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Research paper

Unusual carboxylesterase bearing a GGG(A)X-type oxyanion hole discovered in *Paenibacillus barcinonensis* BP-23

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ABSTRACT

Strain *Paenibacillus barcinonensis* BP-23, previously isolated from Ebro's river delta (Spain), bears a complex hydrolytic system showing the presence of at least two enzymes with activity on lipidic substrates. EstA, a cell-bound B-type carboxylesterase from the strain was previously isolated and characterized. The gene coding for a second putative lipase, located upstream cellulase Cel5A, was obtained using a genome walking strategy and cloned in *Escherichia coli* for further characterization. The recombinant clone obtained displayed high activity on medium/short-chain fatty acid-derivative substrates. The enzyme, named Est23, was purified and characterized, showing maximum activity on *p*NP-caprylate ($C_{8:0}$) or MUF-heptanoate ($C_{7:0}$) under conditions of moderate temperature and pH. Although Est23 displays a GGG(A)X-type oxyanion hole, described as an important motif for tertiary alcohol ester resolution, neither conversion nor enantiomeric resolution of tertiary alcohols could be detected. Amino acid sequence alignment of Est23 with those of known bacterial lipase families and with closely related proteins suggests that the cloned enzyme does not belong to any of the described bacterial lipase families. A phylogenetic tree including Est23 and similar amino acid sequences showed that the enzyme belongs to a differentiated sequence cluster which probably constitutes a new family of bacterial lipolytic enzymes.

1. Introduction

Esterases (EC 3.1.1.–), widely distributed in nature, are a diverse group of hydrolases catalysing the cleavage or formation of ester bonds [1]. Included under the general term of carboxylesterases, the utmost characterized lipolytic enzymes are lipases (EC 3.1.1.3, triacylaglycerol hydrolases) and esterases (EC 3.1.1.1, carboxyl ester hydrolases), which differ both in their kinetics and chain-length substrate preferences [1]. While esterases hydrolyse small ester-containing molecules partially soluble in water like short-chain acylglycerides, lipases have optimal activity towards long-chain triacylglycerides not soluble in aqueous environments [2]. Therefore, esterases display a typical Michaelis–Menten behaviour, whereas most lipases show an interfacial activation when lipids reach an equilibrium between the monomeric, micellar and emulsified states [2].

In recent years there has been a growing interest for carboxylesterases due to their broad array of substrate specificity and versatility in the reactions they catalyse [3–5]. Therefore, these enzymes are considered as powerful biocatalysts with diverse biotechnological applications such as food technology, detergent formulation or the synthesis of optically pure compounds, among other uses in chemical industry [2–6]. Conveniently, carboxylesterases do not usually require cofactors, are quite stable, and may be active in organic solvents [1,7,8]. Although the physiological function of many microbial carboxylesterases remains not clear, some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources, and some may also have a role in detoxification of biocides [9,10].

Both, lipases and esterases, hold the characteristic α/β-hydrolase fold in their tridimensional structure, consisting on a defined alternation of α-helices and β-sheets also found in other hydrolases [11]. These enzymes contain a catalytic triad constituted by a serine, an aspartic or glutamic acid, and a histidine, usually being the first residue embedded in the GXSXG consensus pentapeptide [12]. Bacterial lipolytic enzymes were initially classified into eight families [13], with successive revisions up to ten families described nowadays [12,14]. These classifications are based on amino acid sequence similarity and the presence of conserved motifs, like the GDSL-pattern of family II lipases [12,13].

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Strain *Paenibacillus barcinonensis* BP-23, previously isolated from Ebro's river delta [15] and further classified [16], displays numerous hydrolytic activities. Three cellulases [17–19], five xylanases [20–25] plus two pectinases [26–28] have been isolated, cloned and characterized. Presence of hydrolysis haloes when growing on tributyrin-supplemented plates indicated also the presence of lipolytic activity. Zymogram analysis revealed the presence of at least two enzymes displaying activity on MUF-butyrate. A cell-bound type B carboxylesterase was previously isolated, cloned and characterized [29], showing activity on short-chain length fatty acid substrates, which was further improved for resolution of tertiary alcohols [30]. In this work we describe the identification, cloning and biochemical characterization of a second lipolytic enzyme of the strain, a carboxylesterase designated Est23. Assays on protease activity and tertiary alcohol resolution by Est23 are also reported.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

Wild-type strain *P. barcinonensis* BP-23 (CECT 7022) was grown in Luria–Bertani medium (LB) for 24 h at 30 °C, under aerobic conditions [16]. *Escherichia coli* BL21 Star (DE3) (Novagen®) was routinely cultured overnight at 37 °C in LB broth or on LB agar plates, and was used as the host strain for cloning and expression of lipase-encoding genes. Plasmid pET101/D-TOPO® (Invitrogen®) was used as expression vector.

2.2. DNA manipulation and cloning

DNA manipulations were carried out according to Sambrook [31]. Plasmid DNA was purified using commercial kits (Illustra PlasmidPrep, GE Healthcare, UK). Thermostable polymerases *taq* and *pfu* (Biotools, Spain) were used according to the manufacturers' instructions. PCR amplifications were performed in a GeneAMP PCR system 2400 (Perkin Elmer) using different cycling periods [31]. Genome walking for gene sequence identification was performed as previously described [14,24]. Specific primers FWEst23-Topo (5′-CACCATGCGAAAAAAGATAG), RVEst23-Topo (5′-TTAACTCGATCCGAAATATTCA), and RVEst23-Topo-His (5′-CGCACTCGATCCGAAATATTCA) were used for *est*23 ORF amplification and cloning either including or not a His tag at the C-terminus of the protein, using *P. barcinonensis* BP-23 genome as a template. Amplified DNA was purified through chromatography (Illustra™ *GFX*™), PCR DNA and Gel Band Purification kit (GE Healthcare). For ligation and expression, the pET101/D-TOPO® vector was used (Invitrogen®). To obtain the nucleotide sequences of DNA, PCR amplified fragments were analysed using the ABI PRISM® BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), and the analytical system CEQ™ 8000 (Beckman–Coulter) available at the Centres Científics i Tecnològics of the Universitat de Barcelona. DNA samples were routinely analysed by agarose gel electrophoresis [31], and stained with GelRed™ 0.27% (v/v). Nucleic acid concentration and purity was measured using a Spectrophotometer ND-100 NanoDrop®.

2.3. Bioinformatics tools

Blast searches were routinely performed for DNA or protein sequence analysis [32]. Alignments were performed using the MAFFT (Multiple Alignment Fast Fourier Transform) server (http://mafft.cbrc.jp/alignment/server). BioEdit Sequence Alignment Editor v.7.0.1 [33] was used for restriction pattern determination. The webtool ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify the open reading frames, and the software CONTIG EXPRESS was used to assemble DNA sequences (Vector NTI version 8; Invitrogen, Carlsbad, CA). Identification of putative signal peptide [34] and transmembrane regions was performed through SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM-2.0), respectively. Phylogenetic analysis was conducted using MEGA v5.2 software applying Maximum Likelihood (PALM) method (Tamura 3-parameter model). A bootstrap consensus tree was achieved after 1000 repeats and results were obtained with a cut off of 50% [35]. The DNA sequence of *est*23 was submitted to ENA databank at EBI and given the GenBank Accession number KF373032.1 (UniProt S5XG82).

2.4. Est23 purification

Exponential growth cultures (OD_{600nm} = 0.6) of recombinant clone *E. coli* BL21/pET101Est23 were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37 °C for 3 h. Cells were disrupted by using a French Press, as described, and cell extracts were concentrated using an ultrafiltration unit (Amicon® Ultra centrifugal filter devices) with a 30 kDa cut off membrane [36,37]. The recombinant His_6 tag-Est23 protein was purified from concentrated cell extracts by immobilized metal affinity chromatography (IMAC) using HisTrapHP columns of 1 ml (GE Healthcare) and eluted at a flow rate of 1 mL/min with 50 mM Tris buffer with a 0–300 mM imidazole (pH 7) gradient, on a fast protein liquid chromatography system (ÄKTA FPLC; GE Healthcare). Collected samples were desalted using an ultrafiltration unit with a 30 kDa cut off membrane. Purified esterase and crude cell extracts were analysed by SDS-PAGE [38] after a 2 min treatment at 100 °C. Zymogram analysis was performed as previously described [39]. The Bradford method was used for protein concentration determination [30,40].

2.5. Activity assays

Activity of free enzyme, crude cell extract and supernatant fractions was analysed by measuring the release of methylumbelliferone (MUF) from MUF-derivate fatty acid substrates (Sigma), using a spectrofluorometer (Variant, Spain), as previously described [41,42]. Alternatively, activity was analysed by measuring the release of *para*-nitrophenol (*p*NP) from *p*NP-derivative fatty acid substrates, as previously reported [37,43]. One unit of activity was defined as the amount of enzyme that released 1 mol of *p*NP or MUF per minute under the assay conditions used. Protease activity was assayed using skimmed-milk supplemented agar plates.

Optimum temperature of Est23 was determined by analysis of the activity over a range from 4 to 70 °C at pH 7, using MUF-heptanoate as substrate. Thermal stability of the enzyme was determined by incubating the purified enzyme at temperatures from −20 to 50 °C for 1 h or longer periods (1 month); residual activity was measured under standard conditions once samples reached room temperature. Optimum pH was established by analysing Est23 activity in a pH range from 3 to 10 using Britton–Robinson buffer adjusted to the different pHs [42,44]. To equal the different pH of each reaction, and therefore to avoid the effect of pH on the fluorometric determinations, 100 μL of 2 M Tris HCl (pH 7) were added to each reaction mixture before measuring MUF release [41]. Stability of the enzyme was also determined by measuring the residual activity on MUF-heptanoate after 1 h incubation at different pH. For inhibition studies, activity assays were performed on MUF-heptanoate in the presence of several metal ions, used at 1 mM and 10 mM concentration. Purified Est23 was also incubated in the presence of various concentrations of Triton X-100, SDS, urea or PMSF to measure the effect of such substances on enzyme activity [37,43]. Kinetic parameters (V_{max} and K_m) were