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Carica papaya; latex; lipase; phospholipase A2; *Vasconcellea heilbornii*

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Latex from Caricaceae has been known since 1925 to contain strong lipase activity. However, attempts to purify and identify the enzyme were not successful, mainly because of the lack of solubility of the enzyme. Here, we describe the characterization of lipase activity of the latex of *Vasconcellea heilbornii* and the identification of a putative homologous lipase from *Carica papaya*. Triacylglycerol lipase activity was enriched 74-fold from crude latex of *Vasconcellea heilbornii* to a specific activity (SA) of 57 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ on long-chain triacylglycerol (olive oil). The extract was also active on trioctanoin (SA = 655 $\mu mol \cdot min^{-1} \cdot mg^{-1}$), tributyrin (SA = 1107 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and phosphatidylcholine (SA = 923 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). The optimum pH ranged from 8.0 to 9.0. The protein content of the insoluble fraction of latex was analyzed by electrophoresis followed by mass spectrometry, and 28 different proteins were identified. The protein fraction was incubated with the lipase inhibitor [^{14}C]tetrahydrolipstatin, and a 45 kDa protein radiolabeled by the inhibitor was identified as being a putative lipase. A *C. papaya* cDNA encoding a 55 kDa protein was further cloned, and its deduced sequence had 83.7% similarity with peptides from the 45 kDa protein, with a coverage of 25.6%. The protein encoded by this cDNA had 35% sequence identity and 51% similarity to castor bean acid lipase, suggesting that it is the lipase responsible for the important lipolytic activities detected in papaya latex.

Introduction

Upon wounding, laticiferous plants exude latex, which serves to protect the plant against predators. Latex originates from specialized cells called laticifers. The most important information comes from studies on *Hevea brasiliensis* [1], in which the latex exuded after breaking of the laticifers contains rubber particles, Frey–Wyssling bodies (a possible form of plastid filled mostly with lipids) and lysosomal-like organelles called lutoids, which contain proteins. Mitochondria and nuclei usually remain in the laticifer, but the exuded latex may contain endoplasmic reticulum. The latex usually coagulates almost immediately upon release, unless it is brought to high pH upon collection. Papayas are also laticiferous plants [2], and their latex is a

Abbreviations
BAC, 16-benzyldimethyl-n-hexadecylammonium chloride; EST, expressed sequence tag; GA, gum arabic; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PL, phospholipase; PtdCho, phosphatidylcholine; SA, specific activity; TAG, triacylglycerol; TC4, tributyrin, TC8, trioctanoin; THL, tetrahydrolipstatin.

unique and abundant source of economically interesting enzymes. In *Carica papaya* and *Vasconcellea heilbornii*, proteins represent about 40% of the latex dry weight, whereas the other components remain largely uncharacterized, especially the nonsoluble ones. The protein fraction has been thoroughly studied [3]. It contains mostly water-soluble cysteine proteases such as papain [4], a protein whose 3D structure was one of the first to be elucidated. Its physiological role in defense against predators has been investigated recently: papain shows strong toxicity against lepidopteran larvae, and it prevents them from feeding on the leaves [5]. Several thousand tons of crude papain (mostly crude dried latex) are produced each year and used in various food applications, such as brewery and meat tenderizing, and in the pharmaceutical industry. In addition to proteases, which constitute the vast majority of latex proteins, several glycosyl hydrolases, such as chitinase [6], have been characterized, and strong lipase activity was shown as early as 1925 [7].

Lipases are enzymes that catalyze the hydrolysis of nonsoluble, long-chain triacylglycerol (TAG) [8,9]. They are interfacial enzymes that need to bind to their substrate before they can hydrolyze it. The binding can be strongly influenced by tensioactive agents, salts (especially divalent cations such as calcium), pH, etc. [10]. Some TAG lipases also possess secondary phospholipase (PL) A1 [11,12], galactolipase [13] or cholesterylester hydrolase [14] activities. The active site is composed of a catalytic triad (Ser, Asp/Glu, His). On the basis of their amino acid sequences, several different lipase families have been identified, some of which diverge widely from the others [8]. Only a short, degenerated consensus sequence that surrounds the catalytic Ser and forms the so-called nucleophilic elbow can be determined (PROSITE PS00120). Mammalian digestive lipases and fungal lipases have been extensively studied [8]. By contrast, little is known about plant lipases [15]. Only a few plant enzymes showing true lipase activity [i.e. catalyzing the hydrolysis of long-chain, insoluble TAGs with high specific activity (SA)] have been cloned so far. For plant lipase biochemical properties [15,16], all of the work published has been carried out on nonpure fractions, except for the SDP1 recombinant enzyme [17]. The most documented plant TAG lipases are involved in fat storage breakdown during early postgerminative growth of oil seeds [18,19]. Germination lipases are usually present in trace amounts. Some plant materials, including lattices, have been shown to contain much higher levels of lipase activity [15,20,21]. Seeds of castor bean (*Ricinus communis*) contain a strong acid lipase [16], and this enzyme was the first TAG lipase with high SA to be cloned from plants [20]. The mesocarp of the fruit of oil palm contains the highest level of lipase activity recorded for a plant tissue [21]. The lipase from *Euphorbia* latex has been studied by a few groups [22–25]. It was found to be soluble in organic solvents, and a solvent-based procedure has been used to purify this enzyme [24,25]. Its N-terminal sequence exhibits homologies to ricin B-chain [25]. In Caricaceae, the main work has been carried out on *C. papaya* latex by Giordani *et al*. [23]. These authors showed that the enzyme works best at basic pH, is much more active on short-chain than on long-chain TAGs, and is still fairly active at 55 °C. They also confirmed that the enzyme was not water-soluble. Most studies on this enzyme since then have been concerned with applications in the field of biotransformation of lipids [26]. *C. papaya* lipase is 1,3-regioselective and shows a slight stereopreference for the *sn-3* position of the TAG molecule [27,28]. It also exhibits stereoselectivities and enantioselectivities for certain substrates that might prove interesting for specialty applications [26,28]. More recently, an esterase from the GDSL family has been purified from *C. papaya* latex [29]. This enzyme was found to be very active on short-chain TAGs but showed very little activity on long-chain TAGs and on phosphatidylcholine (PtdCho). A close relative of *C. papaya* is *V. heilbornii* (mountain papaya or babaco), formerly known as *Carica pentagona* [30]. The *Vasconcellea* and *Carica* genera are close enough that hybrids can be obtained under certain conditions [31]. Although the latex composition is quite similar, *V. heilbornii* latex is considered to contain more active proteases than *C. papaya* latex [32]. The biosynthetic capabilities of *V. heilbornii* lipase have been investigated with regard to fat bioconversion [27,33]. Also, it has been shown to be 1,3-regioselective for TAGs, with no stereopreference for one of the two external positions [27]. All work has been carried out on crude latex or on an insoluble fraction, as attempts to purify the lipase have been unsuccessful. Here, we present the biochemical characterization of lipolytic activities present in an enriched fraction of the latex of *V. heilbornii*, and the identification of a candidate lipase responsible for these activities, using a proteomic approach coupled to radiolabeling with a lipase inhibitor.

Results

The lipase activity is not soluble in aqueous buffers

The dried latex of *V. heilbornii* contained about 2100 lipase IU (1 IU = 1 μmol fatty acid released per minute) per gram dry weight when assayed at pH 8.0 with

tributyrin (TC4). This is comparable to the figures obtained for *C. papaya* latex [23,29]. The activity assayed with olive oil as substrate was 300 IU per gram of dried latex at pH 8. The activity was found to be nonsoluble in aqueous buffers (i.e. 50 mM Tris/HCl, pH 7.5). Addition of SDS, Chaps, Triton X-100, Nonidet P40, Brij35 or sodium taurodeoxycholate at twice the critical micellar concentration did not allow us to solubilize the activity (data not shown). Because lipases are known to aggregate with hydrophobic compounds, we delipidated the latex with, successively, acetone, chloroform/butanol mixtures and diethyl ether. About 50% of the activity on TC4 could be recovered in the delipidated powder; however, no activity was detected with olive oil as substrate. This confirms that latex contains an esterase capable of hydrolyzing TC4 and not long-chain TAGs [29]. Assay of the solvent washes for lipase activity indicated that about 30% of the initial activity on olive oil was recovered in the chloroform/butanol (9 : 1, v/v) wash. This property of the lipase prompted us to devise a protocol to obtain enriched fractions of lipase.

Enrichment of lipase activity from babaco latex

Quantification of proteins with the Bradford assay [34] gave inconsistent results, probably because of the presence of interfering substances. The occurrence of such substances has been reported for *Hevea* latex [35]. Therefore, the protein content was determined on the basis of the analysis of amino acids after *in situ* acid hydrolysis. Dried latex contained 39.2% (w/w) proteins. Comparable results were obtained by determining the total nitrogen content of the samples (data not shown). The lipase SA of the dried latex was 0.75 IU per mg protein with olive oil as substrate. The latex powder was then extracted three times with an aqueous buffer (see Experimental procedures). This allowed us to remove water-soluble compounds, which accounted for about 86% ± 1% (w/w) of the dried latex. The nonsoluble protein fraction represented about 1% of total protein (i.e. 4.2 mg when starting from 1 g of dried latex). No lipase activity could be detected in the washes when olive oil was used as substrate. After washes and centrifugations, the final pellet was resuspended in the washing buffer and assayed for lipase activity with olive oil as substrate. It was found to contain about 85% of the initial activity, and the SA was about 60 IU per mg of protein with olive oil as substrate (Table 1). Therefore, this series of washes allows the lipase activity to be enriched about 80-fold. The pellet was lyophilized. Once dried, it appeared to be made of a sticky, resin-like substance. Lipase activity could not be quantified, because it was not possible to disperse and homogenize the sample properly. Hexane extraction of the pellet (about 30 mL per gram of lyophilized pellet) allowed us to solubilize about 50% of the pellet dry mass. The hexane extract obtained when starting from 1 g of dried latex contained 1.7 mg of protein (which represents about 0.4% of the initial protein content). When olive oil was used as substrate, about 32% of the initial activity was recovered in the hexane extract, and the SA was 57 IU·mg^{-1}. The sticky residue not extracted by hexane also contained lipase activity. Therefore, it seems that extraction by hexane is not specific for a particular protein and does not enrich lipase activity. This was confirmed (data not shown) by comparing the electrophoretic profile of proteins from the washed latex with those from the hexane extract and the hexane-insoluble residue: all three profiles were found to be similar. With storage at 4 °C, the activity of the hexane extract was remarkably stable for at least 6 months. We chose to characterize the activities from this extract because of its ease of handling and the stability of the enzyme.

Biochemical characteristics of lipolytic activities

As shown in Fig. 1, the extract hydrolyzed olive oil (57 IU·mg^{-1} protein at pH 8.0), TC4 (1107 IU·mg^{-1} protein

Table 1. Enrichment of lipase activity from babaco latex (from one representative enrichment experiment). The crude latex (latex powder) was washed with an aqueous buffer to remove water-soluble compounds. Hexane was used to extract lipase activities (enzyme extract) from the nonsoluble residue. Olive oil and TC4 were used as substrates. Assay conditions were as in Fig. 1, at pH 8.0. Activities were measured two to four times, and the standard deviation was below 10%. Activity values reported for the hexane extract correspond to the total activity of hexane extract obtained from 1 g of latex.

	Activity (IU·g^{-1} latex powder)		SA (IU·mg^{-1} protein)		
Substrate	Latex powder	Enzyme extract	Latex powder	Enzyme extract	Enrichment factor
Olive oil	300	97	0.77	57	74
TC4	2130	1883	5.44	1107	203
TC8	485	1111	1.24	654	527
PtdCho	75	1033	0.19	608	3198

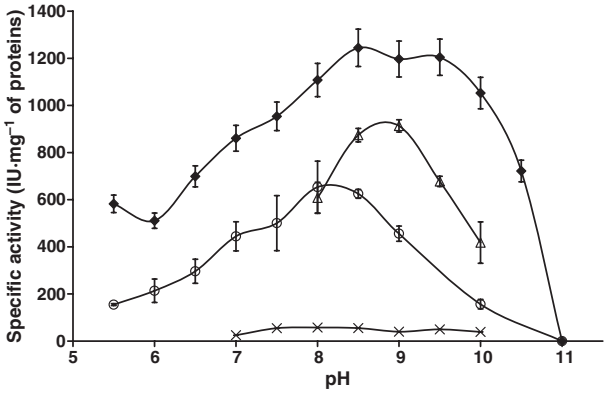

Fig. 1. SA as a function of pH. Measurements of activity were carried out at 25 °C in 2 mM Tris/HCl and 150 mM NaCl (30 mL, final volume). The substrates used were TC4 (500 μL, closed lozenge), TC8 (500 μL, open circle) and olive oil (1 mL emulsified in 9 mL of GA 10%, w/v, crosses). When PtdCho (open triangle) was used as substrate, the reaction mixture (30 mL, final volume) contained 13.3 mM sodium deoxycholate, 8 mM $CaCl_2$ and 1.2% (w/v) PtdCho. Values are the results of three independent assays.

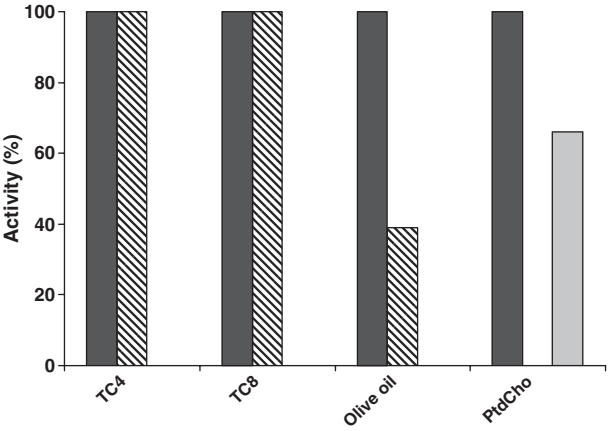

Fig. 2. Effect of EDTA on enzyme activity. Black bars: experiments were carried out as described in the legend to Fig. 1, at pH 9.0, except that the final concentration of GA was 0.33% for assaying lipase activity on olive oil (see Experimental procedures). Hatched bars: EDTA (5 mM) was included in the reaction buffer and $CaCl_2$ was omitted (PL assay). Gray bar (only for PtdCho): 4 min after addition of EDTA (without $CaCl_2$) to the reaction mixture, $CaCl_2$ (10 mM) was added and the activity was recorded. Values are the results of two independent assays; the standard deviation was < 10%.

at pH 8.5), trioactanoin (TC8) (654 IU·mg^{-1} protein at pH 8.0) and PtdCho (923 IU·mg^{-1} protein at pH 9.0). The fatty acids released from PtdCho came almost exclusively from the *sn-2* position, which indicated a PLA2 activity (Table 2). No activity could be detected with cholesteryl-oleate as substrate. For all substrates, the enzyme extract was active at pH values above 7, with optima between pH 8 and pH 9 (Fig. 1). At the optimal pH, the kinetics were linear for at least 10 min for all substrates tested. EDTA reduced the lipase activity measured on olive oil to 60%, and completely abolished PL activity (Fig. 2). Most of the PL activity (about 65%) was restored by calcium chloride (Fig. 2). However, EDTA had no significant effect on the activity when TC4 and TC8 were used as substrates. Tetrahydrolipstatin (THL), a lipase inhibitor that binds covalently to the catalytic Ser of pancreatic lipase [36], was found to inhibit both lipase and PL activities. About 0.3 nmol of THL inhibited 50% of lipase activity when starting from 4.5 IU (Fig. 3), and

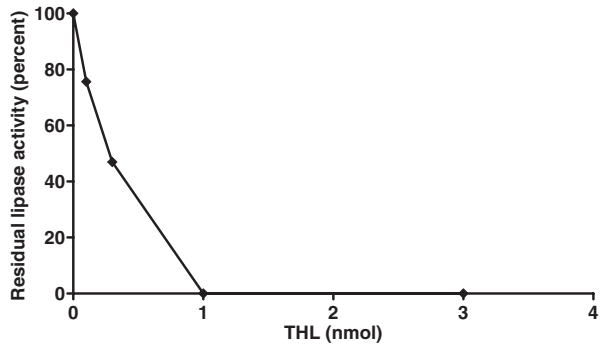

Fig. 3. TAG lipase activity in the presence of THL. Lipase activity was assayed with an enzyme extract (4.5 IU) on olive oil. The enzyme extract was preincubated for 1 h with different amounts of THL. Assay conditions were as in Fig. 1, at pH 9.0. Values are the results of two independent assays; the standard deviation was < 10%.

Table 2. Regioselectivity of PL. The enzymes were incubated for 30 min in a medium containing PtdCho with a radiolabeled fatty acid in position 2. The lipids were extracted from the reaction mixture and separated by TLC. The plate was analyzed and the radioactive lipids quantified with a PhosphorImager. The percentage of release from position 2 was calculated as follows: area fatty acid/(area fatty acid + area lysoPtdCho). Pancreatic PLA2 was used as a positive control, and *T. lanuginosus* lipase as a PLA1. The table shows the results of three independent experiments.

(Phospho)lipase	*V. heilbornii*	Pancreatic PLA2	*T. lanuginosus* lipase
% Release position *sn-1*	3.0 + 2.9	1.5 + 0.2	97.8 + 0.3
% Release position *sn-2*	97.0 + 2.9	98.5 + 0.2	2.2 + 0.3

3 nmol of THL inhibited 90% of PL activity when starting from 4.1 IU.

Identification of proteins that bind to THL

SDS/PAGE analysis of the profile of proteins extracted from crude latex showed only three bands with Coomassie Blue staining (data not shown), with molecular masses ranging from 24 to 30 kDa. On analysis of the proteins extracted from the hexane fraction, whose lipase SA had been enriched 80-fold when compared to crude latex, these three bands still constituted the majority of proteins detected. About 10 additional bands with masses ranging from 35 to 90 kDa could also be detected with Coomassie Blue staining (Fig. 4A). These are likely to be nonsoluble proteins, enriched by the washing procedure. In an attempt to identify the lipase, the hexane extract was incubated with [^{14}C]THL. The proteins were extracted, loaded onto an SDS/PAGE gel and further analyzed by fluorography (Fig. 4B). Two radioactive bands could be detected at 45 and 42 kDa, and also a faint band immediately below the large 24 kDa protein. The gel did not have enough resolution to properly identify the labeled proteins. Therefore, 2D electrophoresis (i.e. IEF followed by SDS/PAGE) was performed (Fig. 5B). Subjection of the gel to fluorography showed two radiolabeled spots at 42 kDa only. Analysis by mass spectrometry (MS)/MS and N-terminal sequencing identified both spots as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The radioactive spot corresponding to the 45 kDa protein could not be detected after 2D electrophoresis. It is likely that the protein could not be resolved by the first-dimension gel or that it did not enter it. MS analysis of a band excised from the 1D gel and an N-terminal sequence detected chymopapain only. The protein extract was then subjected to another type of 2D analysis, using 16-benzyldimethyl-n-hexadecylammonium chloride (BAC) as detergent in the first dimension and SDS in the second (Fig. 5A). Another gel was run using a higher percentage of acrylamide to resolve the low molecular mass proteins (Fig. 5C). Three radioactive spots could be detected at 45 kDa (spot 5, Fig. 5), 42 kDa (spot 8, Fig. 5) and 23.5 kDa (spot 14, Fig. 5). The 45 kDa spot was well resolved; it was subjected to MS/MS analysis and *de novo* sequencing. On screening of the nonredundant GenBank protein database using spectra from MS/MS analysis and SEQUEST software (Sequest Technologies Inc., Lisle, IL, USA), only a contaminating protease could be detected. Screening of the same database with PEPNOVO and FASTS yielded four significant hits. Two proteins with high similarity

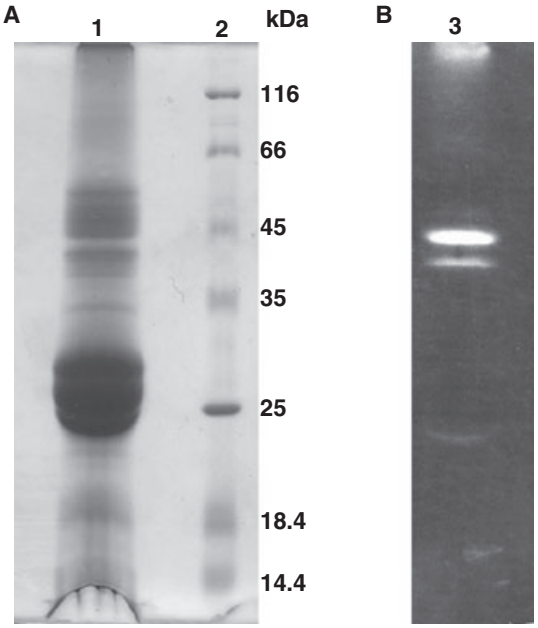

Fig. 4. SDS/PAGE (A) and fluorography (B) of a THL-radiolabeled enzyme extract. The gel was stained with Coomassie Brilliant Blue R-250. (A) Lane 1: protein content of an enzymatic extract (~ 2.5 IU) preincubated with [^{14}C]THL. Lane 2: molecular mass markers. (B) Lane 3: fluorography of lane 1.

to castor bean acid lipase were identified (GenBank accession no. ABK94755, E = 4.6 e−20, 58% identity, 62% similarity, 23% coverage; GenBank accession no. CAO71857–XP002277782.1, E = 1.1 e−07, 47% identity, 62% similarity, 16% coverage) from *Populus trichocarpa* and *Vitis vinifera*, respectively, and two cysteine proteases (GenBank accession no. ABI30271.1, E = 2.8 e−16, 79% identity, 89% similarity, 14% coverage; GenBank accession no. AAB02650, E = 3.4 e−06, 73% identity, 77% similarity, 6.4% coverage) from *V. heilbornii* and *C. papaya*, respectively (Fig. S1). Therefore, it appears that the 45 kDa spot, which binds to radioactive THL, contains a protein that has sequence similarities with castor bean acid lipase.

Identification and cloning of a putative lipase from *C. papaya*

Because *V. heilbornii* is a close relative of *C. papaya*, and that both expressed sequence tag (EST) and genomic resources [37] are available for this organism, we tried to identify the putative homologous lipase from *C. papaya*. Screening of translated ESTs from *C. papaya* by PEPNOVO, using spectra from the 45 kDa spot, identified a few sequences with *E*-values as low as 6.4 e−27. All of these ESTs, together with genomic contigs from *C. papaya* [GenBank nucleotide accession

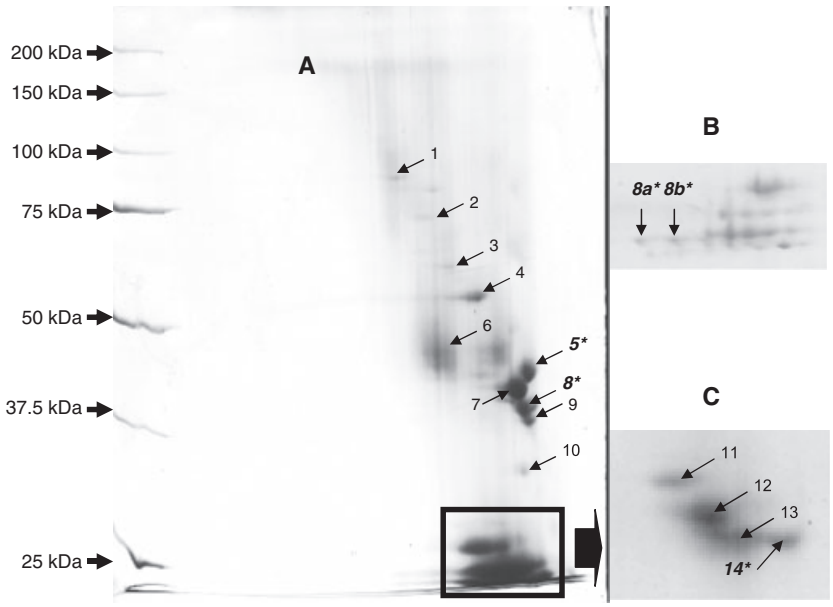

Fig. 5. Polyacrylamide gels used to prepare protein spots for MS analysis. (A) BAC SDS 2D gel (12% acrylamide). (B) Part of an IEF SDS 2D gel. (C) Part of a BAC SDS 2D gel (15% acrylamide). Gels were stained with Coomassie Blue, and small spots were carefully sampled for MS analysis. The spot numbers are identified in Table S1. Spots at 45 kDa (spot 5), 42 kDa (spot 8) and 24 kDa (spot 14) marked with an asterisk (*) are labeled with [^{14}C]THL.

nos. ABIM01012471 (5′-end) and ABIM01012472 (3′-end)] that share more than 95% identity with EST sequence stretches, allowed us to reconstitute the whole mRNA sequence coding for the putative lipase from *C. papaya*. This mRNA was designated *CpLip1*. *CpLip1* cDNA was then cloned by RT-PCR, and its deduced amino acid sequence matched exactly the sequence encoded by the mRNA that we had reconstituted from ESTs and genomic sequences. We reasoned that the *C. papaya* genome might contain other related putative lipases more closely related to *V. heilbornii*'s 45 kDa spot than is CpLip1. We used the *CpLip1*-encoded protein sequence to identify genes coding for related proteins in *C. papaya* whole genome shotgun and EST sequences. We were able to identify three other genes with BLASTX scores above 200. The fourth hit score was below 50. The three candidates were designated *CpLip2–4*. The encoded protein sequences were tentatively determined; *CpLip2* was interrupted by a stop codon in the middle of the sequence, and no ATG codon could be identified for *CpLip4*. One and four ESTs were identified for *CpLip3* and *CpLip1*, respectively. The four protein sequences were included in a yeast protein database and screened using PEPNOVO with the spectra obtained from the 45 kDa protein. However, only the *CpLip1*-deduced protein contained the peptides identified by MS from the latex extract of *V. heilbornii*. The peptides that showed, individually, at least 50% identity with *CpLip1*-deduced protein were, on average, 8.1 amino acids long. Taken together, they had 76.4% identity (83.7% similarity) to the *CpLip1*-deduced protein, with a coverage of 25.6% (Table S1). When the CpLip1 protein sequence was used to screen the GenBank nonredundant database with BLASTP, the two most significant hits were GenBank accession no. ABK94755 (score: 561) and GenBank accession no. XP002277782.1 (score: 546), the two sequences mentioned above that were identified by *de novo* sequencing (Fig. S1). Taken together, these data indicate that *CpLip1* is likely to code for the *C. papaya* protein, homologous to the one we detected in *V. heilbornii*.

CpLip1 codes for a 55 kDa (479 amino acid) protein that has 35% sequence identity (51% similarity) with castor bean acid lipase [20]. This is the most significant hit corresponding to an experimentally identified protein, the second one being a fungal lipase. It contains the residues (Ser293, Asp357 and His451) of a putative catalytic triad and the PROSITE motif of TAG lipases (Fig. 6). Comparison of the deduced amino acid

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ABK94755              QNDQTKYIVTGHSLGGALAILFPAVLAFHDE  307
CAO71857              ANDQTKFLVTGHSLGAALAILFPAILALHEE  309
CpLip1                HNDQVKFILTGHSLGGALAILFPAILFLHEE  307
AAR15173_RcOBL1       DHKNAKFVVTGHSLGGALAILFTCILEIQQE  358
059952.1 LIP_THELA    EHPDYRVVFTGHSLGGALATVAGADLRGN--  184
                       : : : :.******.*** :  . *  :
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Fig. 6. Comparison of the lipase region that surrounds the catalytic Ser residue (PROSITE motif PS00120). Lip_Thela: lipase of *T. lanuginosus*. RcOBL1: castor bean acid lipase. ABK94755 and CAO71857 (XP002277782.1): putative lipases from poplar tree and vine.

sequence with nonplant proteins indicates similarities to *Thermomyces lanuginosus* (and related fungi) lipase only.

The protein contains two hydrophobic stretches; the first one (residues 53–73) is predicted to be a transmembrane helix (according to HMMTOP), and the second one immediately follows the putative catalytic Ser. Neither transit nor signal peptide could be identified with TARGETP (the protein was predicted to be cytosolic, with the highest RC1 score). It appears that CpLip1, as a castor bean acid lipase, is composed of two domains: The N-terminus contains a strongly hydrophobic segment that might allow anchoring to membranes. The C-terminal domain is the lipase active domain.

The MS/MS analysis of the 24 kDa band (Fig. 5C) led to the identification of a protein of unknown function (homologous to *Arabidopsis* At5g01750, GenBank accession no. NP_850751.1), which we designated as CpTSRP (tubby structurally related protein). It is structurally homologous to tubby-like proteins, which contain a domain that binds to phosphoinositides, and also to phospholipid scramblases [38], which are capable of mediating movement of phospholipids across membranes. No obvious PL active site could be inferred from the analysis of the sequence. A soluble protein was overexpressed successfully in *Escherichia coli*, but neither PL nor lipase activities could be detected under the same conditions used to measure these activities in the latex (data not shown).

Identification of major proteins from the nonsoluble fraction

All major spots of the nonsoluble fraction were analyzed by MS/MS, and one by N-terminal sequencing (Table S2; peptides listed in Table S1). All spots were found to be contaminated by Cys proteases and most by Met synthase. Identification was carried out by comparing MS/MS spectra obtained experimentally with theoretical spectra deduced from databases with the use of SEQUEST software. When this approach failed to detect significant identity (at least two peptides) with SwissProt proteins, *de novo* sequencing was carried out and the peptides were used to screen databases. Among the databases used, a translation of *C. papaya* ESTs was found to be the most rewarding. Then, ESTs coding for sequences matching MS peptides were used to screen the SwissProt database. All BLAST scores and similarities between *C. papaya* ESTs and SwissProt closest proteins were high enough for ESTs to be unambiguously assigned to a defined protein or protein family. Only spot 7 could not be firmly identified, as *de novo* data showed only weak similarity to chitinase. However, this similarity was confirmed independently by comparison with an N-terminal sequence.

Screening by MS analysis from the whole extract yielded 12 additional proteins (Table S3; peptides listed in Tables S4 and S5). A similar study was carried out by *de novo* sequencing (Table S3; Figs S2 and S3). Most enzymes identified fell into three classes: (i) defense-related enzymes (proteases, hydrolases, rubber elongation factor and strictosidin synthase); (ii) protein synthesis and processing [a chaperone (heat shock protein 70), protein disulfide isomerase, Met synthase, elongation factor 1 and a ribosomal protein]; and (iii) polysaccharide metabolism. Neither obvious PLA2, nor other possible TAG lipases, could be detected.

Discussion

The lipase is extracted by organic solvents

Lipases are usually stable enzymes that can withstand the denaturing effect of several organic solvents. This property enables them to be widely used as biocatalysts in organic synthesis [9]. This is the case for *C. papaya* and *V. heilbornii* lipases, which remain active in organic solvents [26]. Also, hydrophobic proteins, such as plastid membrane proteins [39] or oleosin [40], are soluble in chloroform/methanol-based mixtures. However, it is difficult to understand how a protein can be soluble in a fully apolar solvent such as hexane. The lipase from *Euphorbia* latex [25] was purified with an organic solvent-based procedure. To explain the apparent solubility of the enzyme, the authors speculated that the lipase might be trapped into reverse micelles. Reverse micelles are micelles made of amphiphilic molecules in which the apolar part faces the outside and the polar part the inside. They are widely used to 'encapsulate' enzymes that catalyze bioconversion reactions in organic solvents [41]. The formation of such structures during homogenization of the insoluble fraction of latex in hexane would largely explain the apparent solubility of *V. heilbornii* lipase in hexane. This is also consistent with the apparent lack of selectivity of hexane extraction towards protein species. However, the existence of such structures remains to be demonstrated. Further speculation on the nature of the amphiphilic molecules susceptible to forming these micelles is hampered by the lack of knowledge on the nonproteinaceous components of papaya latex.

PLA2 activity is detected in latex

All activities show basic pH optima. The activity was highest with the artificial, short-chain TAGs TC4 and

TC8. Lipases are known to hydrolyze those substrates much more efficiently than long-chain TAGs, and a similar preference for short-chain fatty acids has already been reported for *C. papaya* lipase [23,29]. In addition, one cannot exclude the presence of active esterases in the extract. The amount of activity (300 IU per gram of fresh latex) is comparable to what has been reported for *C. papaya* [23] and *V. heilbornii* [27] lipases. The PLA2 activity represents one-quarter of the TAG lipase activity in the crude latex. This is comparable to results obtained with oil palm mesocarp [21]. However, the activity is much higher when assayed from the hexane extract. It is well known that organic solvents can tremendously increase PL activities [42], probably by improving the binding of the enzyme to the substrate. The PLA2 activity is inhibited by THL. If the inhibitory mechanism is similar to that described for pancreatic lipase (i.e. covalent binding to the nucleophilic Ser), then the PLA2 activity that we measure needs to be catalyzed by an enzyme with an active nucleophilic residue, which rules out a classical PLA2 with a catalytic dyad devoid of a nucleophilic residue. PLA2s with an active nucleophilic Ser fall into classes IV (cPLA2) and VI (iPLA2) [43]. The presence of strong PLA2 activity in latex makes sense in view of the main physiological role of latex, which is to protect the plant against pests [3]. The antimicrobial function of PLA2s is well documented [44]. Recently, a PLA activity was also shown in the latex of *Euphorbia* [22]. No obvious PLA2 candidate has been detected by MS analysis of latex major proteins. A possible candidate, CpTSRP, does not resemble known PLs, and the recombinant protein was unable to hydrolyze PtdCho. Therefore, it might be that both PLA2 and TAG lipase activities are borne by the same enzyme. Whereas dual lipase–PLA1 [11] enzymes are well documented, evidence for dual TAG lipase–PLA2 enzymes has been provided only recently [45].

How specific is THL towards lipases?

Lipolytic activities on olive oil and on PtdCho are both inhibited by THL, an inhibitor that binds covalently to the active site Ser of human pancreatic lipase [36,46]. To our knowledge, this is the first time that THL has been reported to inhibit a PLA2. Three bands are labeled with THL. One of them resembles castor bean acid lipase, one of the few plant lipases unambiguously identified up to now (see discussion below). Another protein that binds THL is GAPDH (phosphorylating). Although it may appear surprising that THL binds to GAPDH, the esterase function of this enzyme, in the absence of NAD, is well documented [47]. The active site Cys responsible for the dehydrogenase reaction is known to also be the nucleophilic residue involved in the esterase function. In both cases, an acyl-enzyme intermediate is formed during the reaction. These data indicate that THL might bind to GAPDH through a similar mechanism to that for binding to pancreatic lipase, except that the enzyme nucleophilic residue involved in the reaction is a Cys instead of a Ser. THL is widely considered to be an inhibitor that is rather specific for lipases, because its action on pancreatic lipase and several other lipases is well documented [36]. However, it has also been shown to be active on human acyl-ACP thioesterase, a Ser enzyme [48]. Also, it inhibits an esterase from *C. papaya* [29]. Now, our data suggest that Cys esterases might also be potent targets for THL. This is not linked to the hexane extract, as THL labeling is also obtained with washed latex in the presence of 4 mM Chaps. It is likely that increasing the number of enzymes tested will show that THL has a larger spectrum of action than initially thought.

Identification of a candidate lipase

MS analysis of the 45 kDa spot labeled with [^{14}C]THL indicates that the highest similarity to the characterized enzymes is obtained with castor bean acid lipase. No other protein resembling a TAG lipase could be identified in the nonsoluble fraction of latex. The intensity of the spot on the gel indicates that the protein represents 1.3–4.4% of total proteins, which suggests an SA ranging from 1300 to 4400 IU per mg of pure protein; this value is comparable to that for most characterized TAG lipases. Taken together, these data strongly suggest that we have identified the enzyme responsible for TAG hydrolysis as a member of the castor bean acid lipase structural family.

Using *de novo* sequencing data (from a *V. heilbornii* protein), we searched *C. papaya* genomic and EST resources to identify *CpLip1*, a cDNA coding for the most similar protein from this organism. *C. papaya* and *V. heilbornii* are very closely related species; however, it is difficult to estimate an average percentage of similarity between proteins from the two species, as there are only six protein sequences known from *V. heilbornii*. There are four proteases that show 61.5% identity and 77% similarity to proteins coded by the *C. papaya* whole genome shotgun sequence. Two mutases show 93% identity and 96% similarity. The lipase is abundant in *C. papaya* latex, and CpLip1 is the most frequently represented in EST databases, suggesting that its level of expression is higher than that of other members of the family. Therefore, we can hypothesize that CpLip1 is the *C. papaya* homologous

lipase of *V. heibornii*. However, as *C. papaya* genome coverage is estimated to be 80%, and that the castor bean acid lipase family comprises five members in *Arabidopsis*, we may have missed a member of the family.

CpLip1 codes for a 55 kDa protein. Because most closely similar proteins from poplar, vine and *Arabidopsis* have similar molecular masses, it is likely that this is the case for *V. heilbornii* lipase. It might be that *V. heilbornii* lipase behaves unusually on SDS/PAGE. Another possibility is that the protein is processed from a precursor, as is the case for papain, which is synthesized as a pre-pro-protein. Also, it might be that the putative N-terminal membrane domain is cleaved off by the proteases during experimental processing of the sample, as has been reported for several membrane-bound proteins.

Conclusion

Papaya lipase has eluded identification for a long time. Using an approach based on radiolabeling a protein extract from *V. heilbornii* with a lipase inhibitor, we have identified a protein with high similarity to the family of castor bean acid lipases. Its estimated SA on olive oil is comparable to that of most characterized true lipases. We have identified a gene that is likely to code for a protein of *C. papaya* that is homologous to the *V. heilbornii* putative lipase. As no putative PLA2 could be found, it may be that the lipase identified possesses both TAG lipase and PL activities.

Experimental procedures

Plant material

Babaco latex was collected near Quito in Combaya province, Ecuador. The fresh latex was obtained by making three longitudinal incisions on the epidermis of the unripe fruit. The latex was lyophilized and ground. The latex powder was stored at room temperature.

Fractionation of babaco latex

Latex powder was ground (three bursts of 30 s each at 24 000 r.p.m.), using an Ultra Turrax, in 30 mM Tris/HCl (pH 8.0) and 150 mM NaCl (10 mL per gram of latex). The homogenate was centrifuged at 23 700 ***g*** and 4 °C for 20 min, and the pellet was re-extracted twice again under the same conditions. The final pellet was lyophilized and extracted with hexane (30 mL of hexane per 1 g of dried pellet). The mixture was centrifuged at 4300 ***g*** for 15 min. The hexane phase (used as enzyme extract) was saved and stored at 4 °C.

Delipidation of latex powder

Total delipidation of latex powder was carried out according to [49], using successive treatments with acetone, chloroform/butanol mixtures (9 : 1 and 4 : 1, v/v), chloroform/methanol mixture (9 : 1, v/v) and diethyl oxide.

Measurement of lipolytic activities

Lipolytic activities were assayed by continuous titration of the fatty acids released, using 0.01 M NaOH with a Metrohm pH-STAT, as previously described [21]. The substrate was either TC4, TC8 (500 μL) or olive oil (1 mL) for measurement of lipase activity. The oil was emulsified immediately before use in 10% (w/v) gum arabic (GA). For the calcium/EDTA effect, GA was reduced to 1% in the emulsion, as a 10% GA solution may contain 30 mM calcium. Each assay was performed at 25 °C in 30 mL of reaction mixture containing 2 mM Tris/HCl and 150 mM NaCl. Before measurement of the activity, the latex powder was dispersed in 30 mM Tris/HCl (pH 8.0) and 150 mM NaCl (100 μL per 1 mg), and stored on ice. For all activity tested, the rate of reaction was linear for at least 9–10 min, except at pH values above 9.5, for which linearity could be observed for a shorter time.

PL activity was assayed as described by Abousalham & Verger [50], using PtdCho as substrate. The reaction mixture contained 13.3 mM sodium deoxycholate, 8 mM $CaCl_2$ and 1.2% (w/v) PtdCho. Cholesteryl oleate esterase activity was assayed according to [14]. Inhibition experiments with THL were carried out according to the so-called method A (lipase/inhibitor preincubation method) [51]. The enzyme extract was preincubated for 1 h at room temperature with THL solubilized in hexane, in the presence of 4 mM Chaps. Lipolytic activities were then assayed as described above.

The amount of sample used in the assay was 5–20 μL (hexane extract) and 2–5 mg (latex powder), as the activity increased linearly with the amount of enzyme in these conditions.

For determination of regioselectivity, the extract was incubated (100 μL final volume) under continuous stirring for 15 min at room temperature in 25 mM Tris/HCl (pH 8.0), 8 mM $CaCl_2$, 0.4% (w/v) PtdCho from egg yolk, 6 mM sodium deoxycholate and PtdCho 1,2-di[1-^{14}C]palmitoyl (3.6 kBq, 4.2 GBq·mmol^{-1}). Lipids were extracted with 1-butanol to ensure quantitative recovery of lysoPtdCho as previously described [52] and separated by TLC. The plate was dried and exposed overnight for PhosphorImager (Perkin Elmer, Waltham, MA, USA) analysis.

Protein extraction for PAGE analysis

A phenol-based method gave us the best results in quantitatively extracting proteins for SDS/PAGE analysis. In addition, this procedure was found to remove compounds that

interfere with Coomassie Blue staining. Proteins were extracted either from latex powder or from enzyme extract according to [53]. Latex powder (10 mg) was homogenized in a micropotter with 1 mL of 30 mM Tris/HCl (pH 8.0) and 150 mM NaCl. The content was shaken vigorously by use of a vortexer, and incubated for 30 min on ice. An equal volume of water-saturated phenol (buffered to pH 8.0) was then added. After centrifugation at 12 000 ***g*** for 7 min, the upper phase was re-extracted with fresh phenol. The phenol phases were combined and extracted twice with an equal volume of hexane to remove residual nonpolar lipids. Proteins were precipitated (overnight at −20 °C) from the phenol phase by adding five volumes of cold methanol containing 0.1 M ammonium acetate. The precipitate was collected by centrifugation (20 000 ***g***, 15 min, 4 °C), and the pellet was washed six times with cold methanol containing 0.1 M ammonium acetate and twice with 80% acetone. The pellet was dried and resuspended in Laemmli buffer [54]. Insoluble material was removed by centrifugation at 20 000 ***g*** for 20 min at 4 °C. Proteins were extracted from the hexane fraction by adding an equal volume of water-saturated phenol (pH 8). After vigorous shaking with a vortexer, the two phases were separated by centrifugation (12 000 ***g*** for 7 min) and proteins were precipitated from the phenol phase as described above.

Protein concentration was determined at the Institute of Structural Biology Facility in Grenoble (France), on the basis of the amount of amino acid determined after protein hydrolysis. Because Cys, Met and Trp cannot be quantified, the amount of protein is slightly underestimated.

Protein electrophoresis

SDS/PAGE

Proteins were resuspended in Laemmli buffer [54] and analyzed by electrophoresis on 12% polyacrylamide gels, using standard conditions, except that the SDS concentration in both stacking and resolving gels was 1% (w/v).

2D gels

Proteins were solubilized in 125 μL of loading buffer [7 M urea, 2 M thiourea, 20 mM dithiothreitol, 2% (w/v) Chaps, 2% (w/v) amidosulfobetaine-14, 2% (v/v) immobilized pH gradient buffer]. The sample was used to rehydrate a 7 cm linear Immobiline Dry Strip gel (pH 3–10) overnight. IEF was performed on an IPGphor II at 20 °C. The strip was equilibrated for 15 min in 5 mL of equilibration solution [50 mM Tris/HCl (pH 6.8), 6 M urea, 25% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) dithiothreitol]. The strip was sealed at the top of a 1 mm vertical second-dimension gel with melted 1% agarose that contained 0.5% (w/v) SDS, 25 mM Tris/HCl and 200 mM glycine supplemented with bromophenol blue as a tracking dye.

Separated proteins were stained with Coomassie Brillant Blue R-250.

BAC gels

Two-dimensional BAC SDS/PAGE was carried out according to [55].

Fluorography

Fluorography [56] was carried out by imbibiting the gels in Amplify TM (GE Healthcare, Waukesha, WI, USA). The gels were then dried on a Whatman 3MM paper and exposed for a few days to Hyperfilm (Amersham). Alternatively, dried gels were exposed to a screen and the radioactivity was analyzed with a PhosphorImager.

Estimation of spot intensities

The intensity of all spots was determined with SCION IMAGE for Windows (Scion Corp., Fredrick, Maryland, USA; http://www.scioncorp.com/pages/scion_image_windows.htm), with subtraction of background measured in the bottom left part of the gel. The percentage of a given spot was estimated with the use of two different measures. The intensity of the spot of interest was divided by the sum of all major spots. Alternatively, the intensity of the spot of interest was divided by the whole area that contains proteins (about the left two-thirds left of the gel), to take into account streaks of unresolved proteins.

Amino acid sequencing

The N-terminal sequence of proteins was determined by automated Edman degradation, with a Procise 494 sequencer (Perceptive Biosystems, Framingham, MA, USA).

Protein identification by nanoLC-MS/MS

Gel pieces were digested with trypsin, and the peptide mixture was analyzed by on-line capillary HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LTQ XL Ion Trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Ten microliters of peptide digests were trapped on a 300 μm × 5 mm C18 PepMap column (LC Packings) at a flow rate of 30 $\mu L \cdot min^{-1}$ before being separated on an analytical 75 μm × 15 cm C18 PepMap column (LC Packings) with a 5–40% linear gradient of solvent B in 35 min (solvent A was 0.1% formic acid in 5% acetonitrile, and solvent B was 0.1% formic acid in 80% acetonitrile) at a flow rate of 200 $nL \cdot min^{-1}$. Data were acquired in a data-dependent mode, alternating an MS scan survey over the range *m/z* 500–3500 and three MS/MS scans in an exclusion dynamic mode. MS/MS spectra were

searched by SEQUEST through the BIOWORKS 3.3.1 interface (ThermoFinnigan) against SwissProt (restricted to Viridiplantae), GenBank nonredundant proteins (restricted to Viridiplantae), *Arabidopsis* (TAIR), home-made databases that contain either a six-frame translation of *C. papaya* ESTs or a six frame-translation of *C. papaya* whole genome shotgun sequences. The search parameters were as follows. Mass accuracy of the peptide precursor and peptide fragments was set to 2 and 1 Da, respectively. Only b-ions and y-ions were considered for mass calculation. Oxidation of Met residues (+16 Da) and carbamidomethylation of Cys residues (+57 Da) were considered as differential modifications. Two missed trypsin cleavages were allowed. Only peptides with X_{corr} higher than 1.5 (single charge), 2.0 (double charge) and 2.5 (triple charge) were retained. In all cases, ΔC_n had to be superior to 0.1, and the peptide *P*-value had to be lower than 1.10 e−3. Proteins identified by a unique peptide were rejected. Alternatively, MS/MS spectra were analyzed by *de novo* sequencing with the PEPNOVO algorithm [57], and sequenced tags were subsequently FASTS [58] searched against GenBank nonredundant proteins (restricted to Viridiplantae), home-made databases that contain either a six frame-translation of *C. papaya* ESTs or a six-frame translation of *C. papaya* whole genome shotgun sequences. These databases were constructed using TRANSEQ, which is part of the EMBO open software suite [59], available at http://www.cbib.u-bordeaux2.fr/pise/transeq.html.

Other bioinformatics methods

BLAST searches [60] were carried out at the website of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov). The protein databases used were SwissProt and GenBank nonredundant proteins. The nucleic acid databases used were GenBank nonredundant, ESTs (nonhuman nonmouse, restricted to *C. papaya*) and whole genome shotgun reads (restricted to *C. papaya*).

N-terminal amino acid sequences were used to search GenBank nonredundant proteins restricted to Viridiplantae, using default conditions for short sequences; only the subject sequences that showed similarity at the N-terminus (taking into account possible signal sequences) were retained.

Conserved motifs were searched for in PROSITE [61] (http://expasy.org/prosite/), signal sequences were searched for using TARGETP [62] (http://www.cbs.dtu.dk/services/TargetP/), and transmembrane domains were searched for at HMMTOP [63] (http://www.enzim.hu/hmmtop/).

cDNA cloning

Total RNAs were purified from *C. papaya* fruit peels (1 mm thick) as previously described [64]. First-strand cDNA was prepared with oligodT as primer, with Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The cDNA was amplified with the primers 5′-ACTCAAATCACTAGTATTCTTCCACCA-3′ and 5′-CATTTGAACATAAACATGAACAAATAAGTT-3′ and the following conditions: annealing temperature 55 °C, 25 cycles, Phusion polymerase used according to the manufacturer's instructions. The PCR product was gel purified and cloned into pCR-TOPO Blunt (Invitrogen) according to the manufacturer's instructions. Sequencing was carried out on both strands and subcontracted to Cogenics (France). The EMBL accession number is FR676961.

The CpTSRP ORF was amplified by RT-PCR with the oligonucleotides 5′-GCGCATATGGCCGGATTAAGCTACCTGAC-3′ and 5′-GCGGGATCCTTAGTCGTCGCCACTGCGATC-3′, and cloned in frame into the *Nde*1–*Bam*H1 sites of pET3A. Sequencing was carried out on both strands. The construction was expressed in *E. coli* BL(21)DE3 according to standard procedures, and a crude soluble extract was obtained that contained an abundant 24 kDa protein (absent from the control), which was used to assay lipase activity as described above. The EMBL accession number is FR682666.

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Supporting information

The following supplementary material is available:
Fig. S1. Identification of proteins contained in spot 5 by *de novo* sequencing and screening a GenBank nonredundant protein database restricted to Viridiplantae.
Fig. S2. Identification of proteins contained in the whole insoluble latex fraction by *de novo* sequencing and screening a SwissProt database restricted to Viridiplantae.
Fig. S3. Identification of proteins contained in the whole insoluble latex fraction by *de novo* sequencing and screening a six-frame translated *C. papaya* EST database.
Table S1. List of peptides that identified proteins from 2D gels.
Table S2. Identification of major protein spots of the insoluble fraction of *V. heilbornii* latex.
Table S3. Whole sample proteomic analysis.
Table S4. List of peptides identified by screening a six-frame translated *C. papaya* EST library by SEQUEST.
Table S5. List of peptides identified by screening a nonredundant GenBank library (restricted to Viridiplantae) with SEQUEST, using MS/MS spectra from a whole latex-insoluble protein fraction.

This supplementary material can be found in the online version of this article.

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