Biochimica et Biophysica Acta 1791 (2009) 1048–1056

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Identification and biochemical characterization of a GDSL-motif carboxylester hydrolase from *Carica papaya* latex

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ARTICLE INFO

Article history:
Received 6 May 2009
Received in revised form 8 June 2009
Accepted 16 June 2009
Available online 23 June 2009

Keywords:
Carica papaya
Latex
GDSL
Esterase
Gene
Mass spectrometry

ABSTRACT

An esterase (CpEst) showing high specific activities on tributyrin and short chain vinyl esters was obtained from *Carica papaya* latex after an extraction step with zwitterionic detergent and sonication, followed by gel filtration chromatography. Although the protein could not be purified to complete homogeneity due to its presence in high molecular mass aggregates, a major protein band with an apparent molecular mass of 41 kDa was obtained by SDS-PAGE. This material was digested with trypsin and the amino acid sequences of the tryptic peptides were determined by LC/ESI/MS/MS. These sequences were used to identify a partial cDNA (679 bp) from expressed sequence tags (ESTs) of *C. papaya*. Based upon EST sequences, a full-length gene was identified in the genome of *C. papaya*, with an open reading frame of 1029 bp encoding a protein of 343 amino acid residues, with a theoretical molecular mass of 38 kDa. From sequence analysis, CpEst was identified as a GDSL-motif carboxylester hydrolase belonging to the SGNH protein family and four potential N-glycosylation sites were identified. The putative catalytic triad was localised (Ser^{35}-Asp^{307}-His^{310}) with the nucleophile serine being part of the GDSL-motif. A 3D-model of CpEst was built from known X-ray structures and sequence alignments and the catalytic triad was found to be exposed at the surface of the molecule, thus confirming the results of CpEst inhibition by tetrahydrolipstatin suggesting a direct accessibility of the inhibitor to the active site.

1. Introduction

The latex from *Carica papaya* is recognized as a rich source of plant proteases that are used in pharmaceutical, food (as meat tenderiser, for example) and beverage (for beer chill-proofing) industries. Among them, papain is the most well-known proteinase but chymopapain, caricain (or papaya protease omega) and glycyl endopeptidase (or papaya peptidase B) have been also purified and characterized [1]. These enzymes are commercially available under a crude protease preparation or industrially refined depending on the application. Besides the proteases, other proteins and enzymes have been identified as minor constituents of the water-soluble fraction of the latex [2]. The detection of lipase activity in the papaya latex [3] has triggered a novel interest in this latex for synthetic reactions such as esterification and transesterification [4]. The reason for this is mainly the fact that the enzyme is not soluble and therefore regarded as a "naturally immobilised" biocatalyst [1,5]. The regio-selectivity, stereo-selectivity and typo-selectivity of the crude *C. papaya* latex were studied in both hydrolysis and acyltransfer reactions. In hydrolysis, this biocatalyst is 1,3-regioselective with a slight sn-3 stereo-preference [6]. In addition to these properties, the *C. papaya* lipase can be active at relatively high temperature and has a pronounced stability with its activity unchanged over a broad pH range [3,7,8].

In order to better characterize the *C. papaya* lipase at the molecular level, several attempts were made to solubilise the active enzymes from particulate fractions of latex, but all the trials were however unsuccessful up to now [3,9]. One explanation for this failure might be that the lipase is covalently linked to the polyisoprenoid polymers present in the latex [10] and that classical techniques for protein separation and purification are not well adapted. Several fractions of *C. papaya* latex with lipolytic activity can however be prepared using organic solvents such as hexane, and more recently, with detergents and physico-chemical treatment.

We report here the isolation and identification of a carboxylester hydrolase from *C. papaya* latex. Sequence analysis reveals that it is a

Abbreviations: CpEst, *Carica papaya* esterase; EST, expressed sequence tags; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; HPL, human pancreatic lipase; TLL, *Thermomyces lanuginosus* lipase; THL, Tetrahydrolipstatin; NaTDC, sodium taurodeoxycholate

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doi:10.1016/j.bbalip.2009.06.002

Table 1
Flow sheet of CpEst partial purification.

Purification step	Total activity[a] (IU)	Proteins[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Crude dried latex	25800	5500	4.7 ± 1.7	100	1
Washed latex	25506	1228	20.7 ± 3	98 ± 2	4.4
Partially delipidated latex	18720	nd	nd	72 ± 5	nd
CHAPS-containing extract from sonicated latex	2340	57	41 ± 4	9 ± 3	8.7
Superdex 75	1820	10	170 ± 7	7 ± 2	36

nd, not determined.
[a] International Unit: 1 μmol of fatty acid released per min using TC_4 as substrate.
[b] Proteins were estimated by amino acid analysis.

member of the GDSL-motif enzyme family. Enzyme activity measurements using triglycerides with various chain length and vinyl esters confirmed that this enzyme is an esterase that mainly contributes to the overall lipolytic activity of *C. papaya* latex on tributyrin, but poorly to the activity of the whole latex on the long chain triglycerides.

2. Experimental procedures

2.1. C. papaya latex

Dried *C. papaya* latex was obtained from Biohainaut (Mons, Belgium). Fresh latex was collected from Congo after incisions on the epidermis of the fruit. This latex is usually dried and roughly crushed in order to obtain a crude powder called P_3.

2.2. Lipase activity measurements

A pH-Stat (Metrohm 718 Stat Titrino, Zofingen, Switzerland) was used to measure the lipase activities potentiometrically in a thermostated reaction vessel containing a mechanically stirred emulsion of 0.5 mL tributyrin (Fluka, Paris, France), 0.5 mL trioctanoin (Fluka, Paris, France) or 1 mL olive oil. The olive oil was emulsified immediately before use in 10% gum Arabic as described by Abdelkafi et al. [7]. Each assay was performed in 15 mL of reaction mixture containing 2.5 mM Tris–HCl and 150 mM NaCl. Whatever the triglycerides tested, the amount of substrate available for the enzyme was not limiting during the reaction. Lipase activity was expressed in international units (1 U = 1 μmol FFA released per min). Phospholipase activity was measured as described by Abousalham and Verger [11].

2.3. Determination of protein concentration

Protein concentration was determined using the Bradford assay using bovine serum albumin as the reference protein as previously described [12].

2.4. SDS-polyacrylamide gel electrophoresis

Protein electrophoresis was performed using 12% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gel according to the method described by Laemmli [13]. The apparent molecular masses of proteins were estimated by co-electrophoresis of marker proteins (Biorad, Hercules, CA, USA) with masses ranging from 14.4 to 116 kDa. The protein in sample buffer [0.9 g glycerol, 0.1 mL 1% bromo-phenol blue, 1 mL 10% (w/v) SDS, and 0.1 mL mercaptoethanol] was heated for 5 min in boiling water and applied to the gel. The proteins separated on the SDS-polyacrylamide gel (SDS-PAGE) were stained with Coomassie Brilliant Blue R-250.

2.5. Extraction of lipolytic enzyme from latex

Fifty grams of dried *C. papaya* latex was used as the starting material for each purification procedure. To the 50 g of the *C. papaya* latex was added 100 mL of distilled water. The resulting suspension was centrifuged at 8000*g* for 10 min and the supernatant was discarded. This procedure was repeated five times in order to wash the latex and remove soluble compounds. In a second step, the washed powder was extracted three times with 15-mL acetone. The mixture was centrifuged at 3500 *g* for 10 min and the supernatant discarded. The washed and partially delipidated acetone powder was resuspended in 45 mL of 75 mM Tris–HCl, pH 8, 20 mM CHAPS. The mixture was stirred for 20 min at 4 °C, and then sonicated for 60 min (88155 sonicator, Bioblock Scientific, Freiburg, Germany) and ultra-centrifuged (Sigma 4 K-15, Fisher Bioblock, France) for 45 min at 20000 *g*. The supernatant was separated from the pellet and was submitted to a second step of ultracentrifugation for 90 min at 20000 *g*. The insoluble pellet was discarded and the supernatant was then filtered through a 0.22-μm membrane filter and stored at 4 °C until use. Gel filtration chromatography was performed after loading the previous solution on a Superdex 75 HR 16/60 column (Pharmacia), using 10 mM Tris–HCl pH 8, 150 mM NaCl buffer [14]. The flow rate was adjusted to 1 mL/min with a pressure kept below 0.5 MPa using an ÄKTA™ explorer chromatographic device (Amersham Biosciences, Uppsala, Sweden). The protein elution profile was recorded spectrophotometrically at 280 nm. Fractions with lipase activity eluted from the gel filtration column were pooled.

2.6. Inhibition by THL

Inhibition experiments were carried out as previously described [15]. The enzyme extracted from *C. papaya* latex was pre-incubated with THL (Hoffmann-La-Roche, Basel, Switzerland) for 30 min at an enzyme: inhibitor molar ratio 1:20, in the absence or presence of 4 mM NaTDC in the incubation medium. The residual enzyme activities were then measured on tributyrin as described above. Control experiments with

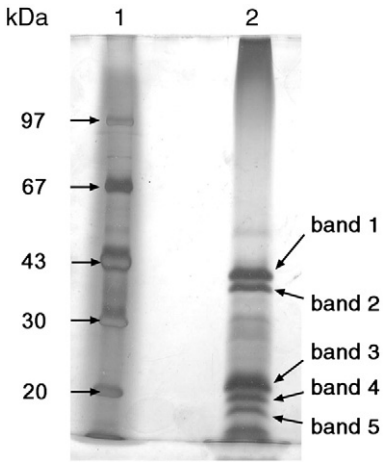

Fig. 1. SDS-PAGE (12%) analysis of the protein fraction with lipolytic activity eluted from gel filtration chromatography. Lane 1, molecular mass markers; lane 2, 100 μL of the pooled fraction of Superdex G-75 chromatography. The gel was stained with Coomassie blue.

Table 2
Carica papaya latex proteins separated by SDS-PAGE (bands 2 to 4) and directly identified by LC/ESI/MS/MS after tryptic digestion.

Band number on SDS-PAGE	Protein	Accession number	Number of unique matched peptides	Sequence coverage (%)	Theoretical molecular mass (kDa)
2	Chymopapain	P14080	13	55	39.38
2	Papaya proteinase IV	P05994	8	34	39.02
2	Papain precursor	P00784	2	5	38.89
3	Glutaminyl cyclotransferase	2IWA	14	49	30.9
3	Endochitinase	P85084	1	5	26.52
4	Caricain	1MEG	12	52	23.28
4	Serine proteinase inhibitor	P80691	4	17	20.63
4	Papaya protease omega	1PPO	2	10	23.27
4	Glycyl endopeptidase	1GEC	2	9	23.30

the well-known lipases from human pancreas (HPL) and *Thermomyces lanuginosus* (TLL) were performed [16].

2.7. Amino acid composition and N-terminal sequencing analyses

Amino acid compositions were determined as previously described by Grzela et al. [17]. The N-terminal sequences of the proteins were obtained using a Procise sequencer Model 610A 2.1 [18].

2.8. In-gel tryptic digestion and protein identification by mass spectrometry

The proteins were separated using 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Bands of interest were manually excised from gels and automatedly treated for destaining, washing, reduction-alkylation and tryptic digestion steps in a robotic workstation EVO 100 (Tecan, Männedorf, Switzerland) as previously described [19] or manually treated as follows. Gel pieces were destained with 50% acetonitrile in 25 mM ammonium bicarbonate, then crushed in Eppendorf tubes, dehydrated with acetonitrile for 5 min, and vacuum-dried. Gel pieces were first fully rehydrated with 15–50 μL of 25 mM ammonium bicarbonate, 10% acetonitrile supplemented with trypsin (5 ng/μL; Promega, Madison, WI) and then overlaid with an equal volume of buffer without trypsin. After a 20–24-h incubation at 37 °C, the incubation volume was adjusted to 100 μL with ultrapure water and acidified with 25 μL of 25% formic acid (5% final concentration). Tryptic peptides were extruded from the gel by adding 125 μL of acetonitrile [19]. After a 15-min-incubation at room temperature, the gel pieces were spun down (8000 *g* for 5 min at room temperature), the supernatant was collected, and the pellet was overlaid with 50 μL of acetonitrile and vortexed to complete peptide extraction. The extracted material was pooled with the supernatant and vacuum-dried. The dried material obtained was solubilised in 10 μL of 5% formic acid, 20% methanol and stored at −20 °C until used.

All samples were analysed by LC/ESI/MS/MS, first on a LCQ-DECA^{XP} mass spectrometer (Thermo Finnigan, Waltham, MA) using a nano-2D liquid chromatography [19], then on a LTQ/FT-Orbitrap mass spectrometer (Thermo Finnigan, Waltham, MA) coupled with pumps and autosampler under standard conditions: capillary temperature, 275 °C; source voltage, 4500 V. Helium was used as the collision gas. Experiments were done in parallel mode (survey at 60 000 resolution and five data-dependent ion trap MS/MS scans (Top 5)). The MS/MS parameters were as follows: isolation width, 3; collision energy, 35%; micro-HPLC Surveyor system (Thermo Fisher); 60-min gradient; and BioBasic C_{18} (Thermo Scientific) column (100×0.32 mm).

Acquired MS/MS spectra were interpreted using Mascot version 2.2.0 (Matrix Science, London, UK) and in-house software. Search parameters were set as follows: enzyme specificity, trypsin; one missed cleavage permitted; fixed modification, carbamidomethylation of cysteine; variable modification, methionine oxidation; mass tolerance for precursor ions, 5 ppm; mass tolerance for fragment ions, 0.3 Da; significance threshold, $p<0.05$; and expect value threshold, 0.001 (to select ions with the highest individual score). Both b and y ion series were used to search against UniProt KB/Swiss-Prot/TrEMBL (database version 51.6; 257 964 sequence entries), non-redundant NCBI (http://www.ncbi.nlm.nih.gov) and the EST *C. papaya* database containing 77 187 entries (EX227656-EX303501). Since contaminations from human (mainly keratins) origin could be present in the

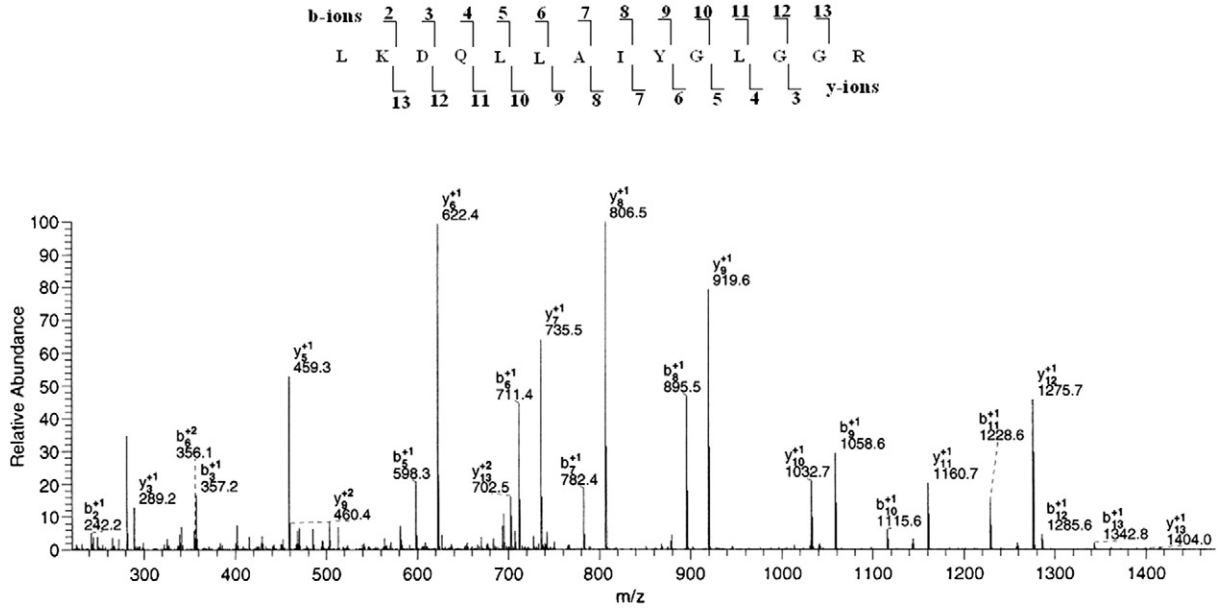

Fig. 2. MS/MS (fragment ion) spectrum of the double charged precursor ion $(M+2H)^{2+}$ at m/z 758.9 corresponding to the LKDQLLAIYGLGGR peptide.

samples analysed, the search in databases was restricted (UniProt KB/Swiss-Prot/TrEMBL, 49887 sequence entries; NCBI nr, 551056 sequence entries). In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides.

2.9. Bioinformatic analysis and structural modeling

Bioinformatic analysis of the *C. papaya* peptide sequences, ESTs, genomic sequences and deduced protein sequences were performed using the following tools. Multiple sequence alignment was performed using the ClustalW algorithm [20]. The peptide sequences were compared with the NCBI (National Center for Biotechnology Information, USA) non-redundant sequence databases and the EST *C. papaya* database containing 77187 entries (EX227656-EX303501) using BLAST [21]. Primary structure analysis was performed using the ExPASy Proteomics tools. The ProtParam tool was used to calculate the theoretical parameters of the protein [22]. The prediction of the signal peptide sequence was performed using the signalP 3.0 application [23]. The prediction of Asn glycosylation (N-glycosylation) sites was performed using NetNGlyc 1.0 Server. A three dimensional structure of CpEst was modeled using the PHYRE PROTEIN FOLD RECOGNITION Server (http://www.sbg.bio.ic.ac.uk/phyre/) [24]. The model was edited using PyMOL [25].

3. Results

3.1. Insolubility of the lipolytic activity from C. papaya latex

Using tributyrin as substrate, a lipase activity reaching 2000 IU/g of dried *C. papaya* latex was detected using a 10% w/v dispersion of latex in deionised water. After centrifugation (3000 *g* for 10 min),

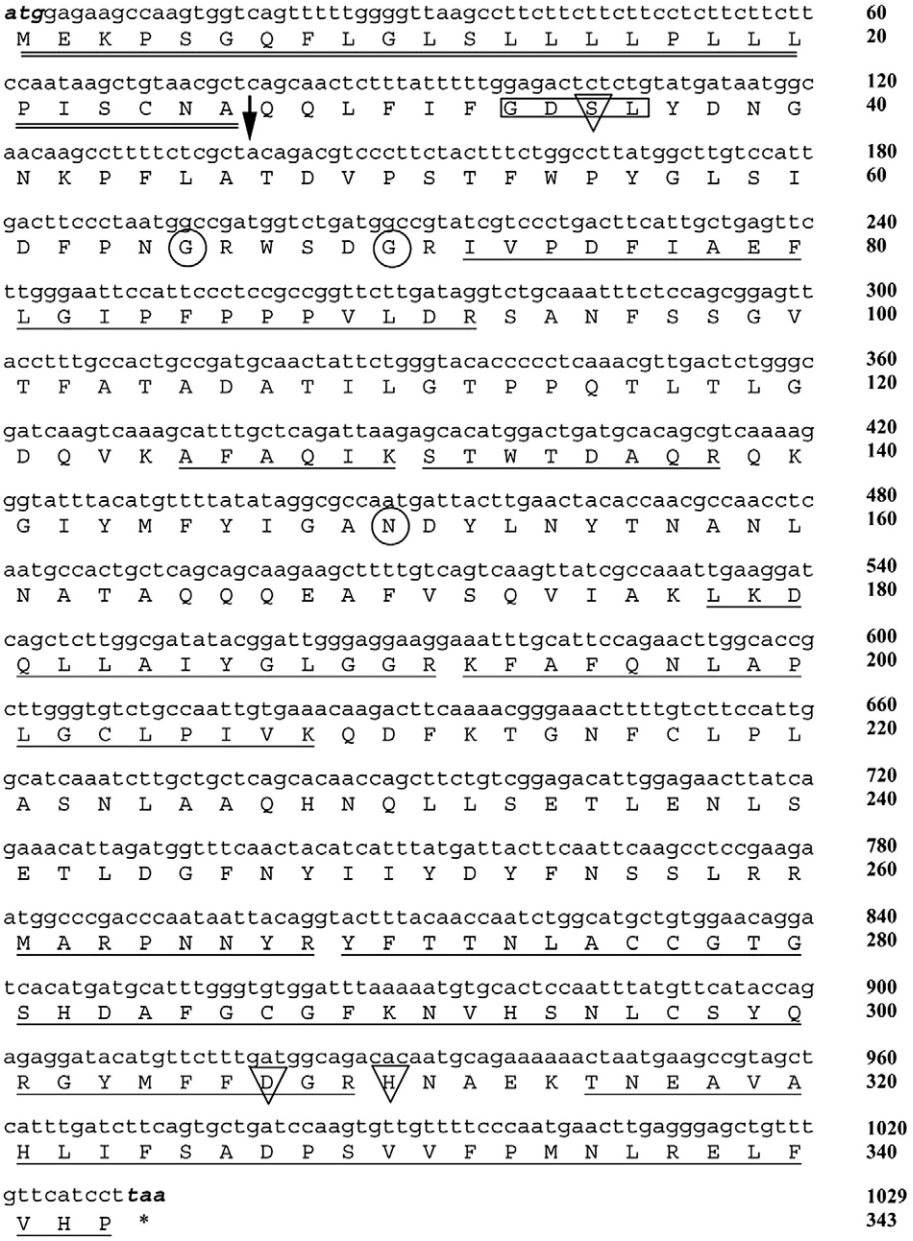

Fig. 3. Open reading frame and amino acid sequences of CpEst. Bold italics mark the start codon (atg) and an asterisk indicates the translation stop codon (taa). The GDSL-motif of CpEst is indicated by an open box. Amino acid sequences obtained by Edman chemistry and by mass spectrometry are underlined. The putative signal peptide is double underlined, with the cleavage site CNA/QQ indicated by an arrow. The amino acid residues involved in the putative oxyanion hole and the catalytic triad typical of esterases are indicated by circles and triangles, respectively.

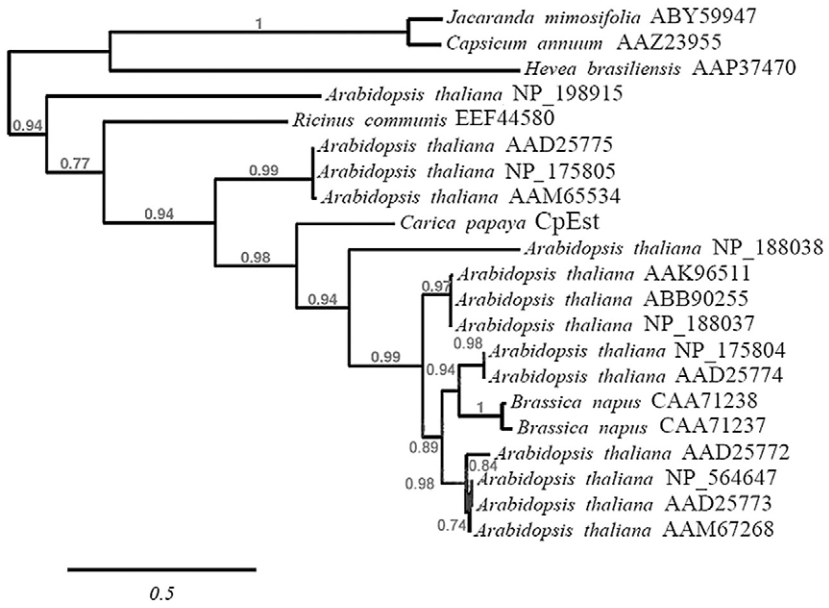

Fig. 4. Dendogram of protein sequence alignments showing the relationship of CpEst with the serine esterase/lipase from the GDSL family. The horizontal line length is an indication of the dissimilarity between the sequences they connect.

lipase activity was no longer detectable in the solution separated from the pellet. In contrast, the particulate (sediment) fraction contained the entire lipolytic activity. This finding is consistent with the fact that the *C. papaya* proteases were usually extracted from latex using water [26] and that no detectable lipolytic activity was found to be associated with these enzymes.

3.2. Partial extraction of the lipolytic activity of C. papaya latex

Dried *C. papaya* latex was used as the starting material for partly extracting proteins with a lipolytic activity. Lipolytic activity in all fractions was monitored as described in Experimental procedures using tributyrin as substrate. After several trials involving various detergents and physico-chemical treatments (ultra-turax, sonication), an optimised procedure for extracting lipolytic activity from washed and partly delipidated *C. papaya* latex was established using the CHAPS detergent and sonication. The dried latex was first washed with water to remove soluble proteins and was then partially delipidated. These steps allowed to recover 72% of the dried latex activity on tributyrin and this activity still remained bound to insoluble material. After the CHAPS+sonication treatment, 9% of the overall activity was recovered in the water phase whereas most of the activity was lost or remained bound to insoluble latex particles. The solution containing lipolytic activity was then loaded on a Superdex 75 gel filtration column. All the lipolytic activity was eluted with the dead volume of the column, indicating the presence of lipolytic enzymes in aggregated particules of high molecular mass. This chromatographic step however allowed to separate additional proteins and after this step, the specific activity of the enzyme preparation was increased from 41 ± 4 to 170 ± 7 U/mg using tributyrin as substrate. The complete purification flow sheet is given in Table 1. At the end, only 7% of the dried latex activity on tributyrin was recovered. The fractions eluted from Superdex 75 and containing the lipolytic activity were pooled and analysed.

3.3. Protein identification

The pooled fractions from gel filtration chromatography containing the lipolytic activity were analysed by SDS-PAGE (Fig. 1). Five main protein bands were separated. These bands were excised from a preparative gel and digested with trypsin *in situ*, and all the resulting peptides were analysed by LC/ESI/MS/MS. The amino acid sequences of these peptides were determined either by manual interpretation of the collision-induced spectra of the major peptide ion or computer-aided fragment-matching algorithms (SEQUEST). With the exception of band 1, all the other bands were identified as known proteins of *C.*

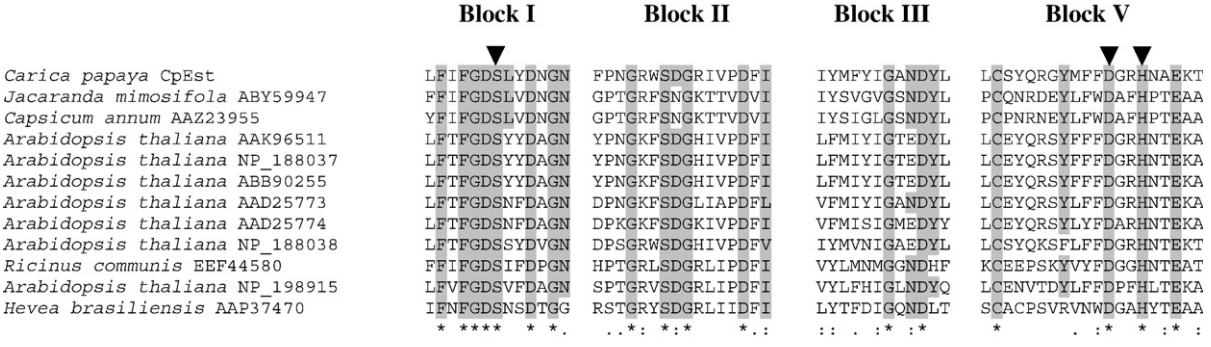

Fig. 5. Multiple amino acid sequence alignment of four peptide stretches from GDSL *Carica papaya* esterase with conserved regions of other homologous plant esterases from the SGNH family identified in NCBI databases (http://www.ncbi.nlm.nih.gov/). The alignment proteins are ABY59947 (*Jacaranda mimosifolia*), AAZ23955 (*Capsicum annuum*), AAK96511 (*Arabidopsis thaliana*), NP_188037 (*Arabidopsis thaliana*), ABB90255 (*Arabidopsis thaliana*), AAD25773 (*Arabidopsis thaliana*), AAD257734 (*Arabidopsis thaliana*), NP_188038 (*Arabidopsis thaliana*), EEF44580 (*Ricinus communis*), NP_198915 (*Arabidopsis thaliana*) and AAP37470 (*Hevea brasiliensis*). The amino acids (Ser^{35} in Bock I, Asp^{307} in Block V and His^{310} in Block V) of the putative catalytic triad are indicated with up-side down triangles. The conserved residues are boxed in grey.

Table 3
Substrate specificity of CpEst and washed *Carica papaya* latex.

Substrates (mM)	Specific activity (U/mg)[a]	
	CpEst	*Carica papaya* latex
Vinyl esters[b]		
Vinyl acetate (108)	218 ± 13	nd
Vinyl propionate (86.6)	254 ± 28	3.55 ± 0.27
Vinyl butyrate (73.6)	272 ± 15	8.09 ± 1
Vinyl laurate (35.9)	32 ± 9	11.78 ± 1.68
Triacylglycerols[c]		
Tributyrin (TC4)	168 ± 9.8	22.37 ± 1.04
Trioctanoin (TC8)	14 ± 3	16.38 ± 3.92
Olive oil (OO)	8 ± 1.3	4.26 ± 1.2
OO/TC4 ratio	0.047	0.19
TC8/TC4 ratio	0.083	0.73
Phospholipids[d]		
Egg phosphatidyl choline	4 ± 0.9	1.1 ± 0.4

[a] Values are means ± SD ($n = 3$). nd, not determined.
[b] Assays with vinyl esters were performed at 37 °C in 2.5 mM Tris–HCl buffer, 150 mM NaCl and at pH 7 to avoid chemical hydrolysis. The number between parentheses after the substrate name corresponds to the concentration used in the assay (mM).
[c] Assays with triacylglycerols were performed at 37 °C in 2.5 mM Tris–HCl buffer, 150 mM NaCl and at pH 9.
[d] Assays with phospholipids were performed at 37 °C in 7.5 mM $CaCl_2$, 13.3 mM NaTDC and at pH 8.

papaya latex (Table 2), some of these bands corresponding to several proteins. Eight *de novo* sequence peptides were identified for band 1 corresponding to a protein of ≃41 kDa (sequences underlined in Fig. 3, 42% coverage). One example is given in Fig. 2, which shows the fragment ion spectrum of the double charged precursor ion $(M + 2H)^{2+}$ at m/z 758.9 corresponding to LKDQLLAIYGLGGR peptide.

The peptide sequences obtained were then used to screen a *C. papaya* EST/genomic sequence data set (EX227656-EX303501; ABIM01000000). First, we identified a genomic scaffold (contig_7319, ABIM01007310, 18903 bp) as well as an EST (EX228849) corresponding to the peptide sequences using TBLASTN (http://blast.ncbi.nlm.nih.gov [27]). The scaffold corresponds to the second chromosome [linkage group 2 (LG2)] of *C. papaya*. Next, we predicted the structure of the identified gene by comparing the *C. papaya* genomic scaffold with the *Arabidopsis thaliana* GDSL-motif lipase protein sequence (NP_175805; 54% sequence identity with EX228849 at the amino acid sequence level) using the Wise2 server (http://www.ebi.ac.uk/Tools/Wise2/ [28]). After manual modification of the most 5′ exon (based on the N-terminal peptide sequence data), we determined a final gene model composed of four introns and five exons (with an appropriate stop codon). Based on these analyses, the protein isolated from *C. papaya* latex (corresponding to band 1) was identified as a GDSL-motif protein and was named CpEst, which stands for *C. papaya* esterase (see the following results on the kinetic properties of CpEst). The predicted cDNA of CpEst and protein sequence (343 amino acids) are presented in Fig. 3. The sequence of EST EX228849 allowed to deduce 217 amino acids out of the 343 residues of CpEst.

3.4. Sequence analysis of CpEst

The predicted CpEst cDNA (1029 bp) corresponds to a 343 amino acid residue protein. The predicted molecular mass of the protein is 38.09 kDa and the theorical isoelectric point (p*I*) is 6.06. CpEst shows a high degree of homology at the protein sequence level with proteins from the GDSL family, particularly those found in a variety of plants (Fig. 4). The deduced amino acid sequence (accession number: P86276) shares 46 to 54% sequence identities with several hydrolases and a putative GDSL-motif hydrolase from *A. thaliana* (accession numbers: AAK96511, 54%; NP_188037, 52%; ABB90255, 54%; AAD25773, 52%; AAD25774, 49%; NP_188038, 47% and NP_198915, 46%), and shares a lower homology with other GDSL-motif proteins from *Jacaranda mimosifolia*, *Capsicum annuum*, *Ricinus communis* and *Hevea brasiliensis*. A search against the Conserved Domain Database [29] (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), revealed that the CpEst amino acid sequence possesses domains conserved in SGNH hydrolases (cd00229) and specifically into SGNH plant lipase-like proteins (cd01837), all of which contain a conserved GDSL domain (pfam00657). Moreover, the CpEst sequence possessed domains conserved in fatty acyltransferase-like (cd01846), and phospholipase/lecitinase/haemolysin protein (COG3240). The critical residues for enzyme activity, assigned by comparison with other SGNH proteins, include the catalytic triad (Ser^{35}, Asp^{307} and His^{310}), which appeared in similar positions to those in other carboxylester hydrolase of this family; the oxyanion hole residues (Ser^{35}, Gly^{65} or Gly^{70}, Asn^{150}). The analysis of CpEst sequence also revealed the presence of five highly conserved peptides stretches/blocks shared with SGNH proteins, previously reported by Upton and Buckley [30] in GDSL-motif lipases (Fig. 5). A 26-residue signal peptide was predicted using the Expasy SignalP V3.0 program and the N-terminal sequence of the mature CpEst is expected to start at residue Q27 (Fig. 3). Sequence alignments of CpEst with homologous GDSL-motif hydrolases of known 3D structures support this hypothesis [31]. The NetNGlyc 1.0 Server predicted four possible N-glycosylation sites at residues 154–156 (Asn-Tyr-Thr), 161–163 (Asn-Ala-Thr), 238–240 (Asn-Leu-Ser) and 255–257 (Asn-Ser-Ser).

3.5. Substrate specificity of CpEst

The lipolytic activity of CpEst was tested towards various triacylglycerols, vinyl esters and phospholipid (Table 3). The highest specific activity of CpEst on triglycerides was measured with tributyrin (168 ± 9.8 U/mg at pH 8) and its specific activities on both trioctanoin and olive oil were much lower (14 ± 3 U/mg and 8 ± 1.3 U/mg, respectively at pH 9), suggesting that CpEst acts rather as an esterase than as a true lipase. Tributyrin has a low solubility in water and CpEst might preferentially act on the soluble fraction of TC4. Confirming this hypothesis, the highest specific activities of CpEst on vinyl esters were measured with the highly soluble short chain esters

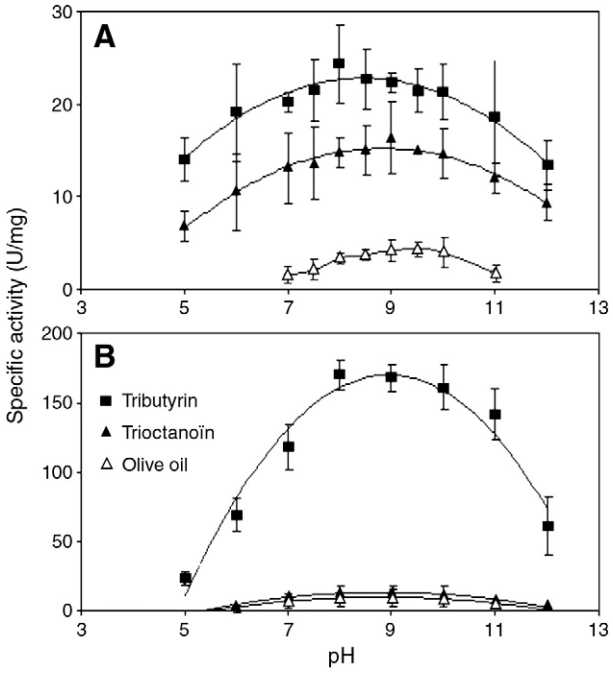

Fig. 6. pH-dependent specific activities of washed *Carica papaya* latex (A) and CpEst (B) on various triglycerides. Activities were measured at 37 °C using olive oil, TC8 and TC4 as substrate. Data are means ± SD of three independent experiments.

Table 4
Inhibition by tetrahydrolipstatin (THL) of washed *Carica papaya* latex, CpEst, *Thermomyces lanuginosus* lipase (TLL) and human pancreatic lipase (HPL).

Enzyme	Residual activity[a] (%)	
	Without NaTDC	With NaTDC
Washed *Carica papaya* latex	46 ± 12	9 ± 1.3
CpEst	10 ± 3	10 ± 2.7
TLL	55[b]	7[b]
HPL	66[b]	8[b]

Residual activities were measured on tributyrin using the pH-stat Technique.
Each enzyme was pre-incubated with THL for 30 min at an enzyme:inhibitor molar ratio of 1:20. The experiments were carried out in the absence or presence of 4 mM NaTDC in the incubation medium. The residual activities of each enzyme were measured as described in Experimental procedures.
[a] From Ben Ali et al. [16].
[b] Values are means ± SD ($n = 3$).

(218 to 272 U/mg; Table 3) and a lower activity was measured with vinyl laurate (32 U/mg). The phospholipase activity of CpEst on egg PC was found to be very low compared to that on tributyrin and vinyl esters (Table 3).

The substrate specificity of CpEst for triglycerides was compared with that of the entire *C. papaya* latex measured after washing the latex with water (Fig. 6). Since the activity of the entire latex might result from a mixture of enzymes having different substrate specificities and pH optima, the enzyme activities were recorded as a function of pH to see if the *C. papaya* latex and CpEst presented similar or different pH-dependent profiles of activity. The first striking observation was that both the olive oil to TC4 activity ratio and the TC8 to TC4 activity ratio were much lower with CpEst than with the latex (Table 3; Fig. 6). This finding indicates that CpEst is not the main enzyme responsible for the lipase activity of *C. papaya* latex on long chain and medium chain triglycerides. Moreover changes in pH optima were observed with olive oil used as substrate: the optimum activity of the latex was observed between pH 9 and 10, whereas that of CpEst was observed between 8 and 9 (Fig. 6). The optimum activities on TC8 and TC4 were observed at pH 9 and 8, respectively, for both the entire latex and CpEst.

At pH values equal to or below 9, the kinetics of free fatty acid release were found to be linear for 5 min at least, whereas linearity was only observed for 1–2 min at pH values above 9, suggesting that *C. papaya* lipases/esterases were less stable at high pHs.

3.6. Effects of the lipase inhibitor orlistat on C. papaya latex and CpEst activities

The inhibitory effects of orlistat (tetrahydrolipstatin, THL) were tested with the washed *C. papaya* latex, CpEst and two other well-known lipases used as controls, human pancreatic lipase (HPL) and *T. lanuginosus* lipase (TLL). As shown in Table 4, the activity of CpEst was reduced by 90% after a pre-incubation of the enzyme with THL for 30 min, at an enzyme:inhibitor molar ratio of 1:20. The absence or presence of bile salts (NaTDC) in the incubation medium had no significant effect on the level of inhibition of CpEst. Similar levels of inhibition were obtained with TLL, HPL and washed *C. papaya* latex but only in the presence of NaTDC in the medium of incubation. In the absence of NaTDC, washed *C. papaya*, TLL and HPL residual activities remained rather high (46, 55 and 66% of the initial value, respectively) after 30 min of incubation.

3.7. Structural modeling

In order to obtain structural insights into CpEst, a 3D structural model was generated using the PHYRE server [32]. The 10 best structural alignments, as provided by the server, show *E*-values ranging from 4.8×10^{-4} for the best hit to $8. \times 10^{-3}$ for the most distant hit. The final model of CpEst (Fig. 7) was built using the X-ray structure of the lipase/acylhydrolase from *Enterococcus faecalis* (PDB code 1YZF; resolution 1.9 Å) that shares 12% amino acid identities with CpEst. This 3D model obtained with an estimated precision of 95% covers the residues 27 to 324. CpEst model very well superimposes onto the 3D structures of several GDSL-motif proteins (PDB codes 1Z8H, 1JRL, 1FXW, 1ESD, 2QOQ, 1ES9, 2HSJ, 3BZW, 2O14). Panel C in Fig. 7 shows the superimposition of CpEst model with the 3D structures of the lipase/acylhydrolase from *E. faecalis* (PDB code 1YZF) and the putative GDSL lipase from *Nostoc* sp. PCC 7120 (PDB code 1Z8H; 2.02 Å resolution).

The structural model of CpEst consists of six α-helices and a central β-sheet core containing four parallel β-strands. The catalytic triad is composed of Ser^{35}, Asp^{307} and His^{310}, a sequence which obeys the Nucleophile-Acid-His rule of α/β hydrolases and SGNH hydrolases. The three residues of the catalytic triad are accessible at the surface of the molecule (Fig. 7B) as observed in most GDSL-motif esterases and this is a striking difference with lipases, in which only the hydroxyl

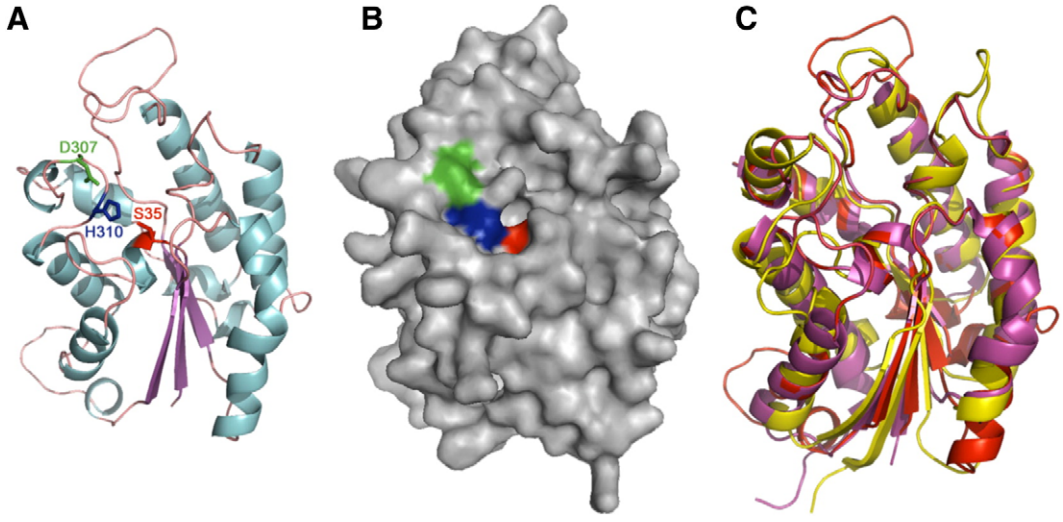

Fig. 7. Structure modeling of CpEst. The 3D model was built based on the X-ray structure of the lipase/acylhydrolase from *Enterococcus faecalis* (PDB code 1YZF). (A) Ribbon model showing the central β sheet composed of 4 parallel strands, surrounded by α-helixes. The catalytic triad (S^{35}-H^{310}-D^{307}) is shown with the side chains colored in red, blue and green, respectively. (B) Molecular surface representation of CpEst showing the accessibility of the residues of the catalytic triad at the surface of the molecule. (C) Superimposition of CpEst (red) model with the 3D structures of the lipase/acylhydrolase from *Enterococcus faecalis* (purple; PDB code 1YZF) and the putative GDSL lipase from *Nostoc* sp. PCC 7120 (yellow; PDB code 1Z8H).

group of the active site serine is exposed to solvent and present at the bottom of a catalytic groove surrounded by hydrophobic amino acid residues forming acyl chain binding sites [33–37]. No site for long chain fatty acid binding could be identified in CpEst 3D model.

4. Discussion

The lipolytic activity of fresh latex of *C. papaya* on the short chain triglyceride tributyrin has been known for several years [3]. Tributyrin is however partly soluble in water and cannot be considered as a true lipase substrate. *C. papaya* latex can however hydrolyse long chain triglycerides and can therefore be considered as a source of lipase activity [7]. This activity is however strictly associated to sedimentable particles of latex and conventional techniques for protein extraction were unsuccessful so far for isolating and identifying lipolytic enzymes from the latex of *C. papaya* [38]. One possible explanation for these findings is that lipolytic enzymes are packed in rubber particles [39].

Using an extraction step with the zwitterionic detergent CHAPS and sonication, it was possible to solubilise some lipolytic activity from *C. papaya* latex for the first time. The enzyme activity remained however associated with high molecular mass particles and was eluted with the dead volume using gel filtration chromatography. SDS-PAGE analysis of the corresponding fractions allowed the identification of five proteins (Fig. 1), four of them corresponding to known proteins from *C. papaya* latex (Table 2). Band 1 (41 kDa) was identified as a GDSL-motif carboxyl esterase after eight *de novo* peptide sequences were generated from tryptic digestion and used to screen *C. papaya* ESTs. Based upon EST sequences and sequence homologies with *A. thaliana* GDSL-motif esterase, a full-length gene with a 1029 bp open reading frame (ORF) was identified in the draft genome of *C. papaya* [40]. The ORF encodes a 343 amino acid residue protein (CpEst) with a theoretical molecular mass of 38 kDa. Since the apparent molecular mass estimated from SDS-PAGE was 41 kDa, CpEst might be glycosylated (four N-glycosylation sites are predicted from sequence analysis), but other post-translational modifications might also be involved and related to CpEst interaction with latex particles. Interestingly Arif et al. [41] reported that a similar GDSL-motif esterase from *H. brasiliensis* latex had an almost identical molecular mass (i.e., 42 kDa) estimated from SDS-PAGE and this mass was nearly 2.5 kDa higher than the mass predicted from the polypeptide sequence [41].

Although CpEst was not completely purified, the protein preparation displayed a high esterase activity on esters containing short chain fatty acids (Table 3) and these biochemical properties supported the identification of CpEst as a carboxylester hydrolase. The activity of CpEst on tributyrin only represented 7% of the dried latex activity on tributyrin (Table 1). The olive oil to tributyrin activity ratio was 0.047 with CpEst versus 0.19 with the crude dried latex (Table 3). Lipase activity on long chain triglycerides was therefore depleted upon purification and CpEst cannot be considered as a true lipase as defined by Verger et al. [42]. These findings are consistent with the fact that most GDSL-motif proteins are esterases and not lipases. Like other GDSL-motif esterases, CpEst contains a catalytic triad (Ser^{35}, Asp^{307} and His^{310}), with the active site serine located near the N-terminus [30]. Recently, a subgroup of this GDSL family, the SGNH hydrolases, was defined based on the presence of four residues (Ser, Gly, Asn and His) strictly conserved in four conserved peptide stretches (Blocks I, II, III, and V) respectively [43]. Each of the four residues plays a key role in the catalytic function of the enzyme. The Ser residue in block I serves as the catalytic nucleophile and proton donor to the oxyanion hole. The Gly residue in block II and the Asn in block III serve as two other proton donors to the oxyanion hole. The His residue in block V acts as a base to make the active site Ser more nucleophilic by deprotonating the hydroxyl group [44,45]. These four peptides stretches/blocks are conserved in CpEst (Fig. 5).

Several 3D structures of enzymes from the GDSL family were obtained and they show several β-strands and α-helices arranged in alternating order and the substrate-binding pocket between the central β-strand and long α-helix appears to be highly flexible [44]. The structural modeling of CpEst shows that the catalytic triad is highly exposed at the surface of the molecule. Experiments with the lipase inhibitor orlistat confirmed that CpEst is a serine hydrolase and that its active site is fully accessible to solvent and inhibitors. Inhibition of CpEst did not require detergents like bile salts to be inhibited. With lipases like TLL and HPL having a lid controlling the access to the active site, detergents are required to promote the opening of the lid and then inhibition [46]. Conversely, lipases with no lid, such as guinea pig pancreatic lipase-related protein 2, do not require bile salts to be inhibited [34]. The inhibitory effect exerted by orlistat in the absence of detergent was previously used as an experimental criterion for predicting the absence of a lid domain in carboxylester hydrolases [16]. The results obtained here with CpEst clearly indicate that there is no lid in CpEst. Inhibition experiments performed with washed *C. papaya* latex suggested however the presence of true lipases with lid in the latex since high inhibition rates were only achieved in the presence of bile salts, as observed with TLL and HPL (Table 4).

Although several GDSL-motif enzymes from *A. thaliana*, *Rauvolfia serpentina*, *Madicagi*, *H. brasiliensis*, *Alopecurus myosuroides*, *J. mimosifolia* have been isolated, cloned and characterized [41,47–52], the knowledge on the GDSL family is still limited. In plants, GDSL hydrolases are generally considered to be mainly involved in the regulation of plant growth and development. Recently research in this field has become more attractive. Possessing multiple functions, GDSL hydrolases generally find applications in food, flavour, fragrance, cosmetic, textile, pharmaceutical and detergent industry [45,53]. The results obtained here on the identification and biochemical properties of a GDSL-motif carboxyl ester hydrolase from *C. papaya* latex are a further step in the characterization of these enzymes in plants. The physiological role of CpEst and its interaction with latex remain however to be elucidated. The complete sequencing of the *C. papaya* genome [40] will certainly accelerate the identification of other esterases and lipases from *C. papaya*.

Acknowledgements

The authors thank Jean-Marie Guigonis and Michel Samson from the Faculty of Medicine (Nice, France) for mass spectrometry analysis. The authors are grateful to Jean-Pierre Andrieu from IBS (Grenoble, France) for the Amino acid composition analysis and Sabrina Lignon (IMM, Marseille, France) for her help on MALDI-TOF analysis. We also thank Peter Van Cauwenberghe, the Managing Director of Biohainaut for his continuous support, and Christophe Collet (CIRAD) and Pierre Michaud (OSEO ANVAR, France) for their help in setting up this project. This study was performed in the framework of the European EUREKA LIPLANT E! 3818 Project (2007–2009) with the financial support of Agence Nationale de Recherche (ANR) in France and the "Direction Générale Opérationnelle — Economie, Emploi et Recherche (DGO6)" in Belgium. We thank Jean-Michel Claverie (the Director of Information Génomique et Structurale and the Director of Institut de Microbiologie de la Méditerranée) for the laboratory space and support provided to H.O. This study was in part supported by the PACA-Bioinfo Bioinformatics Platform, recurrently funded by the CNRS and grants (to J. -M. C.) from the National Genopole Network, the PACA Region and IBiSA.

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