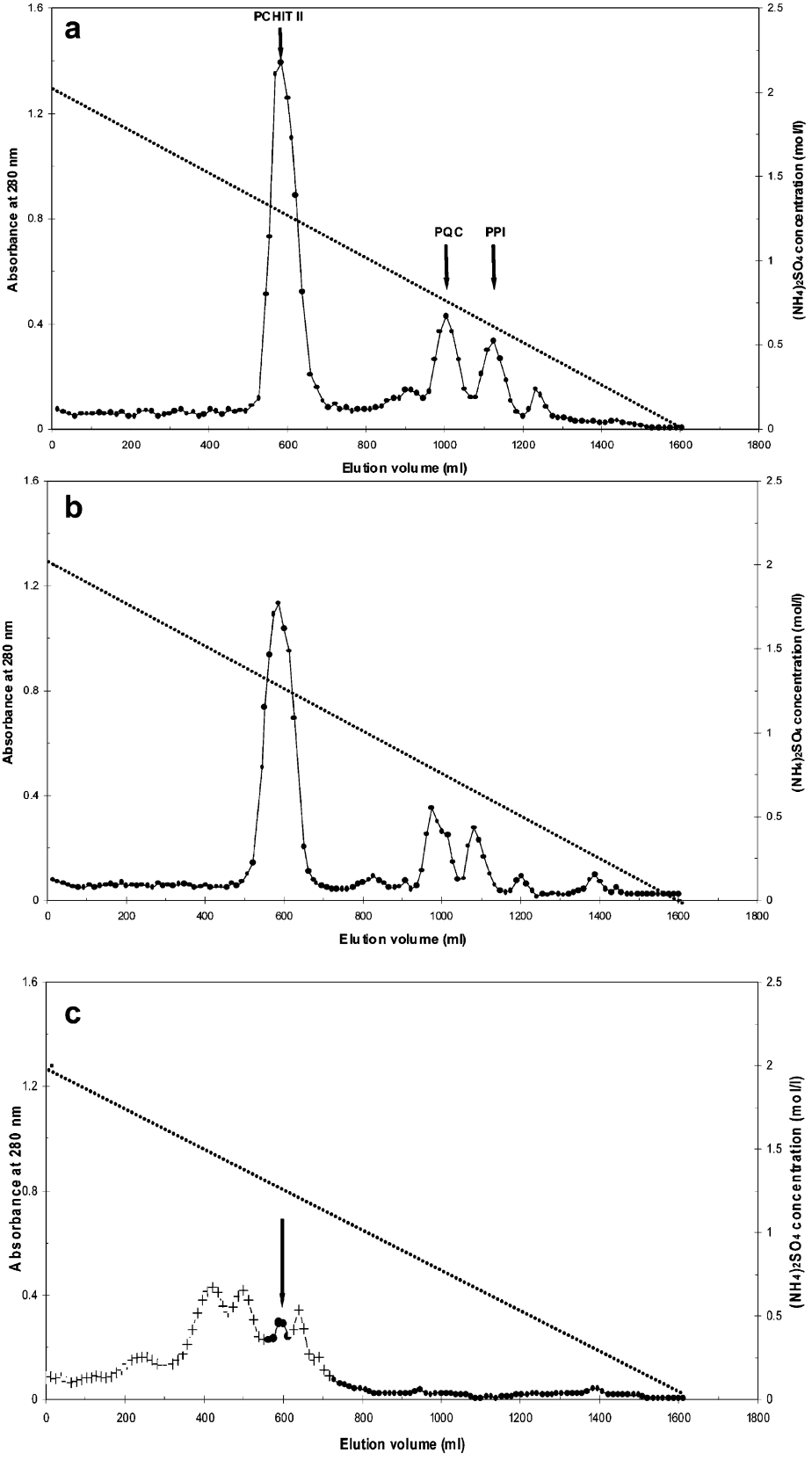



Fig. 3. Hydrophobic interaction chromatography on Fractogel EMD propyl 650 (S) of (a) pool III from commercially available papaya latex, (b) pool III from latex of regularly tapped fruits and (c) pool III from latex of fruits injured for the first time. Fractions of 15 ml were collected at a flow rate of 60 ml/h and were analyzed for $A_{280}$. More details are available under the experimental section.

material, indicated by the "+" symbols, was eluted near to chitinase. Interestingly, this unidentified protein material was absent from the latexes of regularly injured papaya fruits. SDS-PAGE analysis of this material revealed the presence of molecular species migrating with apparent molecular weights ranging from 10,000 to 18,000 Da. Furthermore, neither enzymatic activities (of cysteine proteinase, of chitinase and of glutaminyl cyclase) nor proteinase inhibitory activity could be associated with this material. The identification and characterization of this protein material is currently under investigation. Note that the results obtained with the latex harvested from firstly wounded fruits have been confirmed with other latex samples which were collected from other trees also tapped for the first time.

It is not surprising to see that a chitinase and a proteinase inhibitor were found to accumulate in papaya latex as a result of wounding since they are members of the PR-proteins and are thus implicated in the plant defense reactions. In fact, chitinases, combined or not with other hydrolases such as β-glucanases, are well known to be able, by hydrolysing polymers of β-1,3-glucan and chitin present as main constituents in many fungi, to inhibit the growth of several classes of micro-organisms. In this respect, a β-1,3-glucanase has also been identified in the latex of *C. papaya*. However, this enzyme was only partially purified due to its high proteolytic susceptibility (Wilson, 1974).

On the other hand, it is now well established that proteinase inhibitors are among front line defenses of plants against herbivorous insects and pathogens (Ryan, 1990; Zhao et al., 1996; Koiwa et al., 1997; Rahbé et al., 2003). It is generally admitted that these defensive proteins act by targeting, e.g., the herbivorous insect digestive proteolytic enzymes (Koiwa et al., 1997). Papaya latex contained at least two different proteinase inhibitors. A Kunitz-type trypsin inhibitor (Odani et al., 1996) and a cystatin whose amino acid sequence has been predicted from a cDNA clone using a papaya leaf cDNA library (Song et al., 1995). Until now, papaya cystatin has not been isolated from papaya latex, where it probably forms tight complexes with the cysteine proteinases (Azarkan et al., 2003). One can suggest that these two inhibitors, and the two above-cited hydrolases as well, may be implicated in the plant defense reactions. However, we had no clear evidence for induction of β-1,3-glucanase and cystatin after wounding, probably for the reasons mentioned above. On the other hand, as papaya cystatin is a potent inhibitor of the four cysteine proteinases, including glycyl endopeptidase (Song et al., 1995), its role as a regulator of the proteinase activity may thus not be ruled out.

There are many lines of evidence supporting the present findings. In the case of proteinase inhibitors for example, it has been demonstrated that wounding treatment strongly induced the expression of a trypsin inhibitor gene in the fig tree latex (Kim et al., 2003). Furthermore, high levels of a proteinase inhibitor mRNA accumulate in response to mechanical wounding of maize leaves (Tamayo et al., 2000). Finally, it has been reported that a trypsin inhibitor activity can be induced upon wounding of tobacco and tomato plants (Jongsma et al., 1994) and that several proteinases isoinhibitors can be isolated from leaves of pepper (*Capsicum annuum*) after wounding treatment (Mourra and Ryan, 2001). Futhermore, chitinases can also be induced as a result of wounding. This is the case of a basic class-I chitinase present in the fig latex (Kim et al., 2003), and of a chitinase isolated from grapefruit peel tissue (Porat et al., 1999), as well as an acidic class-III chitinase found in foliar explants of *Coffea arabica* L. (Rojas-Herrera, 2002).

The observation that papaya glutaminyl cyclase was also inducible in the latex harvested from regularly tapped unripe papaya fruits, remained however unexpected. As far as we know, the latex of papaya is the only vegetable source from which a glutaminyl cyclase has been isolated, although several nucleotide sequences for other plant species have already been identified to encode hypothetical papaya glutaminyl cyclase homologues (Dahl et al., 2000).

Compared to bovine and human QCs, the quantity of enzyme that accumulated in the plant latex was very high, even though it constituted only 0.5–1% of the total protein soluble fraction of the papaya latex. For comparison, it has been reported that only 38 micrograms of protein can be obtained when starting from 1085 bovine pituitaries (Pohl et al., 1991). When compared to bovine and human QCs, the amino acid sequence of the plant protein shared no similarity at all (Pohl et al., 1991; Song et al., 1994). This finding suggests that the mammalian and the plant QCs belong to distinct enzyme families assuming, probably, different biological functions. Although the mammalian QCs role is well established in the context of neurotransmitter peptides maturation, the role played by the papaya enzyme remains unknown. The results reported here clearly indicate that the PQC may participate into the wound-induced defense response. How this role is fulfilled by the enzyme must however be clarified. This enzyme is known to catalyze the formation of pyroglutamate residues from glutamine located at the N-terminal end of polypeptide chains with concomitant release of ammonia. As this chemical compound can be toxic for micro-organisms (Banuelos et al., 2000), we can tentatively consider that the emission of gazeous ammonia is a strategy adopted by the plant to defend itself from aggressors. However, this conclusion must be taken with great care and further investigations are necessarily needed to clarify the exact contribution of the PQC enzyme to the plant defense mechanism. Finally, one could also expect PQC to have a role

similar to those of mammalian QCs, which take part in neurotransmitter peptides maturation. Thus, PQC could be implicated in the processing of systemic signal peptides. Other wound-induced proteins have, in fact, already been found to be involved in such processing (Bergey et al., 1996). In papaya latex, this tempting hypothesis could be demonstrated by the identification of systemic signaling peptides and their further purification and characterization. This exciting issue is part of our future research.

## 3. Conclusions

The results of the present investigation clearly demonstrate that regularly tapped papaya fruits accumulate in their latex a series of low molecular weight proteins in addition to three proteins identified as a proteinase inhibitor belonging to the Kunitz-type family, a basic class-II chitinase and a glutaminyl cyclase. Identification of these proteins was based on measurements of enzymatic or inhibitory activities and chromatographic elution profiles. These proteins are either not present at all or only weakly detectable in the latex of fruits that are tapped for the first time. The two first identified proteins are members of the so-called PR-proteins. Their induction after wounding treatment can be explained by the role they can fulfil in the plant defense mechanism. In contrast, the observation that PQC belongs to the wound-induced protein family remains unexplained. As far as we know, this latter result is reported here for the first time.

The PQC enzyme is able to release ammonia as a result of its enzymatic activity. If one recognizes that this chemical compound has a negative effect on micro-organisms, it then becomes obvious to consider a defensive role for this protein.

Taking into account that papaya latex is produced by highly specialized cells known as laticifers that are displayed in all parts of the plant except roots, we still not know, however, whether the accumulation of these proteins upon mechanical wounding is confined to papaya fruits only or if other tissues could be involved as well. Furthermore, it is well known that latex disappears from the fruits upon ripening (Villegas, 1997), possibly because the latex-producing cells cease functioning or breakdown with age. The genes encoding these wound-inducible proteins would also be down-regulated in this tissue. However, this fluid exists in all the other aerial parts of the plant, implying probably that the genes encoding these proteins continue to be expressed in these tissues after mechanical injury. Moreover, we do not know up to now, if the response to wounding is local or systemic nor if the mechanically wounded fruits accumulate the three proteins studied here in a transient manner or not. For this purpose, a comparison can be made between over-wounded fruits and others that have not been re-wounded. All these informations are of crucial importance for a better understanding of the implication of these papaya proteins in the context of plant defense mechanism. Behind this, we could not exclude a possible role of the cysteine proteinases stocked in the papaya latex in the defense process against pathogenic micro-organisms or herbivores. In fact, immediately after wounding, latex coagulation and proteinases activation occur simultaneously, indicating that latex coagulation is a main process contributing to the defense mechanism of the plant acting by sealing the wounded areas and by protecting the plant from pathogens entry and further spread. In addition, as lysis of fungal cell walls often requires degradation of cell wall proteins in addition to hydrolysis of chitin and glucan (Haran et al., 1996; Goldman and Goldman, 1998), it seems reasonable to assume that these endoproteinases can also serve as accessory bio-molecules to antifungal action.

## 4. Experimental

### 4.1. Materials

Commercially available papaya latex was kindly provided by Enzymase International S.A. (Belgium). Papaya (*Carica papaya*) latex was also collected from plants that were regularly tapped for latex production or tapped for the first time. Papaya plants growing in the East part of the Democratic Republic of Congo (ex-Zaire) were selected. The fully grown but unripe fruits were, for that purpose, mechanically incised and the collected latex was immediately blocked with *S*-methylmethane thiolsulfonate before being lyophilized and shipped to our laboratory. It is important to note that the papaya plantations selected to perform this work were from the same region (Mutwanga district).

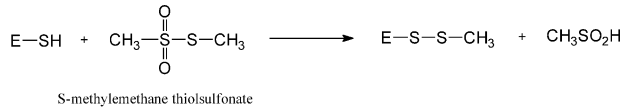
*S*-Methylmethane thiolsulfonate, L-glutamine-*t*-butyl ester (GlntBu), NADH, α-ketoglutaric acid, glutamate dehydrogenase (type VI), bovine pancreatic trypsin (TPCK treated, 13600 units/mg protein), dithiothreitol (DTT) and *N*-α-benzoyl-DL-arginine-*p*-nitroaniline (DL-BAPNA) were purchased from Sigma-Aldrich (Steinhein, Germany).

SP-Sepharose Fast Flow, Precast SDS-PAGE gels (ExcelGels, gradient 8–18%) kit and protein silver staining kit were from Amersham Biosciences. Fractogel EMD propyl 650 (S) was provided by Merck (Darmstadt, Germany). Molecular mass standards for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules CA, USA). All the other chemicals were of analytical grade.

### 4.2. Methods

#### 4.2.1. Sample preparation

Approximately 40 g of lyophilized or spray-dried latex powders were dissolved in water in the presence of *S*-methylmethane thiolsulfonate to block selectively and reversibly the essential thiol functions of the cysteine proteinases according to the reaction (Wynn and Richards, 1995):




S-methylemethane thiolsulfonate

This step is a prerequisite to protect the cysteine proteinases against air oxidation and autolysis, as well as, to prevent proteolytic degradation of the other proteins present in the papaya latex. The resulting suspensions were exhaustively dialyzed (membrane cut off: 3500 Da) against water at 4 °C before to be submitted to centrifugation (40,000×*g*, 4 °C, 30 min). The supernatants, constituting the starting materials, were each divided into six equal volumes ready for use.

#### 4.2.2. Cation-exchange chromatography on SP-Sepharose Fast Flow

Each supernatant aliquot was loaded onto a (30×2.5 cm i.d.) SP-Sepharose Fast Flow column, pre-equilibrated with 100 mM sodium acetate buffer at pH 5.0. The unbounded material was washed away by the pre-equilibrating buffer and the elution of the bound proteins was performed with a linear concentration gradient from 100 to 800 mM sodium acetate buffer at pH 5.0 (total volume 1500 ml, flow rate 60 ml/h) followed by an isocratic elution with 800 mM sodium acetate buffer, pH 5.0 (total volume 300 ml, flow rate 60 ml/h). The chromatographic fractions were analyzed by measurements of absorbance at 280 nm, amidase activity and conductivity. The fractions constituting the last peak (denoted pool III in Fig. 2) were pooled together, concentrated by ultrafiltration (Amicon system, membrane cut off: 3000 Da) and dialyzed (membrane cut off: 3500 Da) against water at 4 °C. This peak constituted the source of papaya glutaminyl cyclase (PQC), of papaya proteinase inhibitor (PPI) and of papaya class-II chitinase (PCHIT II).

#### 4.2.3. Hydrophobic interaction chromatography on fractogel EMD propyl 650 (S)

Solid sodium acetate was added to the concentrated fractions of pool III up to a final concentration of 4 M. This solution was applied onto a (11×3 cm i.d.) Fractogel EMD propyl 650 (S) column pre-equilibrated with a 50 mM Tris/HCl solution at pH 8.0 containing 2 M ammonium sulfate. The unbound material was washed away by the pre-equilibrating buffer and the elution of the bound proteins was carried out by decreasing the ammonium sulfate concentration from 2 to 0 M in a 50 mM Tris/HCl buffer at pH 8.0 (total volume 1602 ml, flow rate 60 ml/h).

The fractions containing the different activities (PQC, PPI or PCHIT II) were separately pooled, concentrated by ultrafiltration (Amicon system, membrane cut off: 3000 Da) and exhaustively dialyzed (membrane cut off: 3500 Da) against water at 4 °C.

#### 4.2.4. Enzyme and inhibitory activities

*4.2.4.1. Amidase activity*. The amidase activity of the cysteine proteinases papain, chymopapain and caricain was measured using DL-BAPNA as the substrate. Each test tube (2 ml) contained 10% DMSO, 1 mM substrate, 2.5 mM DTT, 1 mM EDTA and 50 µl enzyme sample in a citrate–borate–phosphate (100 mM each) buffer at pH 6.8 (Musu et al., 1994). Before starting the reaction by adding the substrate, the enzyme sample was incubated for 15 min at 37 °C in the buffer containing DTT and EDTA. The reaction was stopped with 500 µl of 50% acetic acid. The release of *p*-nitroaniline was determined spectrophotometrically at 410 nm using a $\epsilon_{410} = 8800$ $M^{-1}$ $cm^{-1}$. One unit of activity (nkat) is the amount of proteinase that hydrolyzed one nmol of substrate per second under the above-mentioned conditions (Erlanger et al., 1961).

*4.2.4.2. PQC activity*. Briefly, the PQC enzymatic assay was based on the coupling of ammonia, released during the cyclization of the N-terminal residue of a substrate (here GlntBu), to α-ketoglutarate in the presence of glutamate dehydrogenase (GD) (Zerhouni et al., 1998):

$$NH_3 + \alpha\text{-ketoglutarate} + NADH \xrightarrow{GD} \text{glutamate} + NAD^+$$

In this assay, the released ammonia is proportional to the decrease in absorbance at 340 nm resulting from the conversion of NADH to $NAD^+$ (Bateman, 1989).

*4.2.4.3. Chitinase activity*. Chitinase activity was measured at 37 °C using a suspension of chitin azure (5 mg/ml) as the substrate in a McIlvaine (200 mM sodium phosphate, 100 mM citric acid) buffer at pH 4.6 (Azarkan et al., 1997). Aliquots were removed after different time intervals and centrifuged. The absorbance of the supernatant was monitored at 580 nm.

*4.2.4.4. PPI inhibitory activity*. Inhibitory activity was assayed against bovine trypsin (final concentration 0.15 µM) using DL-BAPNA as the substrate at a final concentration of 1 mM. Briefly, the inhibitor and the serine

proteinase were preincubated for 30 min at 37 °C in a 50 mM Tris/HCl buffer containing 20 mM $Ca^{2+}$ at pH 8.2. Hydrolysis of DL-BAPNA was initiated after addition of the substrate and stopped by addition of 30% acetic acid. Released *p*-nitroaniline was measured spectrophotometrically at 410 nm.

#### 4.2.5. SDS-PAGE experiments

The SDS-PAGE experiments made use of precast gels (ExcelGel, 245×110×0.5 mm, gradient 8–18%) with the Multiphore II kit from Amersham Biosciences. The running conditions were 600 V, 50 mA and 35 W at constant temperature (15±0.1) °C. Protein detection was performed according to the manufacturers' instructions using either Coomassie Brilliant Blue or silver staining procedures. Molecular weight standards were hen egg white lyozyme (14,400 Da), soybean trypsin inhibitor (21,500 Da), bovine carbonic anhydrase (31,000 Da), hen egg white ovalbumin (45,000 Da), bovine serum albumin (66,200 Da) and rabbit muscle phosphorylase b (97,400 Da).

#### 4.2.6. Other spectroscopic and analytical methods

Protein concentrations were determined spectrophotometrically using $\varepsilon_{280}^{0.1\%}$ values of 1.80, 1.30 and 1.56 for PQC, PPI and trypsin, respectively. These values were calculated using the SwissProt ProtParam program (Gill and von Hippel, 1989). PCHIT II concentration was determined using the Modified Lowry Protein Assay Reagent Kit according to the manufacturers' instructions (Pierce, Rockford, USA). Absorbances were measured using a Cary-50 UV–visible Spectrophotometer equipped with a PC. Conductivity measurements were performed at constant temperature (22 °C) with a radiometer conductivity meter CDM3 equipped with a radiometer measurement cell type CDC314.

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