Spontaneous processing of peptides during coagulation of latex from *Carica papaya*

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Abstract

Latex is actively secreted on wounded unripe fruits from *Carica papaya*. We describe the changes in peptide composition by SDS-PAGE analysis of latex from *C. papaya* collected at various times after incision of the unripe fruit. Non random changes in the relative amount of several peptides occur during latex coagulation. The measurement of amidase activity of coagulating latex shows three peaks of activity between 2 and 1200 s. The major activity is found at 1000 s, which is when coagulation is under way. The data from in situ proteolytic activity experiments confirm the presence of active enzyme(s) when latex begins to flow from damaged fruits. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Plants store a variety of fluids, including latexes, resins, gums and mucilages within secretory cells cavities and canals [1]. In latex-containing plants there is strong evidence that this fluid is part of an induced defence mechanism [2,3]. Latex is typically contained within laticifer cells, which are interconnected by ramified structures forming a complex array of tubes throughout the plant. In most species, damage to the canals elicits an abrupt release of secretion.

Latex contains several compounds, whose biological properties confer protection against environmental damage. For instance, latex from *Hevea brasiliensis* L. (rubber tree) contains isoprene monomers [4]. During tapping laticifers are severed, and the latex flows out of the tree, which upon polymerisation and subsequent processing becomes natural rubber.

Instead, latex of *Carica papaya* L., contains a mixture of cysteine endopeptidases [5], such as papain (EC 3.4.22.2) [6], chymopapains A and B (3.4.22.6) [7], papaya endopeptidase III, papaya endopeptidase IV [8,9] and a recently identified one designated as endopeptidase Ω (caricain) [10]. Fresh or dried latex obtained by injuring leaves or fruits of *C. papaya* contains at least some of these proteolytic enzymes. The latex bleeding proceeds for a few minutes until a clot forms around the wounded area. The coagulation process is vital for plant defence against possible pathogen attack. The coagulation in the rubber tree involves aggre-
gation of rubber particles, whereas in latex from C. papaya, the major component is protein and the subsequent clot is mainly made up of proteins. The latter example strongly resembles blood coagulation and clot formation during wounding in mammals [11]. We have proposed that these two phenomena bear some similarities and that latex proteinases may play a role akin to blood coagulation factors endowed with proteolytic activity [12]. We provide here further evidence that cysteine proteinases from C. papaya become activated during clot formation.

2. Material and methods

2.1. Latex collection

Latex was collected at various intervals (1–1200 s) following single wounding of unripe fruits at the plant with a steel razor blade. The sample duplicates obtained from different fruits (100–200 µl sample⁻¹) were collected in eppendorf tubes containing 150 µl water, or 5 mM MMS (Methyl methane thiosulfonate) and dropped into a dry-ice bath, followed by storage at −70°C, until used. Sample duplicates collected in water without MMS were used as controls to determine the inhibitory effect exerted by MMS on proteolysis. Following protein dosage of each sample, equivalent amounts of protein from each duplicate were pooled for electrophoresis analysis.

2.2. Electrophoretic of proteins from latex

Protein determination was carried out by Bradford [13]. The samples (4 µg) were electrophoresed according to Laemmli [14] for 2 h at 110 V, and 15 mA at 8°C. MMS was added to a final concentration of 2.5 mM before sample heat denaturation to prevent degradation during electrophoresis. Acid PAGE was done according to the technique previously described [15]. Protein bands were visualised by silver staining as previously described [16]. The molecular weight standards were bovine albumin (66 000), lactic dehydrogenase (36 500), carbonic anhydrase (29 000), trypsinogen (24 000), and α-lactalbumin (14 200).

For a comparative analysis of the proportion of each peptide in the mix, we scanned each lane by transmission densitometry at 430 nm using a Hitachi laser densitometer supplied with CS 9301PC software. Each latex sample corresponding to the various collection times was run and analysed on the same day to minimise variations within gels. The intensity of a single band on each lane was related to a standard peptide (internal standard) whose concentration remained constant at the various intervals studied. The relative mass of each peptide in the samples was determined by regression analysis of a mix of protein standards run together with the samples.

2. In situ proteolytic activity

The in situ proteolytic activity was determined by 12% SDS-PAGE or acid PAGE following electrophoresis [17]. Protein samples (60 µg) were not denatured by heat before applying onto gels. Briefly, after electrophoresis the gel was rinsed twice with 50 ml buffer containing 0.4 M Tris–Cl pH 8.0 and then a 0.5% agarose solution, containing 0.1 M Tris pH 8.0 and 1.8% casein was applied onto the gel surface. The gel was then incubated at 37°C between 12–48 h to visualise the proteolytic activity. The gel was then photographed with Polaroid film 665 and the negative densitometrically scanned as previously described.

2.4. Latex amidase activity

This procedure measures the enzymatic hydrolysis of BAPNA following incubation 30–40 min at 37°C [18]. Briefly, a proteinase sample (15 µl) was incubated with a solution containing 4 mM cysteine, 25 mM Tris–Cl, 2 mM EDTA pH 8.0 and 4 mM BAPNA in a final volume of 750 µl. The reaction was stopped by addition of 60 µl 60% acetic acid. The release of p-nitroanilide was measured spectrophotometrically at 405 nm.

3. Results

A complex pattern of 5–20 bands of different intensities was observed on each lane of the gel (Fig. 1). To analyse this complex peptide pattern a densitometric scanning of the stained gel was required. To facilitate analysis, bands were grouped into four classes (a, b, c, and d), based on their electrophoretic migration. Group a contained the largest peptides (> 38.5 kDa), group b consisted
Fig. 1. SDS-PAGE of latex fractions obtained at different intervals after fruit tapping. Latex samples were collected at various times following an incision of the unripe fruit as described in Section 2. Aliquots (4 µg) were heat-denatured for 1 min with MMS and loaded on each lane of the gel. The run was performed at 8°C followed by silver staining. Densitometry was done with stained dried gels. A profile similar to that shown in the figure was obtained in triplicate experiments, each using different latex stocks. The first lane of each gel contains the molecular weight standards.

of peptides with a size between 26–38 kDa, group c, contained peptides of intermediate size (22–25 kDa), while group d contained the smallest peptides (12–21 kDa). Samples collected and stored in water showed fewer bands than those collected in MMS, confirming the notion that the sulphydryl modifying reagent efficiently prevents protein degradation before and/or during electrophoresis. Consequently, in controls without MMS, almost no protein remained in the gel after electrophoresis (Table 1). MMS also afforded better inhibition of proteinases during electrophoresis when compared to mercaptoethanol or dithiothreitol (not shown). These are reducing agents commonly used in electrophoresis buffers ([14]).

The relative changes in content of selected peptides during the interval 1–1200 s was obtained by plotting the relative areas of each band against the latex collection times. The summary of these results is shown in Table 1. The data obtained for each peptide were fitted into curves by regression analysis using SigmaPlot software and further classified into six categories: (I) peptides showing an increase that peaked before 2 min, followed by a decrease and stabilisation throughout the remaining period; (II) peptides whose relative amounts showed two to three peaks during the interval 1–1200 s; (III) peptides that decreased until reaching a plateau; (IV) peptides that increased until reaching a plateau; (V) peptides remaining unchanged during the interval; and (VI) peptides that increased their relative amount in a linear or non-linear mode. In addition, other peptides not included in Table 1 displayed variations in their content that could not be fitted into a curve by regression analysis, and therefore they were excluded from further analysis. Based on these data, we concluded that the changes in relative peptide profile observed are non-stochastic.

Next, we evaluated the changes in specific activity of proteolytic enzymes during the period of latex coagulation. The amidase activity of latex samples collected at different intervals was dosed (Fig. 2). Three major peaks (350, 700, and 1000 s) were detected during the period surveyed. Linear extrapolation of the specific activity to zero time in Fig. 2 suggests that the enzyme is already present when latex begins flowing out from the fruit. Its value was estimated to be $5.46 \times 10^{-6} \text{ M} \times \text{min}^{-1} \times \text{mg}^{-1}$ by linear regression analysis.

To verify the presence of proteolytic enzymes in active form at early times following collection, we carried in situ proteolytic activity experiments following electrophoresis. Both acid PAGE and SDS-PAGE gels were screened for enzyme activity. Fig. 3 shows the in situ activity data of SDS-PAGE gels containing samples collected between 2–15 s. Although the enzyme activity was present at each time point, a gradual reduction in caseinolytic activity was observed during the interval analysed. By using molecular weight markers we estimated the relative mass of this activity to be 24–26 kDa. The densitometric analysis of the band shown on Fig. 3 was analysed by densitometry as before. The peak area obtained on each lane was plotted against the time of collection. The result on Fig. 3 reveals an exponential linear decrease of the activity during the period between 2 and 15 s.
Table 1
Changes in the relative content of some peptides during latex coagulation*

<table>
<thead>
<tr>
<th>Peptide groups</th>
<th>Variation</th>
<th>MMS + MMS</th>
<th>H₂O + MMS</th>
<th>H₂O - MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I)</td>
<td>↓↑</td>
<td>d₁₇; d₁₅</td>
<td>a₄₇; a₄₅</td>
<td>a₄₃; a₄₂; b₃₃</td>
</tr>
<tr>
<td>(II)</td>
<td>↓↑</td>
<td>a₅₁; a₄₆; b₇; b₃₀; c₂₅; c₂₃; d₁₆</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(III)</td>
<td>↓↑</td>
<td>a₃₅; a₃₄; b₃₂</td>
<td>a₅₂</td>
<td></td>
</tr>
<tr>
<td>(IV)</td>
<td>↓↑</td>
<td>a₆₁; b₃₆</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V)</td>
<td>↓↑</td>
<td>b₄₆; c₂₅</td>
<td></td>
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</tbody>
</table>

*The relative variation of each band was determined by densitometry and analysed with CS9301PC software (Hitachi) using the less variant peak as standard. The estimated molecular mass (kDa) for each band designated with letters (a–d) is shown as subscript. Samples were collected in H₂O or MMS. MMS was added (+ MMS) or not (− MMS) to the sample buffer. The orientation of the arrows shows the direction of peptide change.

4. Discussion

While a variety of non-defensive functions have been proposed for latex canals and their fluid, including conduction and storage of food [19], its defensive role is supported by many lines of evidence. Plant varieties or individuals with reduced canal systems or decreased titer of chemicals in their latex are more vulnerable to herbivore attack than are plants with normally sized canals and or-latex content [2,20]. The presence of secondary metabolites known to be toxic to insects or animals or the presence of bacteriolytic, proteolytic enzymes [21], furanocumarins [22], vanillic acid [23], aliphatic ketones [24] in various latexes confer protection against pathogens.

In Caricaceae, latex appears to protect by sanitising and sealing the wounded area, thus we searched for biochemical ground to support this notion. We provide evidence that during latex coagulation a number of peptides are being processed in a non-random manner. Many of the changes involve transient increases that after returning to a basal level, further decrease or increase depending on the peptide. In contrast, the relative amount of other peptides increase or remain constant during the period analysed (a₄₁, b₃₆). In same cases, groups of consecutive peptides adopt the same processing profile, such is the case for peptides a₅₇, a₅₅, and a₄₆. It could be argued that an overlapping profile of a₅₇ and a₅₅ is due to crossovers contamination between the two peptides in the gel, but this could not be the explanation for the similar profiles observed between a₅₅ and a₄₆ in which a 11 kDa difference separate them. We attribute this similarity in profile between neighbouring bands, to alternative processing of these peptides, thus for instance peptide a₅₇ or its alternate form a₄₅ undergo the same processing pathway. A similar situation might occur with peptides d₁₇/d₁₅, b₃₄/b₃₃, and c₂₅/c₂₃. It is known that several proteolytic enzymes are components of latex from C. papaya. However, their presence has been generally afforded in non-fresh latex preparations, therefore, we carried this analysis to probe the amidase activity in fresh latex preparation. Unexpectedly, an initial amidase activity was always detected in fresh latex samples (Fig. 2). The zero-time extrapolated specific activity was about (0.25 nkat mg protein⁻¹) 1/3 of the maximal value measured at 1000 s (0.75 nkat mg⁻¹). These figures are in agreement with the data from [10], who reported 1.68 and 1.98 nkat mg⁻¹ for purified proteinase Ω and chymopapain, respectively.

Following a transient decrease in activity, the data show two moderate peaks at 350 and 700 s, followed by a third major peak at about 1000 s. Similar results were obtained with latex collected at different times during the day, different fruit size and before or after plant watering (not shown). All three peaks occur when latex is coagulating, that is to say, when dripping from the fruit is no longer observed. The observed peaks may correspond to a sequential activation of the various proteinases in latex from C. papaya.

The amidase activity at later intervals (1800–2500 s) gradually decreases to attain 40% of the activity detected at 2 s (not shown). Based on these results it is concluded that the proteolytic
activity in commercial latex preparations of \textit{C. papaya} represents the remaining activity following these changes. The presence of active proteolytic enzymes in fresh latex suggests that these enzymes must be kept 'isolated' to prevent undesired proteolysis within the plant.

To confirm the presence of active enzyme(s) at early times following fruit tapping, we dosed the in situ proteolytic activity after SDS-PAGE. The data presented in Fig. 3 confirm the presence of an active enzyme(s) as early as 2 s after fruit injuring. Moreover, in agreement with Fig. 2, the data shown on Fig. 3 confirm the initial steady decrease of this initial activity, suggesting the inactivation of this enzyme(s). The estimated relative mass of this activity is 24–26 kDa. This mobility is shared by papain, chymopapain, papaya proteinase IV and proteinase Ω on SDS-electrophoresis. Alternatively, by running native acid PAGE which discriminate by charge instead of size, it is possible to distinguish between papain, chymopapain and proteinase Ω [10]. By applying this electrophoretic condition the initial proteolytic activity behaves like chymopapain (not shown).

While the primary sequence of chymopapain has been disclosed [7], many reports describe the various isoforms of this protein in latex [25,26,8]. It is possible that the various isoforms represent the different processing steps for chymopapain during latex coagulation.

The presence of papain precursors between 39–24 kDa in \textit{C. papaya} was also demonstrated [27,28]. Molecular cloning of papain, papaya proteinase IV and proteinase Ω confirmed that these enzymes are synthesised as pro-enzymes, and that removal of the pro-region which is essential for correct enzyme folding, is also required for enzyme activation. Interestingly, the size of these pro-enzymes previously reported are like some of the peptides described in Table 1.

The removal of the pro-region can be achieved in vitro by autocatalytic cleavage at pH 4 to yield the active mature enzyme. The significance of this reaction in vivo remains to be demonstrated, since the pH of latex is closer to 6 rather than 4. Also, previous attempts to produce active papain following in vitro translation of larger precursors were unsuccessful [28].

If the pro-region remains in contact with the enzyme after cleavage, it inhibits enzyme activity [29]. This mechanism has not yet been demonstrated in the case of chymopapain, although the pro-regions from papain and proteinase IV cross-react with chymopapain. Molecular cloning of the chymopapain precursor will show whether this enzyme(s) has its own inhibitory pro-region. According to our preliminary data, a chymopapain-like enzyme appears to be active at the time when latex begins flowing out of the fruit. If this is true, chymopapain or an isoform is kept uninhibited in the plant. It is also possible that the activity of chymopapain is controlled by a different mechanism from that described for the other enzymes.

Based on these results it is concluded that a sequential processing of some peptides takes place during latex clotting along with the formation of other peptides and the transient activation of proteolytic enzymes.

It is tempting to establish a parallel between latex and blood coagulation pathways, the latter process evolved by mammals with participation of serine proteinases [11]. The analogies between these two processes are outlined: both phenomena occur as part of a defence mechanism, in either case, a solid clot is produced and proteolytic enzymes are activated while clot is being formed (Table 1 and Fig. 2 in this work), in mammals fibrin is the major component of clot, while our unpublished data reveal that latex clot is com-
posed by 1–2 peptides and that regenerative processes leading to healing of damaged tissue are evident after clot formation, in plants as well as in mammals. In addition, preliminary evidence (not shown) suggests that mitogenic substances present in latex from Caricaceae stimulate cell division when added to mammalian cultured epithelial cells. The fact that latex proteolytic enzymes are cysteine proteinases does not challenge these analogies, as cysteine enzymes might be the products of independent evolution. In this regard, it is worth to note that a group of peptides was isolated in seeds from squash that inhibit the serine proteinases from the blood coagulation system [30], while our attempts to interfere with the blood coagulation process using latex fractions were unsuccessful (unpublished observations). In spite of these analogies, further work is required to establish the role of processing proteinases from latex on clot formation.

A quite different defense mechanism occurs in latex from *H. brasiliensis*. A lectin-like protein called ‘hevein’, the major protein of vacuolar structures in the latex of the rubber tree, is involved in the coagulation of latex by polymerizing rubber monomers [4]. Interestingly, the C-terminal region of ‘hevein’ shares homology with wound-inducible genes. Wound-inducible genes are part of a systemically induced defense mechanism found in leaves from *Lycopersicon esculentum* and *Solanum tuberosum* [31]. These genes encode serine proteinase inhibitors that are expressed in response to leaf wounding. It is not known whether hevein from *H. brasiliensis* is involved in the wound inducible function as well. It will be interesting to find out if the expression of wound-inducible genes involving proteinase inhibitors also occurs in Caricaceae.

Acknowledgements

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References


