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Identification of lipase encoding genes from Antarctic seawater bacteria using degenerate primers: Expression of a cold-active lipase with high specific activity

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ABSTRACT

Cold-active enzymes are valuable catalysts showing high activity at low and moderate temperatures and low thermostability. Among cold-active enzymes, lipases offer a great potential in detergent, cosmetic, biofuel and food or feed industries. In this paper we describe the identification of novel lipase coding genes and the expression of a lipase with high activity at low temperatures. The genomic DNA from Antarctic seawater bacteria showing lipolytic activity at 4 °C was used to amplify five DNA fragments that partially encode novel lipases using specifically designed COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP). All the fragments were found to have a high identity with an α/β-hydrolase domain-containing protein identified by the sequencing of the complete genome of *Shewanella frigidimarina* NCIMB 400. The complete sequence of one of the lipase-coding gene fragments, *lip*E13, was obtained by genome walking. Considering that the other fragments had a high identity to the putative lipase from *S. frigidimarina* NCIMB 400, the complete lipase genes were amplified using oligonucleotide primers designed based on the 5′ and 3′ regions of the coding sequence of the related protein.

This strategy allowed the amplification of 3 lipase-encoding genes of which one was expressed in the periplasm using the *Escherichia coli* BL21(DE3)/pET-22b(+) expression system. The recombinant protein was obtained with activity toward *p*-nitrophenyl caproate showing a high specific activity between 15 and 25 °C.

1. Introduction

A 'true' lipase (EC 3.1.1.3) is defined as a carboxylesterase, which catalyzes the hydrolysis and synthesis of long-chain acylglycerols with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol [1,2]. Bacterial lipases are classified into six families based on amino acid sequence homology [3]. Members of family IV belong to the group of the cold-active lipases. This group is also known as HSL (Hormone Sensitive Lipase) because they display a striking amino acid sequence similarity to the mammalian hormone-sensitive lipases. Family IV lipases contain the active-site serine residue in a consensus pentapeptide GDSAG, which is located close to the N terminus of the protein. They also have another strictly conserved HGGG motif of unknown function, which contains a glycine residue that is part of the oxianion hole, located immediately upstream of the active site consensus motif [3–5].

Lipases are very useful at an industrial level, in the field of biotechnology. Much attention has been paid to the use of lipases of microbial origin [6]. The most significant industrial application of lipases is in the detergent industry in which about 1000 tons of lipases are sold each year [4,6,7]. Other applications are in the food and pharmaceutical industries, biomedical applications, biosensors and biodiesel.

Cold-adapted lipolytic microorganisms produce lipases, which function effectively at cold temperatures with high rates of catalysis in comparison to the lipases from mesophiles or thermopiles, which show little or no activity at low temperature [8]. The determination and characterization of new lipase sequences with activity at low temperatures would be a contribution to the study

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of protein adaptation to cold and could be useful in industrial processes for energy savings.

In this work, the isolation of novel lipase-coding genes from marine Antarctic origin is described. The use of CODEHOP primers allowed the isolation of five DNA fragments that partially encode five lipase enzymes. The DNA sequence of one of these genes was completed by genome walking and three by PCR using specially designed primers. One lipase gene, LipE5, was cloned into an expression vector and integrated into *Escherichia coli*. A recombinant lipase was obtained with high activity at low temperatures.

2. Materials and methods

2.1. Strain, vectors and cloning materials

Seawater was collected from the Chilean Antarctic (Lat 62° 11″S Long 58° 58″W) at King George Island. Psychrotrophic bacteria were isolated on agar plates of marine medium 2216 incubated at 4 °C, as previously described [9]. *E. coli* DH5α was used as a recipient strain for plasmids in general cloning procedures. The pGEM-T Easy vector (Promega) was used as a TA-cloning vector for polymerase chain reaction (PCR) product. PCR products were purified from agarose gel after electrophoresis using the QIAEXII system (QIAGEN). *E. coli* BL21(DE3) and pET-22b were purchased from Novagen.

2.2. Identification of lipolytic bacteria and DNA extraction

For the detection and screening of lipolytic Antarctic seawater bacteria, microorganisms were grown at 4 °C on marine agar media containing Spirit Blue Agar with Lipase Reagent [10]. The lipolytic activity of bacteria grown on agar plates was visualized after 72 h of incubation at 4 °C by the formation of a halo around the colonies. Cells for DNA manipulation were grown in 15 mL of Marine Broth 2216 at 4 °C with shaking (200 rpm). After four days, the cells were harvested by centrifugation at 4000 × *g* for 10 min at 4 °C. The pellet was washed once with 1 mL of TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) and then resuspended in 200 μL of sterile Tris 10 mM pH 8.0 containing triton X-100 (2%), SDS (1%), NaCl (100 mM), and EDTA (1 mM). Then, 200 μL of a 1:1 phenol–chloroform mixture and 0.3 g of glass beads (425–600 μm) were added. After 2 min of vortex mixing, the sample was incubated in ice for 5 min and DNA purification was continued as described by Sambrook [11]. The genomic DNA of lipolytic microorganisms was used as template for the amplification of 1111 bp from the 16S rDNA gene using *Taq* DNA Polymerase (Promega) and as sense and antisense primers the oligonucleotides 5′-AGCTAGTTGGTAGGGTAAAG-3′ and 5′-CCGCGATTACTAGCGATTCC-3′, respectively. The PCR conditions were: 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min and a final additional extension step at 72 °C for 10 min. Purified PCR products were cloned into pGEM-T Easy and sequenced. The sequences were compared with 16S rDNA gene sequences obtained from the GenBank database [12].

2.3. Cloning and sequencing of partial sequences of lipase gene

Primer design was performed by multiple alignments of the amino acid sequences of lipases and esterases active at low temperatures. The sequences were obtained from the Lipase Engineering Database [13,14]. Lipase GGGX class type (named after the consensus sequence), and the super-family of HSL were selected. This latter one was chosen because cold-active lipases are members of this lipase group [7]. The chosen lipase sequence database was the Lipase 2 from *Moraxella* sp., because these protein sequences contain the highly conserved pentapeptide of cold-active lipases, GDSAG. To identify homology areas among the sequences, 62 amino acid sequences were aligned using BioEdit [15]. The resulting alignment was used to make blocks by the BlockMaker program (http://blocks.fhcrc.org/blockmkr/make_blocks.html). Blocks obtained were ready for CODEHOP design [16,17]. The program designed two primers from these consensus regions, a degenerate sense primer (LFCH: 5′-GTCATGATGTACTTCCAYGGNGGNGG-3′) and an antisense degenerate primer (LRCH: 5′-GGTTGCCGCCGGCDSWRTCNCC-3′). Genomic DNA of lipolytic microorganisms was used as a template for amplification of the partial fragment using *Taq* DNA Polymerase. The PCR conditions were: 1 cycle at 94 °C for 5 min; 5 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; 35 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s and a final additional extension step at 72 °C for 10 min. Purified PCR products were cloned into pGEM-T Easy and sequenced.

2.4. Identification of upstream and downstream sequences by genome walking

To obtain the upstream and downstream sequences flanking the partial fragments, a genome walking method previously described was used [18]. Briefly, specific primers were designed from the partial sequence of the lipase genes obtained. The genomic DNA of the isolates was digested with *Hind*III. For the first round of PCR, a forward primer to amplify the 3′ end GW1F (5′-CTGGCTAAACATGGCAACATG-3′) and a reverse primer to amplify the 5′ end GW1R (5′-GGAATTTATGTTCTGGAGCCAG-3′) were used as first specific primers for isolates A19, B2, B9, E5, E13. For the second round of PCR, a forward primer to amplify the 3′ end GW2F (5′-CTGGCTCCAGAACATAAATTCC-3′) and a reverse primer to amplify the 5′ end GW2R (5′-CATGTTGCCATGTTTAGCCAG-3′) were used as second specific primers. To fill the cohesive ends with adenine at the 3′ end, extension was done using *Taq* DNA polymerase and dNTPs. A double-stranded oligo-cassette AdaptT adapter was ligated and elongated with primer AdaptF2 (5′-CACGCGTCGACTAGTACTAGCTT-3′). The adapter was constructed by annealing two unphosphorylated primers AdaptF: (5′-CTAGGCCACGCGTCGACTAGTACTAGCTT-3′) and AdaptR: (5′-AGCTAGTACTAGTCGACGCGTGGCCTAG-3′). GW1R was used for the 5′ end and GW1F was used for the 3′ end. These elongated products were used as PCR templates to obtain the 5′ end with primers AdaptF2 and GW2R. For the 3′ end AdaptF2 and GW2F were used. The amplified fragments were cut from the gel, purified and ligated to the cloning vector pGEM-T Easy for sequencing

2.5. Amplification of lipase genes by specific primers and phylogenetic analysis

A forward primer LipShewF 5′-ATGGATAACATTAATAAGCCAACACC-3′ and a reverse primer LipShewR 5′-TTCATTAAAGCTGTTTAATCCCATTG-3′ were used to amplify lipase-encoding genes from isolates A19, B2, B9 and E5. These specific primers were designed from the α/β-hydrolase domain-containing protein from *Shewanella frigidimarina* (NCBI Reference Sequence: YP_752317). Complete ORFs were amplified directly from the genomic DNA of each isolate, using the proof reading activity Elongase® polymerase (Invitrogen, CA, USA). The PCR conditions were: 1 cycle at 94 °C for 3 min; 25 cycles at 94 °C for 30 s, 55 °C for 45 s and 68 °C for 3 min; and a final additional extension step at 68 °C for 10 min. Purified PCR products were cloned into pGEM-T Easy and sequenced by Macrogen (Korea). Phylogenetic analysis was performed using the novel lipase protein sequences as previously described [9]. Briefly, multiple alignments of sequences from GenBank [12] and calculation of nucleotide substitution rates were performed with BioEdit version 7.0.0 program [15]. Construction and visualization of neighbor-joining phylogenetic trees were done with the MEGA version 5.05 program [19].

2.6. Recombinant expression and purification of LipE5

For the cloning of LipE5, primers pET22ShewF (5′-CCATGGATAACATTAATAAGCCAACACC-3′) and pET22ShewR (5′-CTCGAGAAAGCTGTTTAATCCCATTG-3′) were used to generate a PCR product carrying a *Nco*I restriction site at its 5′ end and *Xho*I site at its 3′ end, respectively (underlined). Complete open reading frames were amplified directly from the genomic DNA of the strain, using the proof reading activity Elongase®. The PCR conditions were: 1 cycle at 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min; and a final additional extension step at 68 °C for 10 min. The purified PCR product was cloned into pGEM-T Easy and sequenced. The PCR product encoding the mature lipase gene was digested with the corresponding restriction enzymes and then cloned into the expression vector pET-22b(+). The recombinant plasmid was mixed with electrocompetent *E. coli* BL21(DE3) cells and electroporated according to the manufacturer's instructions. After growing the cells on LB agar overnight at 37 °C, one colony was selected and grown in 250 mL of LB medium supplemented with 100 μg/mL of ampicillin at 37 °C with shaking. When an optical density of 0.5 at 600 nm was reached, 0.01 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added, and the growth temperature was lowered to 25 °C. After 16 h of cultivation, cells were harvested by centrifugation at 5000 × *g* for 5 min at 4 °C. Proteins were purified from the periplasm as recommended by the handbook for high-level expression and purification of 6xHis-tagged proteins from "The QIAexpressionist" manual using a Ni-NTA Agarose column and visualized by SDS-PAGE using 12% acrylamide gels and Coomassie Blue or silver staining. Protein concentration was determined according to Bradford [20].

An analysis of the toxic effect of different inducer concentrations was performed according to the pET System Manual from Novagen. Cultures with an OD_{600} of 1 were tested on agar plates containing different concentrations of IPTG (between 0 and 1 mM) with or without carbenicillin 200 μg/mL. A dilution of the culture was plated in order to obtain 1×10^7 colonies per plate. Plates were incubated overnight at 20 °C. The number of colonies was determined in order to establish if the recombinant protein was toxic for the cells.

2.7. Enzyme characterization

Two different lipolytic assays were performed: a liquid and an *in situ* activity assay. The liquid lipase activity assay was performed as previously described [9] using *p*-nitrophenyl caproate (*p*-NPC) as substrate. The optimum temperature was investigated in the range of 5–45 °C at pH 8.0 and catalytic parameters were obtained as described before [9]. For the *in situ* lipase assay, Spirit Blue Agar with Lipase Reagent plates [10] were used. Lipolytic activity was visualized by the formation of a clearing zone after 12 h of incubation at 15 °C. Both assays were repeated at least twice for each sample.