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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.

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.

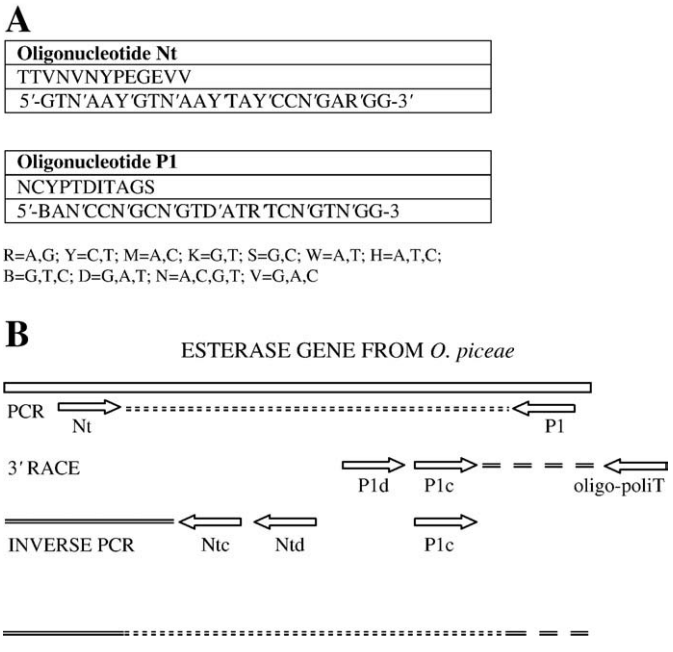

Fig. 1. *O. piceae* esterase cloning strategy. (A) N-terminal sequences of protein and tryptic peptide number 1 (obtained after hydrolyzing protein with trypsin) and degenerated oligonucleotides synthesized for partial sequences. (B) 1000 bp fragment amplified by PCR using these oligonucleotides and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