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Study of a sterol esterase secreted by *Ophiostoma piceae*: Sequence, model and biochemical properties

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ABSTRACT

An extracellular sterol esterase from *Ophiostoma piceae* efficiently hydrolyzes sterol esters, triglycerides and *p*-nitrophenol esters. cDNA was screened with a probe obtained by PCR using as primers oligonucleotides corresponding to the N-terminal and internal mature enzyme sequences and complete sequence was obtained by 3′ rapid amplification of cDNA end (RACE) and inverse PCR. The *O. piceae* esterase gene had a length of 1.8 kbp and lacked introns. A search for proteins with related amino acid sequences revealed around 40% identity with lipases from *Candida rugosa* and *Geotrichum candidum*. Modelling the *O. piceae* enzyme, using the crystal structures of Lip1 and Lip3 from *C. rugosa* as templates, revealed a similar substrate-binding site, but some changes affecting the flap zone and the aromatic region of the tunnel may be responsible for the wide substrate specificity of this interesting sterol esterase. The ability of the new fungal esterase to hydrolyze triglycerides and esters of *p*-nitrophenol and cholesterol was compared with those of commercial lipases and cholesterol esterases showing the new enzyme the highest efficiency hydrolyzing triglycerides and sterol esters in the conditions assayed (in presence of Genapol X-100). Finally, the *O. piceae* gene was successfully expressed in *Pichia pastoris*, as a model organism to express fungal enzymes, resulting in higher levels of esterase activity than those obtained in the *O. piceae* cultures. In spite of its higher glycosylation degree, the recombinant enzyme was able to hydrolyze more efficiently than native enzyme the assayed substrates.

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1. Introduction

Carboxylic ester hydrolases (EC 3.1.1) are a heterogeneous group of enzymes catalyzing the cleavage of ester bonds, including carboxylesterases (EC 3.1.1.1), triacylglycerol lipases (EC 3.1.1.3) and sterol esterases (EC 3.1.1.13). These enzymes preferentially hydrolyze carboxylic esters, triacylglycerol esters and sterol esters, respectively. It is clear that carboxylesterases act on soluble esters of short-chain fatty acids but the triacylglycerol lipases and sterol esterases hydrolyze a much broader substrate range, including water insoluble esters [1], and frequently it is not easy to distinguish them. Lipases are generally strongly activated by water–lipid interfaces, a phenomenon known as "interfacial activation" [2] and the active site of these enzymes usually presents a hydrophobic cavity covered by an amphipathic loop named "flap". Interfacial activation, reported long time ago [3,4], seems to be due to a conformational change on this helical element, making the active site accessible to the substrate. This property is a distinguishing feature of these enzymes, although some lipases have been identified where the flap is absent [5–7]. On the other hand, although some sterol esterases specifically hydrolyze sterol esters [8,9], some of them are also able to efficiently hydrolyze triglycerides. This is true in case of the cholesterol esterase from *Candida cylindraceae* (synonym *Candida rugosa*) [10] known as lipase 3 (Lip3) [11]. This yeast produces several closely extracellular lipases, with a high level of sequence identity (77–88%) that differ in substrate specificity [12].

Lipases are enzymes of biotechnological interest in paper pulp manufacturing and other industrial applications [13,14]. Resinase A® from Novozymes and a lipase from *C. cylindraceae* have been used to decrease pitch problems in mechanical pulping of pine wood [15,16], where triglycerides are the main problematic pitch compounds. However, these preparations are not effective during chemical pulping and total chlorine free bleaching (TCF) of other softwoods or hardwoods (such as *Picea abies* or *Eucalyptus globulus*, respectively) with high levels of sterol esters [17].

In a previous work, we isolated and characterized an esterase from the ascomycete *Ophiostoma piceae* able to hydrolyze *p*-nitrophenol esters, triglycerides and different cholesterol esters [18]. This enzyme is also able to hydrolyze natural mixtures of triglycerides and sterol

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esters present in hardwood and softwood pulp and extractives, its use for pitch biocontrol is included in a patent [19] and it has been successfully immobilized on Dilbeads™ [20]. In this paper, we report: i) the sequencing and molecular characterization of the new fungal esterase, ii) a model for the three-dimensional structure of this enzyme based on its homology with previously crystallized lipases, iii) a comparison of the native enzyme with commercial lipases and cholesterol esterases and iv) the successful expression of the *O. piceae* esterase gene in *Pichia pastoris* and the preliminary characterization of the recombinant enzyme.

2. Materials and methods

2.1. Fungal strains and plasmid

Ophiostoma piceae (CECT 20416) was grown in glucose–peptone medium [18] at 28 °C and 150 rpm. *Escherichia coli* DH5α (Stratagene®), used for cloning and plasmid propagation, was grown in Luria–Bertani medium [21] at 37 °C and 150 rpm. *P. pastoris* GS115 (Invitrogen™) was used as host strain for expressing the *O. piceae* mature esterase sequence under the transcriptional control of the AOX1 promoter. The vectors pGEM-T Easy (Promega) and pPIC9 (Invitrogen™) were used for cloning and expressing the esterase gene respectively.

2.2. DNA and mRNA extraction

O. piceae mycelium was harvested after 15 day-old cultures under the conditions described above, frozen in liquid nitrogen, and the genomic DNA was isolated by phenol:chloroform:isoamylic alcohol (25:24:1) extraction and isopropanol precipitation [22]. The RNA was obtained from 7-day-old cultures using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc.). Polyadenylated RNA was purified using an mRNA purification kit (Pharmacia).

2.3. Primer design and preparation of DNA probe

A specific DNA probe was prepared by Polymerase Chain Reaction (PCR) using the degenerate primer pair corresponding to N-terminal sequences of the mature protein and the internal peptide P1 (obtained after protein hydrolysis with trypsin). The reaction was carried out using 0.1 μg of DNA, 400 pmol of each primer and 2.5 U of Amplitaq DNA polymerase (Applied Biosystems). The PCR products were separated on 0.8% agarose gels in TAE buffer and purified (QIAquick PCR purification kit, Qiagen) before cloning into pGEM-T Easy.

2.4. Cloning of 3′ and 5′ ends of the esterase gene

Rapid amplification of cDNA ends (RACE) was used to clone the 3′ end of the esterase gene (5′/3′ RACE, 2nd generation kit, Roche). The first-strand cDNA was synthesized using polyadenylated RNA (2 μg) as a template and a poly dT oligonucleotide as primer. First-round PCR was performed with esterase specific primer P1d and PCR anchor primer at 55 °C (5×) and 60 °C (30×) as annealing temperatures. Second-round PCR was carried out with esterase specific primer P1c and PCR anchor primer, using diluted and purified first-round PCR products (MicroSpin™ S-400 HR, Amersham Biosciences) as templates, at 70 °C (30×) for annealing, decreasing each successive cycle by 0.6 °C. The 5′ end was cloned by inverse PCR [21] using PvuI restriction enzyme and the esterase specific primers Ntd and P1c at 60 °C (30×) as the annealing temperature. Another PCR reaction was performed using diluted and purified PCR products from the latter reaction as templates, and the oligonucleotides Ntc and P1c as primers. The reaction was carried at 67 °C (35×) for annealing, decreasing each successive cycle by 0.3 °C. The esterase specific primers were designed from the nucleotide sequence of the previously cloned genomic DNA probe. The PCR products were separated and purified as described above.

2.5. DNA sequencing and sequence analysis

DNA sequencing of the fragments was carried out in an ABI PRISM 377 automatic sequencer (Perkin Elmer). The GCG package, BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and EXPASY programmes (http://www.expasy.org) were used for analysis, alignment and comparison of sequences.

2.6. Molecular modelling of esterase

The theoretical molecular structure of *O. piceae* esterase was generated by homology modelling using the atomic coordinates of *C. rugosa* Lip1 (PDB entries 1TRH, 1LPM, 1LPS, 1LPN, 1LPO and 1LPP) and *C. rugosa* Lip3 (PDB entries 1CLE and 1LLF) as templates. Comparative modelling and energy minimization were carried out with ProModII and Gromos96 programmes at the Swiss-model server [23]. The DeepView/Swiss-PdbViewer programme was used for visualization and analysis of pdb files [24]. The secondary structure elements were calculated using the PALSSE programme [25].

2.7. Expression of O. piceae esterase in P. pastoris

The genomic DNA from *O. piceae* was isolated [22] and used as template to amplify by PCR the esterase mature sequence with primers which incorporated an EcoRI site in 5′ and a NotI site in 3′. The purified amplicon was cloned in pGEM-T Easy vector and subcloned in pPIC9 expression vector downstream of the AOX1 promoter and in frame with the α-Mating Factor secretion signal peptide from *Saccharomyces cerevisiae*. *P. pastoris* spheroplasts were produced and transformed with the linearized recombinant DNA following Invitrogen's instructions.

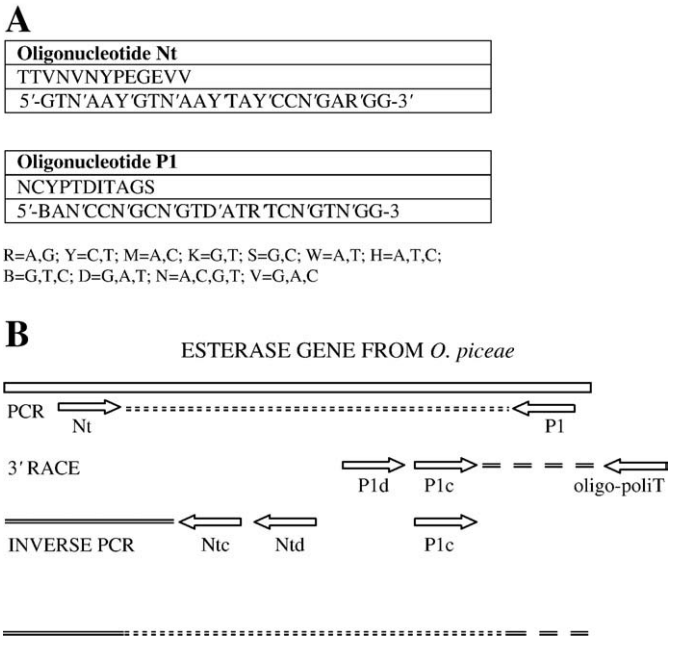

Fig. 1. *O. piceae* esterase cloning strategy. (A) N-terminal sequences of protein and tryptic peptide number 1 (obtained after hydrolyzing protein with trypsin) and degenerated oligonucleotides synthesized for partial sequences. (B) 1000 bp fragment amplified by PCR using these oligonucleotides and genomic DNA, and nucleotide sequences of 3′-end and 5′-end of the esterase DNA cloned by RACE and Inverse PCR methods, respectively.

Mut$^+$ phenotype in transformed colonies was confirmed by detecting normal growth of transformants both on MM (minimal methanol) and MD (minimal dextrose) plates whose formulation was 1.5% agar, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol for MM and 2% glucose for MD. Five positive transformants were cultivated in shake flasks on BMMY media at 28 °C and 250 rpm for 8 days. The medium composition was 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB without amino acids

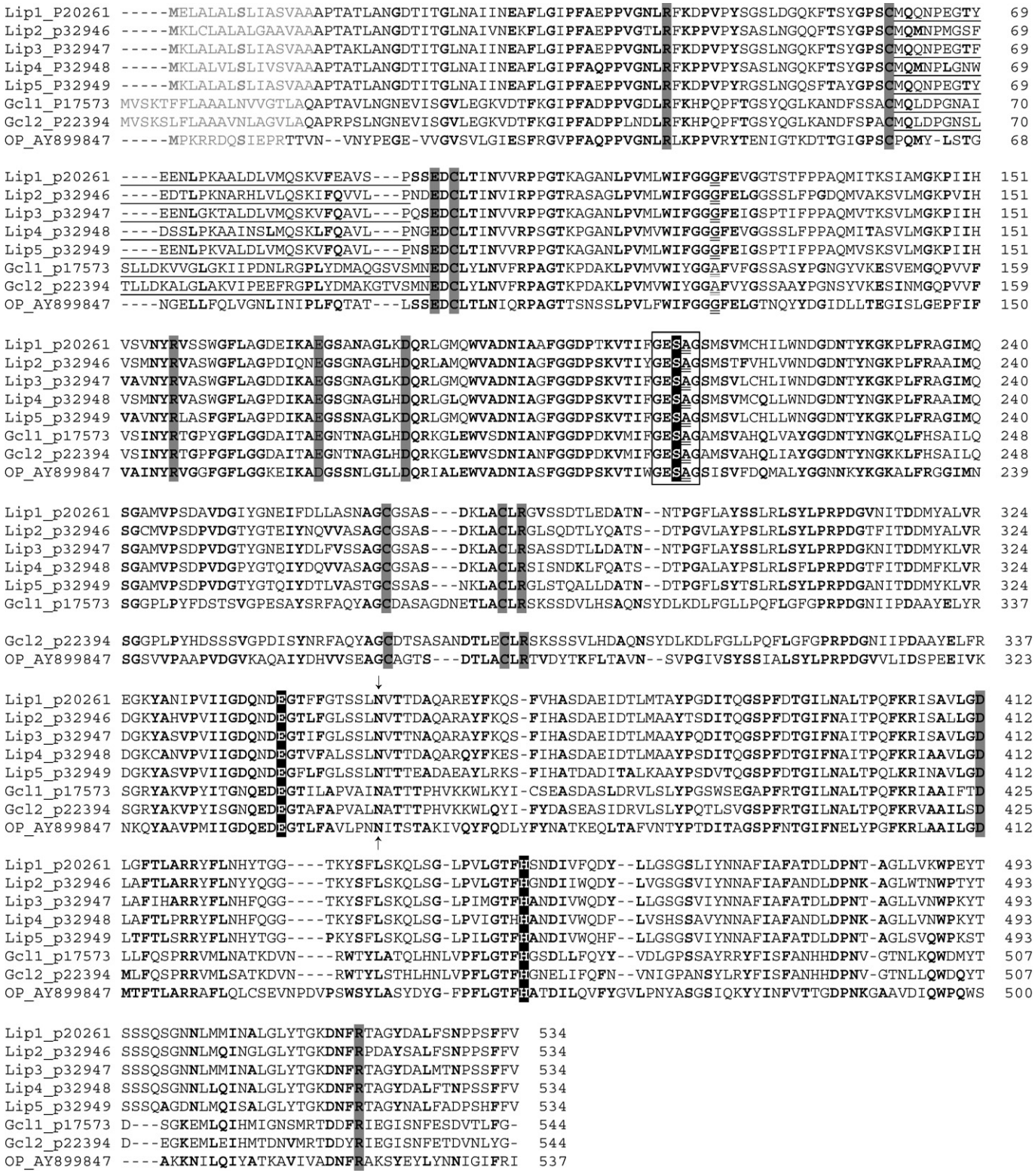

Fig. 2. Multiple alignment of lipase amino acid sequences. *C. rugosa* (Lip1–Lip5), *G. candidum* (Gcl 1 and Gcl 2) and esterase from *O. piceae* (Op). Residues conserved in the sequences are in bold characters. The signal peptide is shown in grey and the flap region is underlined once. The catalytic amino acids are shown on a black background and the residues implicated in the formation of salt and disulfide bridges are shown on a grey background. The amino acids that formed the oxyanion hole are underlined twice. The arrows mark a potential N-glycosylation site. The conserved sequence which included catalytic serine is boxed. The GenBank entries are shown.

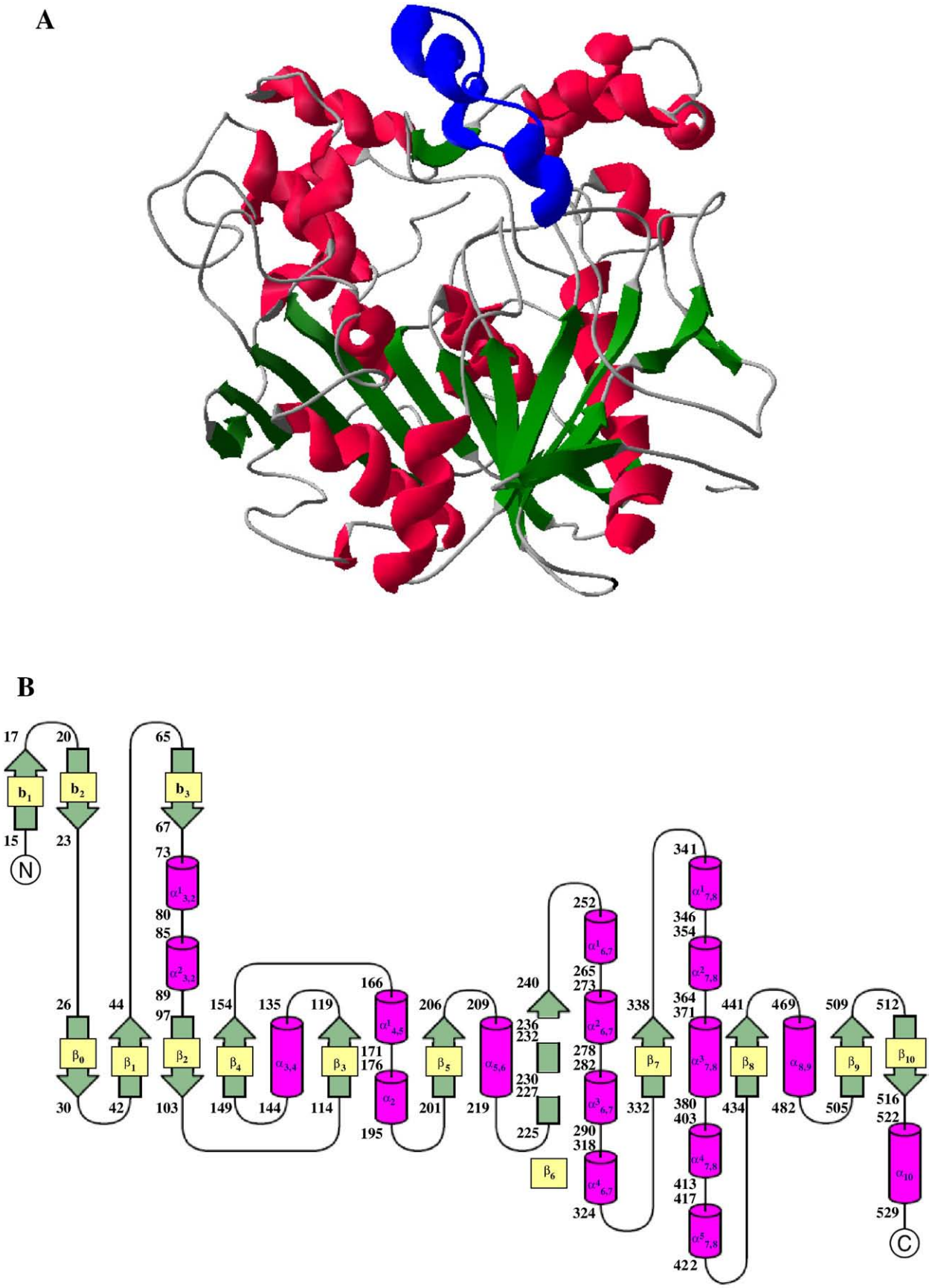

Fig. 3. Overall structure of *O. piceae* esterase. (A) Model of *O. piceae* esterase: The α-helices and β-strands are represented by red helical ribbons and green arrows, respectively. Residues corresponding to the flap region in *C. rugosa* lipases are marked in blue. (B) Representation of esterase topology with the secondary structure elements identified: The α-helices and β-strands are represented by fuchsia cylinders and green arrows, respectively. The nomenclature follows that described by Cygler (1993).

and with ammonium sulphate, 4×10^{-5}% biotin and 0.5% methanol (the methanol was added every 24 h to maintain the induction), as is indicated in Invitrogen´s manual (2002). The same medium with glycerol (1%), but without methanol, was used to obtain the preinocula.

2.8. Enzyme assays

The hydrolysis of *p*-nitrophenyl butyrate (*p*NPB) and *p*-nitrophenyl palmitate (*p*NPP) was assayed spectrophotometrically by *p*-nitrophenol ($\varepsilon_{410}=15200\ M^{-1}\ cm^{-1}$), release at pH 7.0 [18]. One unit of activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per minute under the above conditions.

The hydrolysis of tributyrin, triolein and cholesterol esters was assayed titrimetrically at pH 7.0 and 25 °C in a pH-stat, in the presence of 0.15 M NaCl and 5% (v/v) polyoxyethelene 10-tridecyl ether (Genapol X-100) [18]. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of free fatty acid per min.

Comparative studies of the hydrolysis of triglycerides and esters of *p*-nitrophenol and cholesterol were carried out under the above conditions but 1% (v/v) Genapol X-100 was added for hydrolysis of *p*-nitrophenol esters. The following commercial enzymes, lipases from *Aspergillus oryzae* (Fluka), *Candida antarctica* (Novozymes), *C. rugosa* (Novozymes), *Humicola lanuginosa* (Novozymes) and Resinase A® (Novozymes) and *Rhizomucor miehei* (Fluka), and cholesterol esterases from *C. rugosa* (Roche), *Pseudomonas fluorescens* (Roche) and *Pseudomonas* sp. (Sigma), were compared in these studies.

2.9. Enzyme purification and hydrolysis

The *O. piceae* esterase was purified from 15-day-old cultures on glucose–peptone–yeast extract medium supplemented with 0.5% olive oil [18]. Recombinant protein was purified following the same procedure from 7-day-old cultures growing on BMMY medium. The molecular mass of the denatured enzymes was determined by SDS/PAGE using 7.5% polyacrylamide gels, and the content of N-linked carbohydrates was estimated by the molecular mass of both native and recombinant esterases before and after deglycosylation with Endo-H from Roche.

Samples of native deglycosylated protein (200 μg) were hydrolyzed with trypsin (10 μg) in 0.4 M NH_4HCO_3 (37 °C for 24 h). The tryptic peptides were separated in a C_{18} column (Spherisorb s5ods2, 25×4.6 mm, 250 Å, hichrom), using 0–70% (v/v) acetonitrile gradient in 0.1% (w/v) trifluoroacetic acid at 1 ml min^{-1} flow rate, and detected at 214 nm.

3. Results

3.1. Amino acid sequence analysis

An extracellular sterol esterase from the ascomycete *O. piceae*, able to hydrolyze triglycerides and esters of *p*-nitrophenol and cholesterol, was previously purified to homogeneity in a single chromatographic step on a HiTrap Octyl Sepharose FF cartridge [18].

The strategy used to obtain the probe for the gene of this enzyme, as well as the processes for obtaining the complete genomic sequence of the gene are described in Fig. 1. Degenerated oligonucleotides from N-terminal sequences of the mature protein and a tryptic peptide (P1) were used as PCR primers using genomic DNA as template. A fragment of around 1 kb was amplified by PCR with a significant identity sequence with fungal lipases (BLAST analysis ~30–40%). The 3′ and 5′-end sequences were obtained using the RACE and Inverse PCR methods, respectively. The complete cDNA sequence was obtained using cDNA, obtained after RT-PCR reactions from mRNA when maximal expression of the gene was detected and using the primers Nt and E3 (the last designed from 3′ sequence). The same primers were used to amplify genomic DNA and the alignment of both DNA and cDNA sequences showed that this gene lacked introns. The DNA nucleotide and deduced amino acid sequence of the esterase gene, were submitted to the GenBank/EBI data bank with the accession number AY899847.

This esterase gene has an open reading frame of 1647 bp that encodes for a protein of 549 amino acids. The first 12 amino acids correspond to a predicted signal sequence, and the N-terminal sequence deduced from the amino acid sequence is in agreement with that previously obtained from the purified protein [18]. The molecular mass deduced from the amino acid sequence (58.2 kDa) was similar to the value estimated by MALDI-TOF and SDS-PAGE of the deglycosylated enzyme [18].

The analysis of the *O. piceae* amino acid sequence showed maximal identity with lipases from *C. rugosa* (44%) [26,27] followed by *G. candidum* (37%) [28,29] and cholesterol esterase from *C. rugosa* (43.4%) [30]. The homology with other lipases from filamentous fungi, such as *A. oryzae* [31] and *Humicola lanuginosa* [32], was very low (4 and 10%, respectively).

Multiple amino acid sequence alignments of lipases from *C. rugosa* and *G. candidum* and esterase from *O. piceae* are shown in Fig. 2. The analysis of these sequences reveals conserved residues important for their structure, as well as the essential catalytic residues although significant differences exist in their substrate specificity. Most of the lipase/esterase enzymes contain the Ser-Asp-His triad in the catalytic site [33]. However, in *O. piceae* the catalytic triad was identified as Ser208, Glu340, and His453. The acid residue is also substituted by a Glu in the lipases from *C. rugosa* [12] and *G. candidum* [34]. The oxyanion hole, also connected to the catalytic activity, shows the GGGX sequence type [35] (Gly121–123, Phe 124). With respect to the glycosylation sites, only one possible site has been identified in the *O. piceae* esterase sequence, in the residue Asn350, contained in a consensus sequence Asn-Xaa-Ser/Thr [36].

3.2. Molecular model

The molecular model of *O. piceae* esterase was obtained using the crystal structures of Lip1 and Lip3 from *C. rugosa* as templates. This model (Fig. 3A) shows an α/β hydrolase fold and consists of a major 11-stranded mixed β-sheet, a small and nearly perpendicular N-terminal three-stranded β-sheet and 11 α-helices. The major β-sheet, which forms the protein core, exhibits a pronounced twist, the first and last strands being almost perpendicular to one another. The catalytic triad is located in highly conserved positions [37]: Ser208 after strand β_5, Glu340 after strand β_7 and His453 after

Table 1
Specific activities (U/mg protein) of *O. piceae* esterase and commercial enzymes on *p*-nitrophenol esters, triglycerides and cholesterol esters.

		*p*NPB	*p*NPP	TB	TO	CB	CO	CE
A. oryzae	Lip	0.6	1.3	74.9	18.3	0.1	0.0	0.1
C. antarctica	Lip	2.2	0.0	22.3	0.2	0.2	0.2	0.2
C. rugosa	Lip	0.0	0.0	0.0	0.0	0.4	0.4	0.5
H. lanuginosa	Lip	0.5	1.4	75.1	16.2	0.0	0.0	0.0
Resinase A®	Lip	0.4	1.3	73.9	13.3	0.0	0.0	0.0
R. miehei	Lip	0.5	3.3	54.7	1.4	0.3	0.2	0.2
O. piceae		23.0	29.0	133.0	78.3	17.0	53.4	15.3
C. rugosa	Che	0.0	0.0	126.0	40.4	0.4	10.1	0.4
P. fluorescens	Che	0.0	0.0	132.0	31.0	8.1	7.2	9.2
Pseudomonas sp.	Che	72.1	7.6	116.2	74.4	1.5	26.1	3.2

Reactions were carried out using 1 mM substrate in presence of Genapol X-100 (1% for *p*-nitrophenol esters and 5% for the rest of substrates).
The abbreviations Lip and Che correspond to enzymes commercialized as lipases and cholesterol esterases, respectively; *p*-nitrophenyl butyrate = *p*NPB; *p*-nitrophenyl palmitate = *p*NPP; Tributyrin = TB; Triolein = TO; Cholesteryl butirate = CB; Cholesteryl oleate = CO; Cholesteryl estearate = CE.

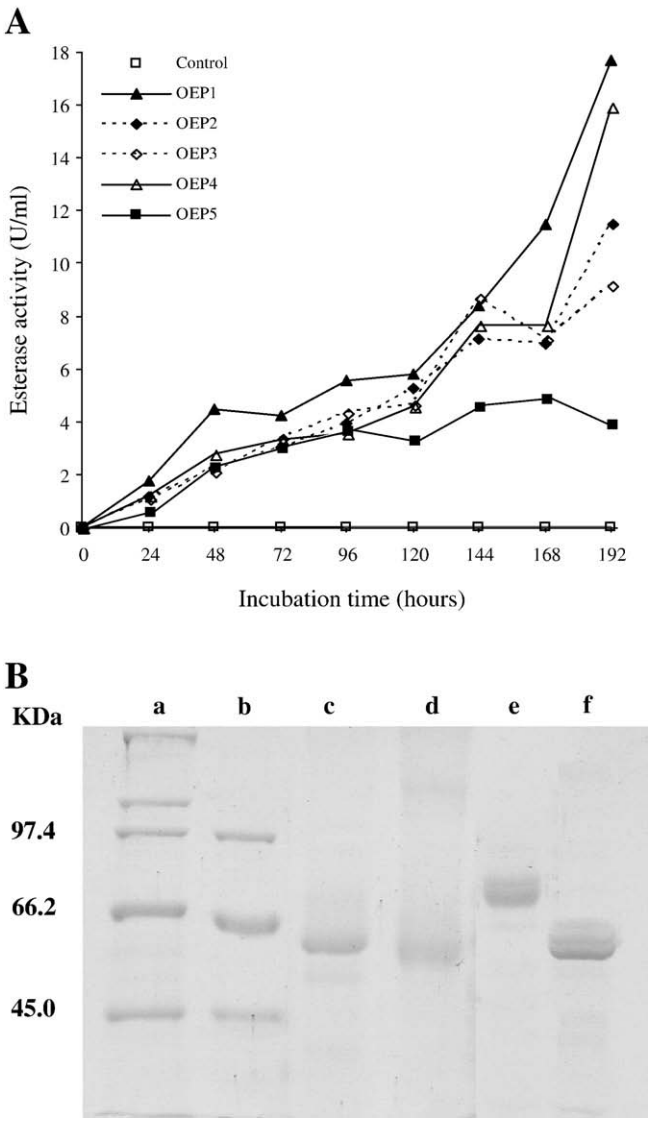

Fig. 4. Heterologous expression of *O. piceae* esterase in *P. pastoris*. (A) Analysis of different *P. pastoris* transformant colonies: OEP1 (▲), OEP2 (♦), OEP3 (◇), OEP4 (Δ), OEP5 (■) and the GS115 strain transformed with the parental pPIC9 vector as control (□) (mean values from triplicate determinations; all standard deviations were below 7–10% of mean values). (B) SDS-PAGE of purified native and recombinant esterase before and after deglycosylation. Lane a: High Molecular Weight Marker; lane b: Low Molecular Weight Marker; lane c: native enzyme; lane d: native enzyme treated with Endo-H; lane e: recombinant enzyme; lane f: recombinant enzyme treated with Endo-H.

strand β_8. The components of the oxyanion hole, Ala209 and Gly123, are located after strand β_5 and between strand β_3 and helice $\alpha_{3,4}$, respectively.

The analysis of the secondary structure elements in the *O. piceae* esterase is shown in Fig. 3B. This structure is practically identical to the Lip1 and Lip3 structures from *C. rugosa* [12], although it shows slight differences in the b_3-β_2 region. Two α-helices are suggested in the molecular model of *O. piceae* esterase ($\alpha^1_{3,2}$ and $\alpha^2_{3,2}$), whereas only one α-helix, corresponding to the flap region, has been reported in the structure of *C. rugosa* lipases ($\alpha_{3,2}$).

3.3. Comparison with commercial enzymes

The specific activity of the *O. piceae* enzyme and other commercial lipases and cholesterol esterases on different substrates is shown in Table 1. Due to the low solubility of some of the substrates assayed, such as triolein and some cholesterol esters (16 and 18 C atoms), the reactions were carried out in the presence of the non-ionic detergent Genapol X-100.

The commercial lipases were only slightly active on *p*-nitrophenol esters and triglycerides under the condition assayed, but the enzymes commercialized as cholesterol esterases were able to hydrolyze the cholesterol esters and triglycerides. Tributyrin was the substrate preferentially hydrolyzed by most of the enzymes. Under the conditions used, the highest specific activity on most of the substrates analyzed corresponded to the *O. piceae* esterase.

It is well known that Resinase A® is an effective lipase hydrolyzing triglycerides in mechanical pulp [15,16,38] although our results on triglyceride hydrolysis, in the presence of Genapol X-100, indicated a low yield. Studies on the detergent effect on Resinase A® and *O. piceae* esterase activities using tributyrin, partially soluble in aqueous solutions, showed that the highest activity of the commercial enzyme was without detergent (2200 and 140 U/mg for Resinase A® and *O. piceae* esterase, respectively). However, the presence of detergent slightly affected the activity of *O. piceae* (95% activity) but reduced drastically the activity of the Resinase A® (3% activity).

3.4. Heterologous expression of the O. piceae esterase

The recombinant plasmid pPIC9OPE was linearized and used to transform the *P. pastoris* GS115 strain as a model organism for heterologous expression. The integration of the vector at the AOX1 locus in the yeast guarantees the genetic stability of the transformants. The correct phenotype (Mut^+) in transformed colonies was shown by being able to use efficiently methanol as a sole carbon source. Five different transformants were analyzed for esterase production in shaken flasks with BMMY medium (Fig. 4A). The GS115 strain, transformed with the parental vector pPIC9, was used as a negative control and did not produce any esterase activity. The highest levels of esterase activity on *p*NPB were obtained by the OEP1 transformant, but all of the transformants obtained showed higher levels of activity on *p*NPB than those obtained with *O. piceae* growing in a basal medium with glucose and 0.5% olive oil as inducer [18].

The recombinant enzyme was purified from the OEP1 transformant by a single chromatographic step on a hydrophobic interaction column after concentration of the culture liquid by ultrafiltration. Esterase activity was retained in the column after the decreasing 0.5 M $(NH_4)_2SO_4$ gradient and the enzyme was eluted as a single protein peak by applying 0.2% reduced Triton X-100. SDS-PAGE analysis of the dialyzed recombinant protein showed a unique electrophoretically homogeneous enzyme with a molecular mass around 76 kDa, more glycosylated than that of the native enzyme (28 and 8% N-linked carbohydrate, respectively). Both native and recombinant enzymes showed the same molecular mass (60 kDa) after deglycosylation (Fig. 4B).

Preliminary kinetic studies showed the ability of the recombinant enzyme to hydrolyze much more efficiently than the native enzyme from *O. piceae* triglycerides and *p*-nitrophenol and cholesterol esters (Table 2).

Table 2
Efficiency of native and recombinant esterase from *O. piceae* (U/mg) hydrolyzing 5 mM triolein, *p*-nitrophenyl butyrate (*p*NPB) and cholesteryl oleate (CHOL).

	*p*NPB	Cholesteryl oleate	Triolein
Native OPE	449.67 ± 4.10	79.84 ± 2.02	171.63 ± 2.03
Recombinant OPE	1335.51 ± 36.65	319.0314286 ± 17.86	698.08 ± 39.70

Reactions were carried out in presence of Genapol X-100 (1% for *p*-nitrophenyl butyrate and 5% for triolein and cholesteryl oleate).

4. Discussion

O. piceae is a sap-staining fungus that produces melanized hyphae on wood and solid or liquid medium, under stationary conditions, but yeast-like cells in liquid medium under shaking conditions [39]. The gene that codifies the esterase/lipase enzyme secreted by this fungus in liquid medium lack introns. Although the presence of introns is usual in filamentous fungi, the absence of introns in lipases from eukaryotic organisms has also been described in *C. rugosa* [40] and *G. candidum* [41] Both, *C. rugosa* and *G. candidum* showed also the same catalytic triad and glycosylation sites that the enzyme from *O. piceae* [29,42,43].

Taking into account the molecular model of *O. piceae* esterase, we propose a similar substrate-binding site for this enzyme and *C. rugosa* lipases. According to previous reports [34,44], it is formed by an extensive hydrophobic pocket with a narrow internal tunnel which includes a phenylalanine-rich region and an aliphatic-rich region. Fig. 5 shows a detail of the substrate-binding site of the *O. piceae* esterase model, including the possible position of cholesteryl linoleate, based on *C. rugosa* lipase crystal structures. The model substrate-binding site shows slight differences with those previously reported in *C. rugosa* lipases [10,12]. The replacement of Ser450 in Lip1 by Ala450 in Lip3 could be related to the activity change of this enzyme from triacylglycerol esters to cholesterol esters in *C. rugosa* lipases. In the esterase from *O. piceae*, this position is occupied by alanine (A454), which is consistent with its high activity on cholesterol esters. In addition, the aliphatic region of the tunnel only showed minor changes in the three *C. rugosa* isoenzymes and the *O. piceae* esterase, but some differences that could affect substrate specificity have been detected in the aromatic region (Table 3). The higher phenylalanine content in Lip1 affects its substrate specificity [12]. Lip1 hydrolyzes preferably triacylglycerol esters whereas Lip2, a minor enzyme in the crude extracts [45], and Lip3 were more efficient on cholesterol esters. The higher activity of Lip2 on cholesterol esters than Lip3 and the higher similarity of *O. piceae* esterase with Lip2 could be related to the higher activity of this enzyme on these substrates.

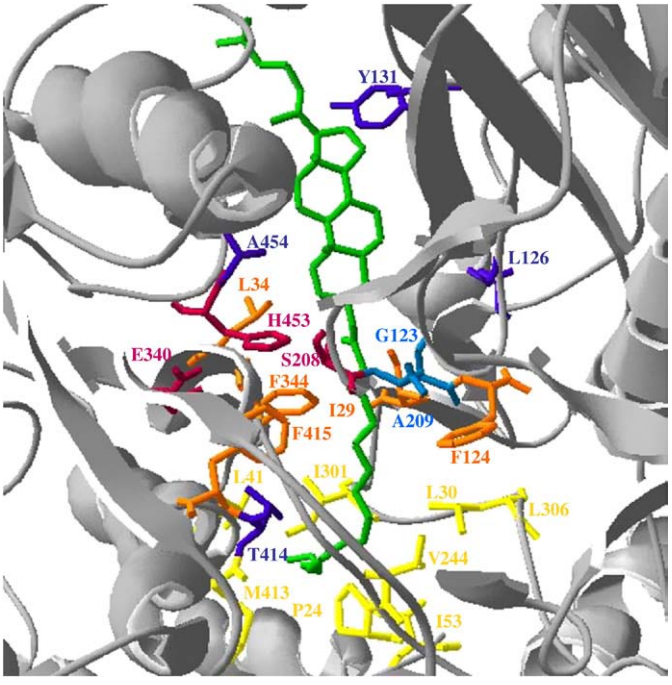

Fig. 5. Detail of substrate-binding site in the model of *O. piceae* esterase showing the possible position of cholesteryl linoleate as substrate (green): catalytic triad (dark red), oxyanion hole (blue), aromatic rich region binding pocket (orange), aliphatic-rich region binding pocket (yellow) and other residues potentially affecting substrate specificity (dark blue).

Table 3
Amino acid similarities and differences in the phenilalanine-rich region of the hydrophobic tunnel at the active site from *C. rugosa* lipase isoenzymes Lip1, Lip2 and Lip3 and *O. piceae* esterase.

O. piceae esterase	Lip1	Lip2	Lip3
Phe 124	Phe 125	Phe 125	Phe 125
Ile 295	Phe 296	Val 296	Phe 296
Leu 343	Phe 344	Leu 344	Ile 344
Phe 344	Phe 345	Phe 345	Phe 345
Phe 415	Phe 415	Phe 415	Phe 415

To analyze the biotechnological interest of the new esterase, the native protein was compared with other commercial enzymes reported as lipases, esterases or cholesterol esterases. Due to the low solubility in water of most of the substrates tested, the study was carried out in presence of Genapol X-100, reported previously as most effective surfactant to solubilise long-chain fatty-acid cholesterol esters preserving the activity of *O. piceae* esterase [18]. Although these conditions were not the optimal for the commercial enzymes, the presence of surfactant is necessary to carry out these kinetic studies and could be useful to know more about the properties of this new fungal esterase. The highest activity of *O. piceae* esterase on different substrates assayed and its stability in presence of Genapol, as well as in presence of other detergents such as polydecanol or Triton X-100 [46], suggest an interesting property to take into account for the use of this esterase in different biotechnological processes. However, these results do not allow concluding that the *O. piceae* enzyme is the best also under more "natural" conditions. In fact, in aqueous solutions and presence of Ca^{2+} Resinase A® hydrolyses tributyrin much better than the *O. piceae* enzyme [47].

The expression of the *O. piceae* enzyme in the methylotropic yeast *P. pastoris* was successful (around 17-fold more than obtained in the native strain). This heterologous system was used previously to obtain high levels of other lipases and esterases [48–50]. The purified recombinant protein confirms that the esterase from *O. piceae* is able to hydrolyze efficiently triglycerides and esters from *p*-nitrophenol and cholesterol. The highest activity of the recombinant enzyme on triglycerides and esters of *p*-nitrophenol and cholesterol (around 3–4 times more) is difficult to explain. Preliminary assays, using cholesteryl oleate as substrate, show similar efficiency of both glycosylated and deglycosylated enzyme (after treatment with Endo-H) (data not shown). This result suggests that other factors, such as protein folding in *P. pastoris*, could affect the efficiency of the recombinant enzyme, although additional studies are necessary to support this theory.

In a previous work, following the recommendations of the International Nomenclature Committee, the *O. piceae* esterase was included in the sterol esterases group (E.C. 3.1.1.13) [18]. This enzyme showed significant sequence identity with Lip2 and Lip3 of *C. rugosa*, the latter initially described as a cholesterol esterase although it has recently been included in the wider lipase family of this yeast [12]. In spite of this, the results obtained in the comparative specificity studies carried out in this work show that only commercial cholesterol esterases, no lipases, are able to hydrolyze both triglycerides and cholesterol esters. In this sense, we consider that the *O. piceae* enzyme and other enzymes with high activity on cholesterol esters and glycerides should be kept in the sterol esterase enzyme-group.

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