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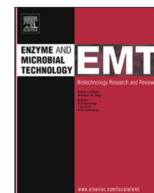

Cloning and protein expression of the *sn*-1(3) regioselective lipase from *Cordyceps militaris*

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ABSTRACT

In this study, the gene of a novel lipase with *sn*-1(3) regioselectivity (*i.e.*, *sn*-1 or *sn*-3 specific) from *Cordyceps militaris* was successfully expressed by a heterologous expression system. Total RNA was extracted from *C. militaris* and then single-stranded cDNA was synthesized. The resulting *C. militaris* lipase (*CML*) gene was inserted in *Escherichia coli* expression plasmids [pET-29b(+), pET-26b, and pColdIII] to construct plasmids encoding *CML*, which were then transformed to *E. coli* strains BL21 (DE3), C43 (DE), C41 (DE3), and Origami (DE3) for protein expression. Although the recombinant *CML* expression level was high, it was overproduced in the form of inclusion bodies. Under a specific condition, the soluble form of the recombinant *CML* was detected using Western blot analysis; however, no enzyme activity was observed. To overcome the lack of post-translational modifications in recombinant *CML*, a baculovirus-insect expression system was introduced for eukaryotic lipase expression. pDualBac was used as the transfer vector, and the *CML* gene was fused under the control of the polyhedrin promoter. After generating the recombinant baculovirus, the active form of *CML* was successfully produced and its kinetic parameters were determined.

1. Introduction

Lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) is a class of enzymes that catalyze the hydrolysis of triacylglycerol and/or esterification between glycerol and fatty acid [1]. The biotechnological applications of lipases include modification of lipids, synthesis of bioactive esters [2,3], and production of biopolymers and biodiesel [4,5]. Because of this versatility, considerable scientific and economic interest has been devoted to the potential applications of lipases with distinctive characteristics.

With regard to positional specificity, lipases have significant selectivity for the *sn* position of glycerol backbone (*i.e.*, regioselectivity), which can be classified into two typical categories [6]. One type of lipases acts preferentially on ester bonds at the *sn*-1 and *sn*-3 positions of triacylglycerols (*i.e.*, *sn*-1,3 regioselectivity), and the other on ester bonds at all positions (*i.e.*, random position). Interestingly, there are few lipases that act at only a single position (*i.e.*, *sn*-1(3) regioselectivity). Hence, there has been an increasing interest in the development of lipases with distinguishing regioselectivity, which could be utilized to the biotechnological applications with specific purposes such as selective synthesis of valuable intermediates in lipid modification and/or specific interesterification at desirable positions of triacylglycerols [7].

In a bid to develop lipases with unique regioselectivity, our research group screened 39 agricultural products and selected *Cordyceps militaris* lipase (*CML*) as a promising candidate for the *sn*-1(3) regioselective lipase. In the previous study on purification and characterization of the *CML*, it was revealed that the purified *CML* possesses a unique *sn*-1(3) regioselectivity to triacylglycerol and high stability at a broad pH-range (Manuscript ID: BTPR-18-0128, in progress).

In academic fields, *sn*-1(3) regioselective lipase is essential to identify the fatty acids esterified at *sn*-1(3) position of glycerol backbone, facilitating positional analysis for various lipid derivatives [8]. In

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the lipid industry, additional purification steps are inevitably required when using random position or *sn*-1,3 regioselective lipases that result in heterogeneous products. Contrary to this, *sn*-1(3) regioselective lipase can be utilized for high-purity production of structured lipids with a high efficiency. Hence, due to the fact that *sn*-1(3) regioselective lipase will be beneficial for both academic (*e.g.*, *sn*-1(3) fatty acid composition analysis in triacylglycerol) and industrial purposes (*e.g.*, cocoa butter equivalents, human milk fat substitutes, and other structured lipids), an efficient expression system for the production of *CML* is needed for practical applications.

Partial peptide sequences of the purified *CML* were obtained from our previous study, exhibiting a high degree of homology (> 99%) with a putative extracellular lipase from *C. militaris* (GenBank accession number XM_006666068.1). On the basis of these findings, the present study aimed to clone and express the *sn*-1(3) regioselective lipase to solve the issue of low protein yield (2.47%) from the purification of native *C. militaris*. Therefore, in this study, we performed heterologous expression of the gene of *CML* in *Escherichia coli* and baculovirus-insect expression systems.

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) expression system has been extensively used for the high-level expression of a wide variety of mammalian and eukaryotic genes in insect cells [9]. Moreover, the baculovirus–insect expression system provides a eukaryotic environment suitable for proper post-translational modifications (PTMs) of expressed proteins, which leads to that the resulting proteins are almost identical to native proteins and have a catalytic activity [10,11]. AcMNPV can enter cells of taxonomically diverse insects and further express genes under the control of a viral promoter. For these reasons, a baculovirus system, under the control of a polyhedrin promoter, was employed to construct and characterize the recombinant *CML*.

2. Materials and methods

2.1. Construction of recombinant plasmids in the Escherichia coli system

2.1.1. Strains, plasmids, and media

E. coli DH5α, which was used as the strain for plasmids with genes that encoded the recombinant proteins, was obtained from Invitrogen (Carlsbad, CA, USA) and grown in Luria-Bertani (LB) [12] medium at 37 °C. The vectors pET-29b(+), pET-26b(+) (Novagen, Billerica, MA, USA), and pColdIII (Takara, Otsu, Shiga, Japan) were used for enzyme expression in *E. coli* BL21 (DE3) (Novagen), C43 (DE3), C41 (DE3), and Origami (DE3) strains. The LB medium was used for growing the *E. coli* strains at 37 °C. In addition, LB agar containing 100 μg/mL ampicillin and 20 μg/mL kanamycin was used for screening recombinant clones.

2.1.2. Total RNA extraction and cDNA synthesis

Total RNA from *C. militaris* was isolated using the RNeasy® plant mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA was finally eluted with 50 μL RNase-free water. The synthesis of single-stranded cDNA was conducted using a Power cDNA synthesis kit (iNtRON Biotechnology, Seongnam, Gyeonggi, Korea). cDNA was synthesized using total RNA, oligo dT primer, RNase inhibitor, 5 × RT buffer, dNTP, DTT, and AMV RT enzyme.

2.1.3. cDNA cloning of the gene encoding the C. militaris lipase

2.1.3.1. Subcloning. PCR products were amplified using CML-out-F/R primers (Supplementary data Table S1) and were purified in 0.8% agarose gels using a gel extraction kit (Takara); they were then subcloned into a T-blunt vector using the T-Blunt PCR Cloning Kit (SolGent). The cloned plasmids were sequenced using the M13 forward/reverse (M13 F/R) primer (Bioneer Co., Ltd, Daedeok, Daejeon, Korea).

2.1.3.2. Sequencing of the CML gene. To amplify the *CML* gene, specific primers (Supplementary data Table S1) were designed according to the DNA sequences of lipase from *C. militaris*. The primers were synthesized by Macrogen (Geumcheon, Seoul, Korea). Amplification was performed with a synthetic single-stranded cDNA as the template using the primeSTAR HS DNA polymerase (Takara) and a thermocycler (Bio-Rad, T100™), which was performed with the following cycles: initial denaturation, 1 cycle at 95 °C for 5 min and 27 cycles at 98 °C for 10 s; annealing, 50 °C for 15 s; elongation, 72 °C for 2 min; and final elongation, 72 °C for 5 min. The amplified DNA was sequenced using the ABI 3730 DNA analyzer (Bioneer Co., Ltd).

2.1.4. Construction of expression plasmids in the E. coli system

2.1.4.1. Construction of the plasmids pET29-CML-C_6His and pET29-CML-MBP. To construct the expression plasmid pET29-CML-C_6His (vector, gene, tag), the *CML* gene (1,743 bp) was amplified by PCR and gel extraction was performed to obtain the correct fragment sizes. The DNA fragments from the PCR products were then digested by *Nde*I and *Xho*I and were ligated with a fragment of pET29b(+), which was digested by the same restriction enzymes (Supplementary data Fig. S1). To construct the expression plasmid pET29-CML-MBP (vector, gene, and tag), the *mal*E gene that encodes the maltose-binding protein (MBP), and *CML* gene were amplified by PCR. The amplified *mal*E gene fragment (1,176 bp) was inserted in the *Nde*I and *EcoR*I sites of the pET29b(+) plasmid, and the amplified *CML* gene, digested by both *EcoR*I and *Xho*I, was ligated with the fragment of pET29b(+), which was digested by the same restriction enzymes. The recombinant plasmids pET29-CML-C_6His and pET29-CML-MBP were transformed to *E. coli* strain competent cells for lipase expression.

2.1.4.2. Construction of the plasmids pET26-CML-C_6His and pET26-CML-MBP. To construct the expression plasmid pET26-CML-C_6His, the *CML* gene (1,743 bp) was amplified by PCR and gel extraction was performed to obtain the correct fragment sizes. The DNA fragments from the PCR products were then digested by *Nco*I and *Xho*I and were ligated with a fragment of pET26b(+), which was digested by the same restriction enzymes (Supplementary data Fig. S2). To construct the expression plasmid pET26-CML-MBP, the *mal*E and *CML* genes were amplified by PCR. The amplified *mal*E gene fragment (1,176 bp) was inserted in the *BamH*I and *EcoR*I sites of the pET26b(+) plasmid, and the amplified *CML* gene, digested by *EcoR*I and *Xho*I, was ligated with the fragment of pET26b(+), which was digested by the same restriction enzymes. The recombinant plasmids pET26-CML-C_6His and pET26-CML-MBP were transformed to *E. coli* strain competent cells for lipase expression.

2.1.4.3. Construction of the plasmids pColdIII-CML-C_6His and pColdIII-CML-MBP. To construct the expression plasmid pColdIII-CML-C_6His, 6 × histidine [13] added at the C-terminal (1,743 bp) of the *CML* gene; the *CML* gene was amplified by PCR, and gel extraction was performed to obtain the correct fragment sizes. The DNA fragments from the PCR products were then digested by *Nde*I and *Xba*I and were ligated with a fragment of pColdIII, which was digested by the same restriction enzymes (Supplementary data Fig. S3). To construct the expression plasmid pColdIII-CML-MBP, the *mal*E and *CML* genes were amplified by PCR. The amplified *mal*E gene fragment (1,176 bp) was inserted in the *Nde*I and *EcoR*I sites of the pColdIII plasmid, and the amplified *CML* gene was then digested by *EcoR*I and *Xba*I and was ligated with the fragment of pColdIII, which was digested by the same restriction enzymes. The recombinant plasmids pColdIII-CML-C_6His and pColdIII-CML-MBP were transformed to *E. coli* strain competent cells for lipase expression.

2.1.5. Expression of the C. militaris lipase in the E. coli system

Each *E. coli* strain, which was transformed with pET-29b(+), pET-26b(+), and pColdIII, was grown in 100 mL LB medium, containing 20 μg/mL kanamycin and 100 μg/mL ampicillin, at 37 °C with shaking

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