A Thesis for the Degree of Master of Science

cDNA cloning and expression of the *sn*-1 specific triacylglycerol lipase from *Cordyceps militaris*

붉은색 동충하초 (*Cordyceps militaris*) 유래 *sn*-1 specific triacylglycerol lipase의 cDNA클로닝 및 발현

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Park, Jung Ha

Department of Agricultural Biotechnology

Seoul National University

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지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

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박정하의 석사 학위 논문을 인준함

위 원 장 최 영 진 (인)

부위원장 장 판 식 (인)

위 원 하 남 출 (인)

Abstract

In the preliminary study, it was revealed that a lipase from *Cordyceps militaris* possesses a *sn*-1 positional specificity. Few lipases are active at the only *sn*-1position of the triglyceride structure, whereas most lipases act preferentially on ester bonds at the *sn*-1,3 or random position. *Sn*-1 positional specific lipase is significant in the academic and industrial aspects. In this study, cDNA cloning and searching for an optimal expression system of *sn*-1 positional specific lipase from *Cordyceps militaris* is the objective to solve a low level of protein yield (2.47%) from a purification of native *Cordyceps militaris.* The ESI-MS/MS analysis was applied to identify the purified lipase from *Cordyceps militaris*. As a result, it was found out a high level of homology with extracellular lipase from *Cordyceps militaris*. Total RNA extraction from *Cordyceps militaris* was followed by the synthesis of single-stranded cDNA. A gene of *Cordyceps militaris lipase* (CML) was amplified with an approximate size of 1,743 bp by PCR and used for subcloning to obtain a cDNA sequence of *Cordyceps militaris lipase* (CML). Amplified CML gene was inserted into plasmids (1) pET-29b(+) (2) pET-26b(+) (3) pColdⅢ to construct recombinant plasmids, and recombinant plasmids were transformed into *E. coli* strains BL21(DE3), C43(DE3), C41(DE3) and

Origami(DE3) for protein expression. However, it was shown that the expression level of the target protein was very high in some cases, but the recombinant protein was overproduced in the form of inactive inclusion bodies in most cases. Although the soluble form of recombinant protein was detected using western blotting analysis, no enzymatic activity was shown. To overcome this drawback, baculovirus system was introduced for the eukaryotic lipase (CML) expression. pDualBac was used as a transfer vector and the CML gene was fused under the control of polyhedrin promoter. After generation of the recombinant baculovirus, lipase activity was shown compared to wild type AcMNPV in *p*-nitrophenyl palmitate assay. Kinetic parameters were determined to characterize the recombinant protein, CML. The values of V_{max}, K_m and k_{cat} of the recombinant CML lipase expressed in the baculovirus were 0.934 μmol·min^{-1}·mg^{-1}, 0.162 mM, and 0.79 min^{-1}, respectively. The catalytic efficiency (*k*cat/Km) was calculated at the value of 4.87 min^{-1}·mM^{-1}.

Keywords: *sn*-1 specific triacylglycerol lipase; *Cordyceps militaris*; protein identification; cDNA cloning, protein expression system; *Escherichia coli*; baculovirus; bApGOZA; insect cells

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Ⅰ. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids (Sharma, Chisti, & Banerjee, 2001). Biotechnological applications of lipases include lipid modification, synthesis of bioactive esters (Davies, Green, Kelly, & Roberts, 1990; Dheeman, Henehan, & Frías, 2011), and production of biopolymers and biodiesel (kirilovaYoo, Simkhada, Cho, Park, Kim, Seong, et al., 2011; Sandoval, Rivera, Barrera-Rivera, & Martínez-Richa, 2010). Because of their wide-ranging significance, lipases remain a subject of intensive study (Beer, McCarthy, Bornscheuer, & Schmid, 1998; Mileto, Brocca, Lotti, Takagi, Alquati, & Alberghina, 1998). Research on lipases is focused particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance (Beer, McCarthy, Bornscheuer, & Schmid, 1998; Mileto, Brocca, Lotti, Takagi, Alquati, & Alberghina, 1998). Lipases can be classified according to positional specificity on which recent researches have focused (J. H. Choi, Kim, Hong, Kim, Kim, Kim, et al., 2012; Romero, Pera, Olivaro, Vazquez, & Baigori, 2012).

There has been an increasing interest in the development of a novel

source of lipase with distinguishing positional specificity which could be applied to the specific synthesis of valuable intermediates in lipid hydrolysis (kirilovaYoo, et al., 2011).

In the preliminary study, it was revealed that the lipase from *Cordyceps militaris* possesses the *sn*-1 positional specificity. This is almost non-existent property of lipase in the nature and generally *sn*-1,3 positional specific lipases or non-specific lipases (random) have been reported to be common. The importance of *sn*-1 positional specific lipase is largely divided into academic and industrial aspects. First of all, it can be applied to the analysis technique of *sn*-1 free fatty acids and it will be academically highly significant analysis to distinguish *sn*-1 and *sn*-2 positional free fatty acids in the oil and fat industry. Moreover, *sn*-1 positional specific lipase can be useful in terms of synthesis of structured lipids by producing high purity materials with high efficiency. It is normally required additional purification steps causing heterogeneous products when using random or *sn*-1,3 specific lipases. Therefore, it is worthy of finding an over-production system of *sn*-1 positional specific lipase both in the academic and industrial aspects.

The ESI-MS/MS analysis by electrospray ionization quadrupole-time of flight mass spectrometry (ESI-Q-TOF MS) was applied to identify the

purified lipase from *Cordyceps militaris*. As a result, partial peptide sequences were obtained and found out a high degree of homology with extracellular lipase from *Cordyceps militaris*. Based on this information, cloning and searching for an optimal expression system of *sn*-1 positional specific lipase from *Cordyceps militaris* is the objective of this study to solve a low level of protein yield (2.47%) from a purification of native *Cordyceps militaris*.

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) expression system has been extensively used for expression of a wide variety of mammalian genes, including human ones. In addition to the high-level expression in insect cells, the baculovirus-insect expression system provides a eukaryotic environment that is suitable for the proper post-translational modifications of the expressed proteins to take place. The expressed proteins are usually post-translationally modified such that they are almost identical to the native proteins (Jha, Pal, Nakhai, Sridhar, & Hasnain, 1992; Jones & Morikawa, 1996; Kost, Condreay, & Jarvis, 2005; Nakhai, Pal, Sridhar, Talwar, & Hasnain, 1991; Sridhar, Awasthi, Azim, Burma, Habib, Jain, et al., 1994). AcMNPV enters cells of taxonomically diverse insects and can express genes under viral promoter control.

In this study, I describe the construction and characterization of the recombinant *Cordyceps militaris* lipase (CML) in a baculovirus system under the control of the polyhedrin promoter.

Ⅱ. Materials and Methods

1. Identification of lipase from *Cordyceps militaris*

1.1. In-gel digestion

Proteins were subjected to in-gel trypsin digestion (Matthias Wilm, Houthaeve, Breitt, & Mann, 1996). Protein spots were cut from gels and de-stained with 100 μL of de-staining solution (50% methanol/distilled water) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 μL of acetonitrile and dried in a vacuum centrifuge. The procedure was repeated three times. The dried gel pieces were rehydrated with 20 μL of 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Promega, Madison, WI, USA) for 45 min on ice. After removal of the solution, 70 μL of 50 mM ammonium bicarbonate was added. The digestion was performed overnight at 37°C.

1.2. Desalting and concentration

Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis. A

column consisting of 100-300 nL of Poros reverse phase (20-30 μm bead size, PerSeptive Biosystems) was packed in a constricted gel loader tip (Eppendorf, Hamburg, Germany). A hypodermic syringe was used to force liquid through the column by applying a gentle air pressure. The peptide mixture (30 μL) from the digestion supernatant was diluted with 30 μL in 5% formic acid, loaded onto the column, and washed with 30 μL of 5% formic acid. For analyses by ESI-MS/MS, peptides were eluted with 1.5 μL of methanol/distilled water/formic acid (50:49:1, v/v/v) directly into a pre-coated borosilicate nano-electrospray needle (EconoTip^{TM}, New Objective, Woburn, MA, USA).

1.3. Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

ESI-MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (AB Sciex Instruments, Framingham, MA, USA). The source temperature was room temperature and the potential of 1 kV was applied to the pre-coated borosilicate nano-electrospray needles (EconoTip^{TM}) in the ion source combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nL/min). The cone voltage was 40 V and the quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The

collision gas was argon (Ar) at a pressure of 6^{-7} x 10^{-5} mbar and the collision energy was 25-40 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter.

2. Construction of recombinant plasmids in *E. coli* system

2.1. Strains, plasmids, and media

Escherichia coli (*E. coli*) DH5α, used as a strain for plasmids with genes encoding recombinant proteins, was obtained from Invitrogen (Carlsbad, CA, USA) and grown in LB medium at 37°C. pET-29b(+), pET-26b(+) (Novagen, Billerica, MA, USA) and pColdⅢ (Takara, Otsu, Shiga, Japan) was used for enzyme expression in *E. coli* BL21(DE3) (Novagen), C43(DE3), C41(DE3) and Origami(DE3) strains. Lauria Bertani (LB) medium was used for growing *E. coli* strains at 37°C. LB agar containing 100 μg/mL ampicillin and 20 μg/mL kanamycin was used to screen the recombinant clones.

2.2. Total RNA extraction & cDNA synthesis

Total RNA from *Cordyceps militaris* was isolated using the RNeasy® plant mini kit (Qiagen, Valencia, CA, USA). All procedures followed the manufacturer's instruction. RNA was finally eluted with 50 μL of RNase-free water. The synthesis of single-stranded cDNA was carried out using Power cDNA synthesis kit (Intron biotechnology, Seongnam, Gyeonggi, Korea). Synthesis of cDNA was done with total RNA, Oligo dT primer,

RNase inhibitor, 5x RT buffer, dNTP, DTT and AMV RT enzyme.

2.3. cDNA cloning of gene encoding a *Cordyceps militaris* lipase

2.3.1. Subcloning

PCR product was amplified using CML-out-F/R primers (Table 1). PCR products were purified from 0.8 % agarose gels using the Gel Extraction kit (Takara) and subcloned into a T-blunt vector using a T-Blunt PCR Cloning Kit (SolGent). Cloned plasmids were sequenced using M13F/R primer by Bioneer Co., Ltd (Daedeok, Daejeon, Korea).

2.3.2. Sequencing of the *Cordyceps militaris* lipase gene

To amplify the lipase gene, specific primers (Table 1) were designed according to DNA sequences of lipase from *Cordyceps militaris* submitted to National Center for Biotechnology Information (NCBI) and were constructed by Macrogen (Geumcheon, Seoul, Korea). Amplification was carried out using a synthetic single-stranded cDNA as a template using a primeSTAR HS DNA polymerase (Takara). Thermocycler (Bio-Rad, T100^{TM}) was used for the PCR amplification and the PCR was performed with 1 cycle at 95°C for 5 min for initial denaturation; 27 cycles at 98°C for 10 sec, annealing at 50°C for 15 sec, and elongation at 72°C for 2 min, and a final elongation cycle at 72°C for 5 min. Amplified DNA was sequenced using a ABI 3730

DNA analyzer by Bioneer Co., Ltd.

2.4. Construction of expression plasmids in *E. coli* system

2.4.1. Construction of plasmids pET29-CML-C6His, pET29-CML-MBP

To construct the expression plasmid pET29-CML-C6His (vector, gene, tag), CML (*Cordyceps militaris* lipase) gene (1,743 bp) was amplified by PCR and performed gel extraction to obtain a correct size of fragments. The DNA fragments from PCR products digested by both *Nde* Ⅰ and *Xho* Ⅰ were ligated with the fragment of pET29b(+) digested by the same restriction enzymes. To construct the expression plasmid pET29-CML-MBP (vector, gene, tag), malE gene and CML gene was amplified by PCR. The amplified malE gene fragment (1,176 bp) was inserted into the *Nde* Ⅰ and *EcoR* Ⅰ sites of pET29b(+) plasmid and amplified CML gene digested by both *EcoR* Ⅰ and *Xho* Ⅰ were ligated with the fragment of pET29b(+) digested by the same restriction enzymes. Recombinant plasmids, pET29-CML-C6His and pET29-CML-MBP, were transformed into *E. coli* strains competent cells for lipase expression.

2.4.2. Construction of plasmids pET26-CML-C6His, pET26-CML-MBP

To construct the expression plasmid pET26-CML-C6His, CML gene (1,743 bp) was amplified by PCR and performed gel extraction to obtain a correct size of fragments. The DNA fragments from PCR products digested by both *Nco* Ⅰ and *Xho* Ⅰ were ligated with the fragment of pET26b(+) digested by the same restriction enzymes. To construct the expression plasmid pET26-CML-MBP, malE gene and CML gene was amplified by PCR. The amplified malE gene fragment (1,176 bp) was inserted into the *BamH* Ⅰ and *EcoR* Ⅰ sites of pET26b(+) plasmid and amplified CML gene digested by both *EcoR* Ⅰ and *Xho* Ⅰ were ligated with the fragment of pET26b(+) digested by the same restriction enzymes. Recombinant plasmids, pET26-CML-C6His and pET26-CML-MBP, were transformed into *E. coli* strains competent cells for lipase expression.

2.4.3. Construction of plasmids pColdⅢ-CML-C6His, pColdⅢ-CML-MBP

To construct the expression plasmid pColdⅢ-CML-C6His, CML gene added 6xHistidine of the C-terminal (1,743 bp) was amplified by PCR and performed gel extraction to obtain a correct size of fragments. The DNA fragments from PCR products digested by both *Nde* Ⅰ and *Xba* Ⅰ were ligated with the fragment of pColdⅢ digested by the same restriction enzymes. To construct the expression plasmid pColdⅢ-CML-MBP, malE gene and CML gene were amplified by PCR. The amplified malE gene fragment (1,176 bp) was inserted into the *Nde* Ⅰ and *EcoR* Ⅰ sites of pColdⅢ plasmid and amplified CML gene digested by both *EcoR* Ⅰ and *Xba* Ⅰ were ligated with the fragment of pColdⅢ digested by the same restriction enzymes. Recombinant plasmids, pColdⅢ-CML-C6His and pColdⅢ-CML-MBP, were transformed into *E. coli* strains competent cells for lipase expression.

Table 1. Primers used for sequencing and construction of the expression plasmid in *E. coli* system

Primer name	**Sequence (5'→3')**
CML-out-F	5'-CAC TTG AGC ACG CAA ACG-3'
CML-out-R	5'-GAT GTG TAT CTC ACT TGA CC-3'
CML-inner-F	5'-GCG CAG GCC ATT TTC GAT G-3'
CML-inner-R	5'-CAT CGA AAA TGG CCT GCG C-3'
M13-F	5'-CAG GAA ACA GCT ATG AC-3'
M13-R	5'-GTA AAA CGA CGG CCA G-3'
pET29-CMLHis-F	5'-AAG GAG ATA TAC ATA TGA AAT TCT CAC TTG TGG CTC T-3'
pET29-CMLHis-R	5'-GGT GGT GGT GCT CGA GGA AGT AGA GTA CCT CCG TAT-3'
pET29-CMLMBP-F	5'-AAG GAT TTC AGA ATT CAA ATT CTC ACT TGT GGC TTC-3'
pET29-CMLMBP-R	5'-GGT GGT GGT GCT CGA GTT AGA AGT AGA GTA CCT CCG-3'
pET29-malE-F	5'-AAG GAG ATA TAC ATA TGA AAA TAA AAA CAG GTG-3'

pET29-malE-R	5’-GAC GGA GCT CGA ATT CTG AAA TCC TTC CCT-3’
pET26-CMLHis-F	5’-CCG GCG ATG GCC ATG GCC AAA TTC TCA CTT GTG GCT CT-3’
pET26-CMLHis-R	5’-GGT GGT GGT GCT CGA GGA AGT AGA GTA CCT CCG TAT-3’
pET26-CMLMBP-F	5’-AAG GAT TTC AGA ATT CAA ATT CTC ACT TGT GGC TCT-3’
pET26-CMLMBP-R	5’-GGT GGT GGT GCT CGA GTT AGA AGT AGA GTA CCT CCG-3’
pET26-malE-F	5’-GAA TTA ATT CGG ATC CAT GAA AAT AAA AAC AGG TGC AC-3’
pET26-malE-R	5’-GAC GGA GCT CGA ATT CTG AAA TCC TTC CCT CGA TC-3’
pColdⅢ-CMLHis-F	5’-ATG AAT CAC AAA GTG CAT ATG AAA TTC TCA CTT GTG GCT CTG-3’
pColdⅢ-CMLHis-R	5’-AGC AGA GAT TAC CTA TCT AGA GTG GTG GTG GTG GTG GTG GAA GTA GAG TAC CTC CGT AT-3’
pColdⅢ-CMLMBP-F	5’-AAG GAT TTC AGA ATT CAA ATT CTC ACT TGT GGC TCT-3’
pColdⅢ-CMLMBP-R	5’-AGA TTA CCT ATC TAG ATT AGA AGT AGA GTA CCT CCG-3’
pColdⅢ-malE-F	5’-ATC ACA AAG TGC ATA TGA AAA TAA AAA CAG GTG-3’
pColdⅢ-malE-R	5’-CGA CAA GCT TGA ATT CTG AAA TCC TTC CCT-3’

2.5. Expression of the *Cordyceps militaris* lipase in *E. coli* system

Each strains of *E. coli* cells transformed with pET-29b(+), pET-26b(+) and pColdⅢ were grown in 100 mL of LB medium containing 20 μg/mL kanamycin and 100 μg/mL ampicillin at 37°C with shaking until the absorbance at 600 nm was approximately 0.5. Subsequently, expression of the lipase gene was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM - 1 mM and incubating at 37°C for optimum hours. In case of cold-shock vector, pColdⅢ, cells were cultured at 15°C for 24 h after adding IPTG to a final concentration of 1 mM. After centrifugation at 5,000 xg for 20min at 4°C, pellets were suspended in 50 mM Tris-HCl buffer (pH 8.0) with 100 mM NaCl, and then disrupted by sonication. The cell components were separated into soluble and insoluble fractions by centrifugation at 14,000 xg for 20 min. The supernatant was used for lipase assay and SDS-PAGE analysis.

3. Construction of a recombinant baculovirus

3.1. Insect cells and baculovirus

The *Spodoptera frugiferda* cell line, Sf9, was maintained continuously in TC-100 medium (WelGene) supplemented with 10% FBS (WelGene) at 27°C and sub-cultured every 3-4 days. The bApGOZA (Je, Chang, Choi, Roh, Jin, O'Reilly, et al., 2001) was used as a parental virus for the production of recombinant baculovirus and Wt AcMNPV-C6 strain (O'Reilly, Miller, & Luckow, 1994) was used as a control virus. All viruses were propagated and measured it's titer in Sf9 cells.

3.2. Total RNA extraction & cDNA synthesis

Total RNA extraction and cDNA synthesis process were performed according to the procedure described in materials and methods.

3.3. Construction of a recombinant baculovirus

3.3.1 Construction of a baculovirus transfer vector containing CML gene

In order to construct a baculovirus transfer vector, pDualBac expressing CML under the polyhedrin promoter, about 1.7 kb of CML gene was amplified using specific primers. The pDualBac-CMLF (5'-CCTGCAGGCC TCGAGATGAAATTCTCACTTGTGGC-3') and pDualBac-CMLR (5'-A GATTCGAACTCGAGTTAGAAGTAGAGTACCTCCG-3') primers include *Xho* Ⅰ restriction site. Restriction sites were added at both ends (*Xho* Ⅰ at the 5' end and 3' end) for cloning into the transfer vector, pDualBac. Plasmid DNA from the subcloned T-vector was used as a template to amplify lipase gene. The PCR was carried out with 1 cycle at 95℃ for 5 min for initial denaturation; 27 cycles at 98℃ for 10 sec, annealing at 50℃ for 15 sec, and elongation at 72℃ for 2 min, and a final elongation cycle at 72℃ for 5min. The desired fragment was purified with the QiaexⅡ gel extraction kit (Qiagen) and ligated with purified PCR products and the transfer vector treated with restriction enzymes using In-fusion HD cloning kit (Clontech). Transfer vector pDualBac-CML was constructed expressing CML gene under the control of polyhedrin promoter.

3.3.2 Generation of a recombinant baculovirus

Recombinant baculovirus expressing CML gene under the control of the polyhedrin promoter was constructed by co-transfection of corresponding a transfer vector, pDualBac-CML (J. Y. Choi, Kim, Wang, Tao, Liu, Roh, et al., 2012) with bApGOZA DNA (Je, et al., 2001) into Sf9 cells. About $1x10^6$ Sf9 cells were seeded in the 60 mm diameter tissue culture dish and incubated at 27°C for 30 min to allow the cell attach. Transfection was carried out using the Cellfectin™ (Invitrogen, USA) transfection reagent according to the manufacturer's instructions. Then, CML gene was analyzed by PCR using specific primer.

4. SDS-PAGE

SDS-PAGE was performed using 12% acrylamide gels followed by the method of Laemmli (Laemmli, 1970). Protein concentration was determined according to the Bradford method (Kruger, 1994) with BSA as a standard. Expressed soluble fractions was diluted with the Laemmli sample buffer (Laemmli, 1970) and boiled for 3 min before loading. Bio-Rad protein marker was used as a broad range protein standard to estimate the molecular weight of the proteins. Protein sample was isolated on Hoefer SE 250 mini-

gel system (GE Healthcare) at room temperature at a current of 20 mA. The proteins were stained with Coomassie Brilliant Blue G-250 (Bio-Rad).

5. Western blot analysis

For Western blotting analysis, purified proteins separated by SDS-PAGE were transferred onto the polyvinylidene fluoride (PVDF) membrane using a mini trans-blot electrophoretic transfer cell (Bio-Rad). To detect his-tagged recombinant protein, His-probe (H-3) monoclonal antibody (Santa Cruz) and goat anti mouse lgG-HRP (Santa Cruz) were used as first and second antibody, respectively.

6. Determination of lipase activity

Lipase activity was measured using a spectrophotometric method (Wrolstad, Decker, Schwartz, & Sporns, 2005) with modification. The substrate solution was prepared by dissolving 0.07545 g *p*-nitrophenyl palmitate in 100 mL distilled water with 1 g Triton X-100 and 0.017 g SDS and then heating the mixture in a water bath at 65°C. The substrate solution (2.5 mL) was added into 2.5 mL Tris-HCl buffer (pH8.0) and the mixture

was pre-incubated for 10 min. Lipase-catalyzed hydrolysis was initiated by adding 0.1 mL of enzyme solution to the reaction mixture pre-incubated in a water-bath at 40°C with magnetic stirring (800 rpm). The absorbance was measured at 400 nm to analyze the *p*-nitrophenol produced from *p*-nitrophenyl palmitate. One unit of activity was defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol per minute.

Ⅲ. Results and Discussion

1. Protein identification

The ESI-MS/MS analysis by electrospray ionization quadrupole-time of flight mass spectrometry (ESI-Q-TOF MS) was applied to identify the purified lipase from *Cordyceps militaris*. The peptide mixture from a proteolytic digestion was separated (Fig. 1). Three other peptides with multiple charges such as $[M+2H]^{2+}$ ion of *m/z* 664.9, $[M+2H]^{2+}$ ion of *m/z* 739.9, and $[M+3H]^{3+}$ ion of *m/z* 898.8 were found and MS/MS spectra were acquired for each fragmented peptide (Fig. 2). To validate protein identification by MS/MS analysis, *de novo* sequencing was performed and sequences of these peptides were obtained (Table 2). A database search indicated a high degree of homology to extracellular lipase from *Cordyceps militaris*, which had not been characterized before. The identified protein was previously only known as the 'predicted' value discovered by genomic sequencing. The value 'predicted' is used for entries without evidence at protein, transcript, or homology levels. As a consequence, homology search showed the presence of extracelluar lipase from *Cordyceps militaris* at the protein level.

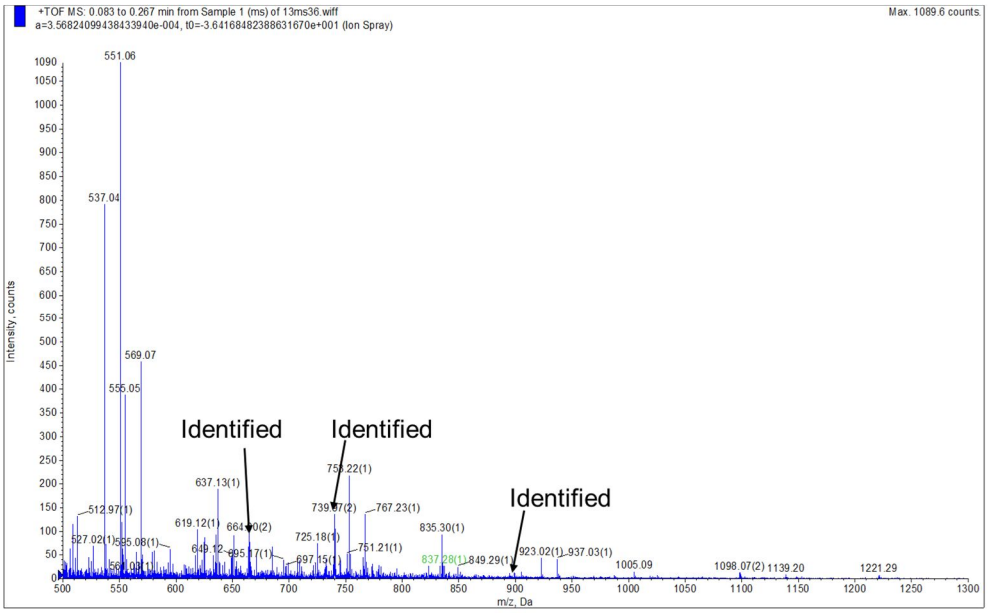
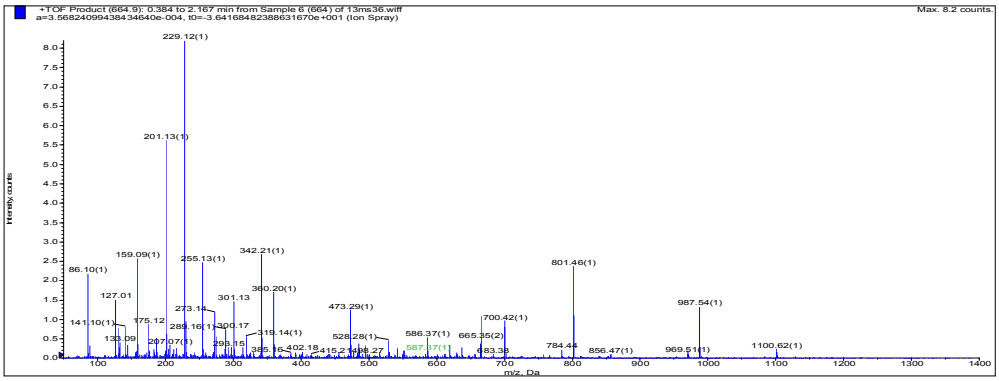
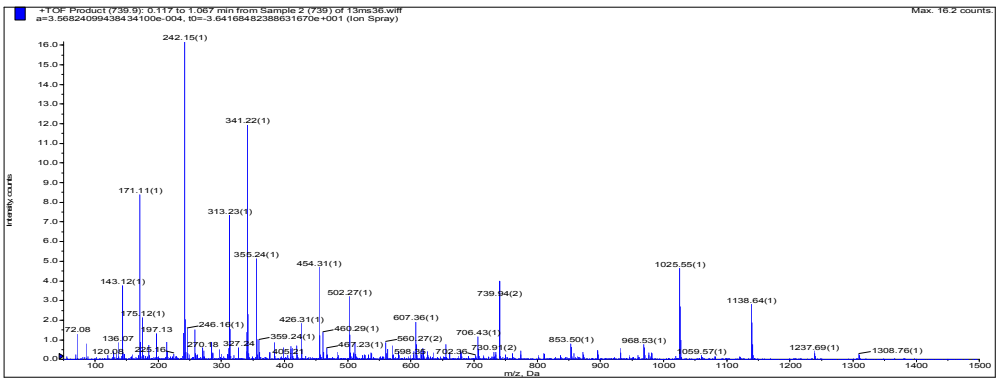


Fig. 1. Mass spectrum of the purified lipase from *Cordyceps militaris*.

(A)


+TOF Product (664.9): 0.384 to 2.167 min from Sample 6 (664) of 13ms36.wiff
a=3.56824099438434640e-004, t0=-3.64168482388631670e+001 (Ion Spray)
Max. 8.2 counts.
Intensity, counts
m/z, Da
229.12(1)
201.13(1)
159.09(1)
255.13(1)
342.21(1)
86.10(1)
127.01
360.20(1)
301.13
801.46(1)
987.54(1)
273.14
473.29(1)
700.42(1)
175.12
289.16(1)
300.17
315.14(1)
586.37(1)
665.35(2)
141.10(1)
133.09
267.07(1)
293.15
528.28(1)
784.44
856.47(1)
969.51(1)
1100.62(1)

(B)


+TOF Product (739.9): 0.117 to 1.067 min from Sample 2 (739) of 13ms36.wiff
a=3.56824099438434100e-004, t0=-3.64168482388631670e+001 (Ion Spray)
Max. 16.2 counts.
Intensity, counts
m/z, Da
242.15(1)
341.22(1)
171.11(1)
313.23(1)
355.24(1)
454.31(1)
1025.55(1)
143.12(1)
502.27(1)
739.94(2)
1138.64(1)
175.12(1)
607.36(1)
72.08
246.16(1)
426.31(1)
460.29(1)
706.43(1)
197.13
136.07
359.24(1)
560.27(2)
853.50(1)
968.53(1)
1237.69(1)
1308.76(1)

(C)

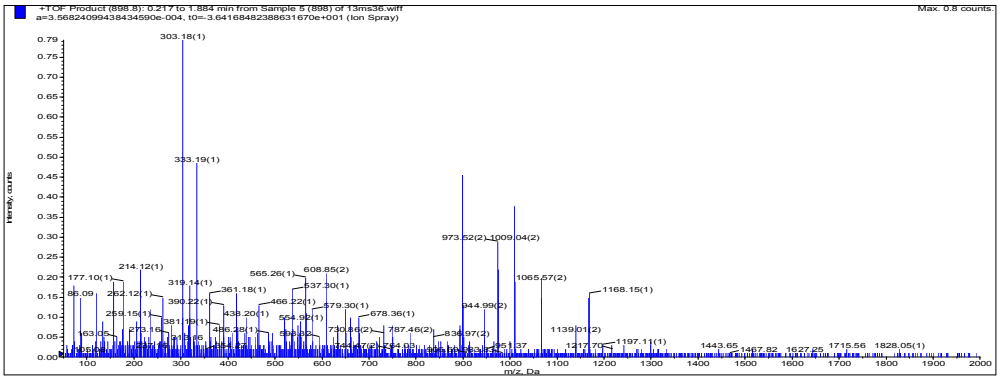

+TOF Product (898.8): 0.217 to 1.884 min from Sample 5 (898) of 13ms36.wiff
a=3.56824099438434590e-004, t0=-3.64168482388631670e+001 (Ion Spray)
Max. 0.8 counts.
Intensity, counts
m/z, Da
303.18(1)
333.19(1)
973.52(2)
1009.04(2)
214.12(1)
177.10(1)
565.26(1)
608.85(2)
1065.57(2)
319.14(1)
537.30(1)
1168.15(1)
86.09
361.18(1)
262.12(1)
390.22(1)
466.22(1)
944.99(2)
579.30(1)
259.15(1)
438.20(1)
678.36(1)
554.92(1)
1139.01(2)
163.05
730.84(2)
787.46(2)
836.97(2)
1197.11(1)
1443.65
1715.56
1828.05(1)

Fig. 2. ESI-MS/MS spectrum of the fragment $[M+2H]^{2+}$ ion of *m/z* 664.9 (A), $[M+2H]^{2+}$ ion of *m/z* 739.9 (B), $[M+3H]^{3+}$ ion of *m/z* 898.8 (C) from the tryptic digest of *Cordyceps militaris* lipase.

Table 2. Peptides identified by ESI-MS/MS analysis

Precursor (*m/z*)	Peptide (mass)	Identified protein	Database searched peptide (sequence coverage %)	Similar proteins in data base of NCBI	MASCOT SCORE
664.9	1327.8	Extracellular lipase, putative	^{547}DLLWTNLLANR^{557} (1)	EGX96254[1)]	62
739.9	1477.8	Extracellular lipase, putative	^{449}VAAVIGDFVFTLAR^{462} (2)	EGX96254	67
898.8	2693.4	Extracellular lipase, putative	^{192}VGGFGFLAGSEVLEDGSTNLGLRDQ R^{217} (4)	EGX96254	71

[1)] Protein information could be found on the web site http://www.uniprot.org

2. Cloning and sequencing of the CML gene

The DNA sequences were determined by ABI 3730 DNA analyzer using the sub-cloned plasmid as a template. M13 forward/reverse primers were used for initial sequencing, then a series of clone-specific primers were synthesized based on determined nucleotides in previous sequencing and used for next sequencing subsequently (Fig. 3). Nucleotide sequence of the CML gene showed that it consisted of 1,743 bp using primers M13F/R and contained one open reading frame ORF of 1,743 bp. The ORF's amino acids sequence of the lipase was encoded 579 amino acids with a calculated molecular mass of 64.02 kDa. No nucleotide sequence corresponding to a typical signal peptide was detected. Verified CML gene sub-cloned in T-blunt vector from the sequencing was used as a template for PCR. Amplification of CML gene using primers (Table 2) yielded a fragment with an approximate size of 1740 bp (Fig. 4). Gel extraction and DNA clean-up processes were performed and cloned into vectors (Table 3) followed by transformation of *E. coli* DH5α. Recombinant plasmids were verified the presence of lipase gene using DNA electrophoresis and sequenced for comparing with the verified cDNA sequence of *Cordyceps militaris* (Fig. 5) in the final process.

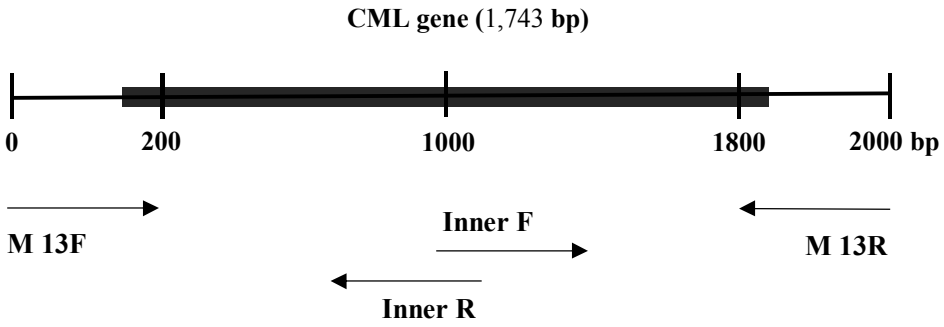


Fig. 3. A schematic view of sequencing process in the case of CML gene. Approximately 1.7 kb of insert encoding a lipase gene of *Cordyceps militaris* was sequenced with M13F/R primers at first, and then, a series of synthetic primers were used for the next sequencing for the complete nucleotide sequence of the CML gene in both forward and reverse sides. M13F/R, the primers used for initial sequencing; Inner F/R, a set of primers used for next sequencing in both sides.

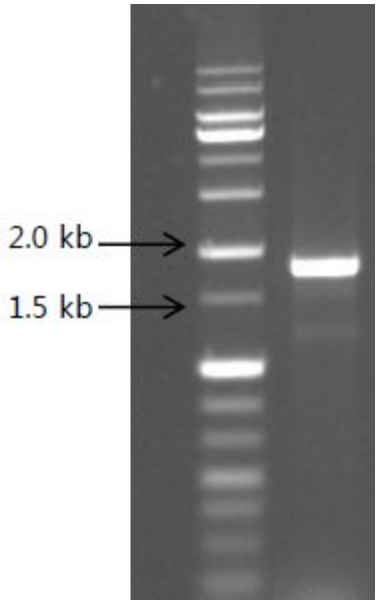


Fig. 4. Amplified CML gene by PCR (98°C, 10 sec denaturation; 50°C, 15 sec annealing; 72°C, 2 min elongation; 27 cycles, primeSTAR HS DNA polymerase (Takara).

Table 3. Characteristics of plasmids for CML expression in *E. coli* and baculovirus system

Host	Vector	Promoter	Antibiotic resistance	Tag
E. coli	pET-29b(+)	T7	Kan	C-terminal 6xHis (Histidine) MBP (Maltose-binding protein)
	pET-26b(+)	T7	Kan	C-terminal 6xHis (Histidine) MBP (Maltose-binding protein)
	pColdⅢ	cspA	Amp	C-terminal 6xHis (Histidine) MBP (Maltose-binding protein)
Baculovirus	pDualBac	Polh.	Amp	C-terminal 6xHis (Histidine) N-terminal 6xHis (Histidine)

atgaaattctcacttgtggctctggccactctcccgttttatgctgtcgcagcgcctcat
M K F S L V A L A T L P F Y A V A A P H
tgcccgggacaacccaatggtccgcccagggccgcggaagtgagggtcgatatacctggc
C P G Q P N G P P R A A E V R V D I P G
gccacaggcggcacagtcattggagtcgtcaccgaagttgagagcttcaacggcatcccc
A T G G T V I G V V T E V E S F N G I P
tacgctgactgcccgtccggtgcccttcgacttcgaccgccgaggaagctctcgcgcgcg
Y A D C P S G A L R L R P P R K L S R A
ctcggcgtcttcaacgccacggccgccgcgccggcctgtccccagatgccgcccaacacc
L G V F N A T A A A P A C P Q M P P N T
accgaagtgctgctgccgccgcttctcggcaaggacatgacgcccgagttctggccagac
T E V L L P P L L G K D M T P E F W P D
gacctcatccggggccaggaagactgcctcaccgtcaacgtgcagcggcccaagggcacc
D L I R G Q E D C L T V N V Q R P K G T
cgtgagggcgcccgcctgcccgtgctcttctacatctttggaggcggcttcacggccggc
R E G A R L P V L F Y I F G G G F T A G
gccaccagcgccaacaatgccgaaaagttcttgcggttcgccgaggcgcagcagaagccg
A T S A N N A E K F L R F A E A Q Q K P
cacccgttcatctttgtcggcgtcaactaccgcgtcggcgggttcgggttcctcgccggc
H P F I F V G V N Y R V G G F G F L A G
agcgaggttctcgaggacggcagcaccaacctcgggctccgcgaccagcggatgggcctc
S E V L E D G S T N L G L R D Q R M G L
gagtgggtggcggacaacattgcctactttggcggcgacccggacagagtgaccatctgg
E W V A D N I A Y F G G D P D R V T I W
ggccagtcgtccggctccatctccgtgtttgaccagctcgcgctctacaacggcaacgca
G Q S S G S I S V F D Q L A L Y N G N A
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T Y K Q K P L F R S A I M N S G S V I P
acggagcgggtcgactcgcatcgggcgcaggccattttcgatgccgtcgtcgaagcggcc
T E R V D S H R A Q A I F D A V V E A A
aactgcagcgagccggccacctccaagctggactgtcttcgaaatgcctcctttccgacc
N C S E P A T S K L D C L R N A S F P T
ttctatcgcgcggcaaactcggtcccgcgcatcttggacaactcgtctctcgccatctcc

```
 F  Y  R  A  A  N  S  V  P  R  I  L  D  N  S  S  L  A  I  S
tacctgccgcggcccgacggggagctgctggcggacagtcccgaggtccttgccaacacg
 Y  L  P  R  P  D  G  E  L  L  A  D  S  P  E  V  L  A  N  T
ggcaactactatgccgttcccgccatcctcaccaatcaggaggacgaaggaaccctgttc
 G  N  Y  Y  A  V  P  A  I  L  T  N  Q  E  D  E  G  T  L  F
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 A  F  A  Q  R  H  V  N  D  T  D  S  L  V  D  Y  L  K  E  T
ttttcgacaaggcgacaagggagcaggtcgccggcctcgtggatacatacccagccgat
 F  F  D  K  A  T  R  E  Q  V  A  G  L  V  D  T  Y  P  A  D
tcggccgatggaagcccgttccggactggtgatcagaacgagtggtacgaggaagcgtac
 S  A  D  G  S  P  F  R  T  G  D  Q  N  E  W  Y  E  E  A  Y
ggtgccggcaaaggcttcaagagggtcgcggctgtgataggcgactttgtcttcacgctg
 G  A  G  K  G  F  K  R  V  A  A  V  I  G  D  F  V  F  T  L
gcccgtcgcctggcgcttgatggcatggccacttcgcatccgacggtgcctctttggtcc
 A  R  R  L  A  L  D  G  M  A  T  S  H  P  T  V  P  L  W  S
tctctgaactccatggcccacgggattgtgggcttctacggtacgggacacggtgcagat
 S  L  N  S  M  A  H  G  I  V  G  F  Y  G  T  G  H  G  A  D
gtcaacatgattttgaaggcattggcatacctgcgctgactaccaggtcgtactatctg
 V  N  M  I  F  E  G  I  G  I  P  A  L  T  T  R  S  Y  Y  L
aatttcctatatactgccgacccgaataatggtaccacggaatttagacagtggccaaag
 N  F  L  Y  T  A  D  P  N  N  G  T  T  E  F  R  Q  W  P  K
tggacgccacagggtagggatttgctgtggactaacctttggcgaatagagacttgaag
 W  T  P  Q  G  R  D  L  L  W  T  N  L  L  A  N  R  D  L  K
gatactttcaggaatgatagctatacattcttgaaggagaatacggaggtactctacttc
 D  T  F  R  N  D  S  Y  T  F  L  K  E  N  T  E  V  L  Y  F
taa
 -
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Fig. 5. The nucleotide sequence and deduced amino acid sequence of CML gene.

3. Expression of the CML in *E. coli* system

A number of expression systems were tested in order to find out an ideal expression system in *E. coli*. All expression systems attempted in *E. coli* were summarized in table 4.

3.1. Plasmid pET29-CML-C6His and pET29-CML-MBP

To achieve high level expression of CML, pET vector system was used as the expression vector which harbors a strong promoter, T7. pET29-CML-C6His, 6xHis(histidine) residues were fused to the C-terminus of CML, was transformed into *E. coli* strains BL21(DE3), C43(DE3) and Origami(DE3). In case of BL21(DE3) and C43(DE3), SDS-PAGE analysis showed that the expression level of the target protein was very high, but the recombinant protein was overproduced in the form of inactive inclusion bodies. Although the soluble form of recombinant protein was detected using western blotting analysis, no enzymatic activity was shown. In case of Origami(DE3), no expression was found in SDS-PAGE.

Maltose-binding protein (MBP) was tested as a fusion partner because of its ability to enhance the solubility and promote the proper folding. The constructed expression vector was transformed into *E. coli* BL21(DE3) and

C41(DE3). In case of BL21(DE3), recombinant protein was overproduced in the form of inactive inclusion bodies. Although the soluble form of recombinant CML was detected using western blotting analysis, no enzymatic activity was shown. No expression was shown when using the C41(DE3).

3.2. Plasmid pET26-CML-C6His and pET26-CML-MBP

pET26-CML-C6His, 6xHis(histidine) residues were fused to the C-terminus of CML, was transformed into *E. coli* strains BL21(DE3), C43(DE3) and C41(DE3). Large amount of the recombinant protein was overproduced in the form of inclusion bodies in all strains and no enzymatic activity was shown. Maltose-binding protein (MBP) was tested as fusion partner and the constructed expression vector was transformed into *E. coli* BL21(DE3) and C41(DE3). Although the soluble form of recombinant protein was detected using western blotting analysis, no enzymatic activity was shown.

3.3. Plasmid pColdⅢ-CML-C6His and pColdⅢ-CML-MBP

pColdⅢ vector that drive the high expression of cloned genes upon induction by cold shock was used to improve their solubility and stability (Qing, Ma, Khorchid, Swapna, Mal, Takayama, et al., 2004). pColdⅢ-CML-C6His, 6xHis(histidine) residues were fused to the C-terminus of CML, was transformed into *E. coli* strains BL21(DE3), C41(DE3), C43(DE3), Origami(DE3). No expression was shown in the BL21(DE3), C41(DE3) and C43(DE3). Low level of expression of recombinant protein was found in SDS-PAGE but it was overproduced in the form of inactive inclusion bodies. Maltose-binding protein (MBP) was tested as fusion partner and the constructed expression vector was transformed into *E. coli* BL21(DE3), C41(DE3) and Origami(DE3). Although the soluble form of recombinant protein was detected using western blotting analysis in the BL21(DE3) and C41(DE), it was overproduced in the form of inactive inclusion bodies. No expression was shown when using Origami(DE3) strain.

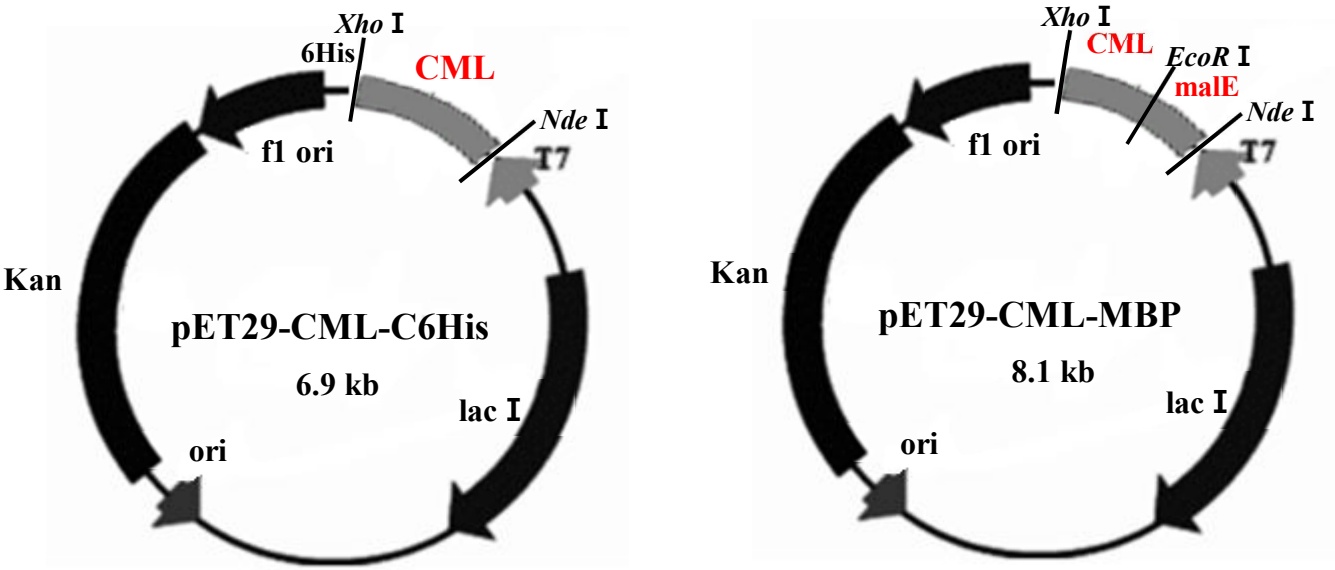

Fig. 6. Construction map of expression vectors, pET29-CML-C6His, pET29-CML-MBP.

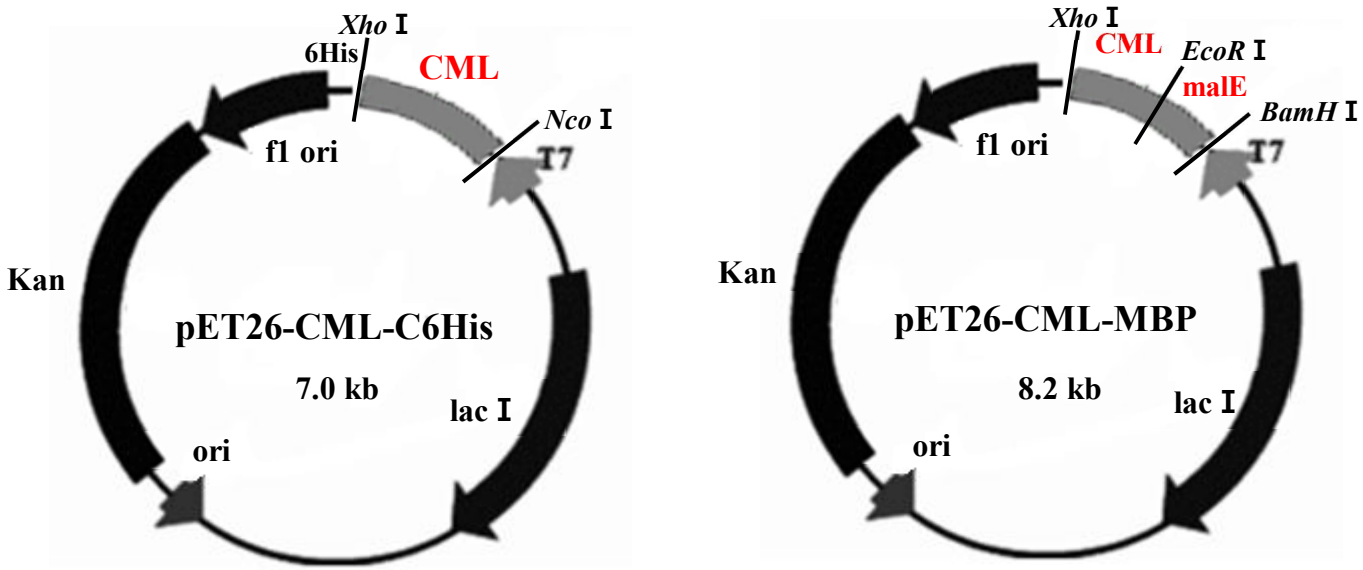

Fig. 7. Construction map of expression vectors, pET26-CML-C6His, pET26-CML-MBP.

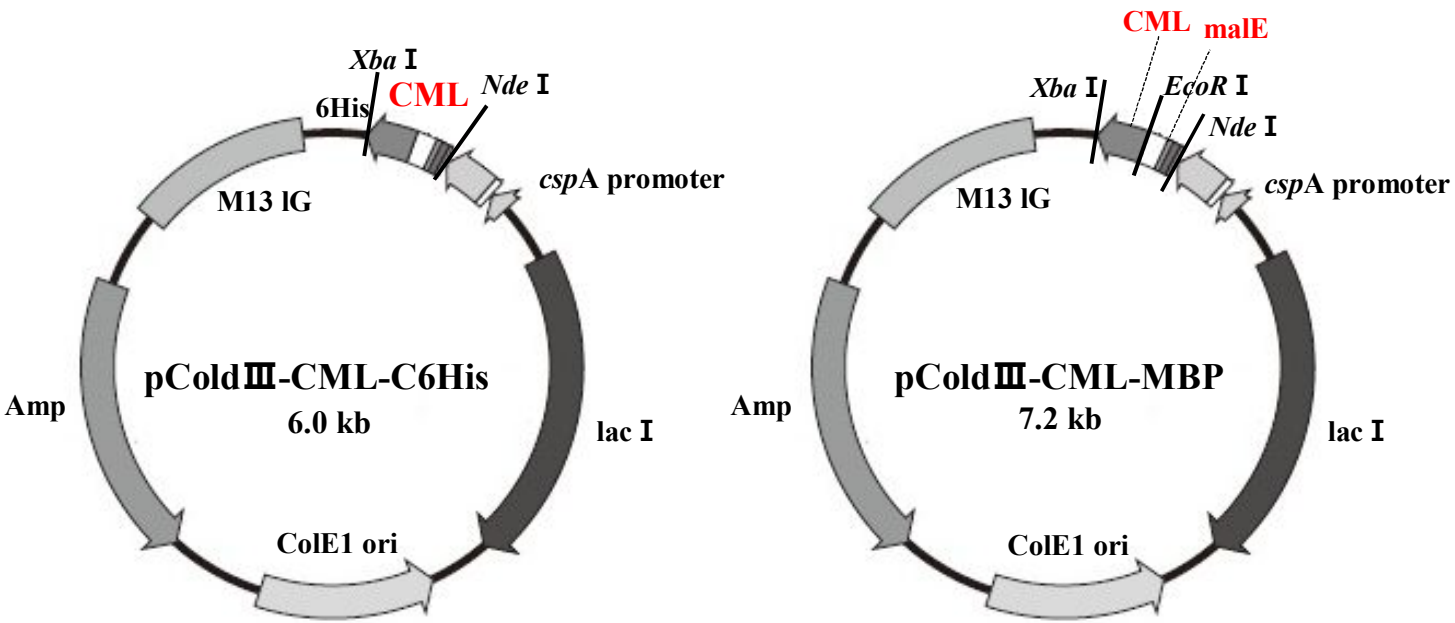

Fig. 8. Construction map of expression vectors, pColdⅢ－CML-C6His, pColdⅢ-CML-MBP.

Table 4. Tried protein expression system in *E. coli* and expression pattern

Host	Plasmid name	Strain	Expression	Solubility	Activity
E. coli	pET29-CML-C6His	BL21(DE3)	++	S < I	N
		C43(DE3)	++	I	
		Origami(DE3)	-		
	pET29-CML-MBP	BL21(DE3)	++	S < I	N
		C41(DE3)	-		
	pET26-CML-C6His	BL21(DE3)	++	I	
		C41(DE3)	+	I	
		C43(DE3)	+	I	
	pET26-CML-MBP	BL21(DE3)	+	S < I	N
		C41(DE3)	+	S < I	N
	pColdⅢ-CML-C6His	BL21(DE3)	-		
		C41(DE3)	-		
		C43(DE3)	-		
		Origami(DE3)	+	I	

pColdⅢ-CML-MBP	BL21(DE3)	++	S < I	N
	C41(DE3)	+	S < I	N
	Origami(DE3)	-		

1) ++, high level of expression; +, low level of expression; -, no expression

2) S, soluble; I, insoluble

3) N, no activity was shown

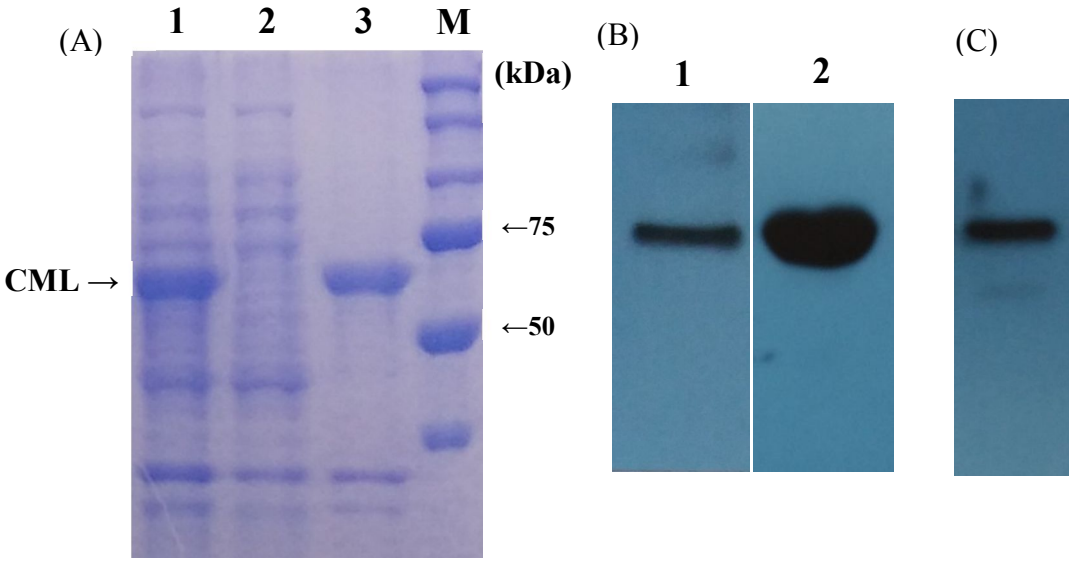

Fig. 9. SDS-PAGE and western blot analysis of CML expression in recombinant *E. coli* BL21. (A) Lane 1: molecular weight marker. Lane 2: cell lysate of *E. coli* BL21 containing CML. Lane 3: soluble cytoplasmic proteins. Lane 4: insoluble cytoplasmic proteins. (B) Western blot analysis of His-tagged CML. 1: soluble cytoplasmic protein detection. 2: insoluble cytoplasmic protein detection. (C) Soluble MBP-fusion protein containing CML after purification.

4. Construction of a recombinant baculovirus

In order to generate a recombinant baculovirus, a transfer vector, pDualBac-CML was constructed as shown in Figure 10. In this transfer vector, the CML gene was fused under the control of polyhedrin promoter. To confirm of CML clone in the pDualBac CML transfer vector, sequencing was performed using an ABI 3730 DNA analyzer by Bioneer Co., Ltd (Daejeon, Korea). The recombinant baculovirus was generated by cotransfection of bApGOZA DNA and pDualBac-CML. The structure of the recombinant virus was confirmed by PCR analysis (Fig. 11). The recombinant baculovirus was purified by plaque assay on Sf9 cells according to the method of O'Reilly *et al*. (1992). To confirm of the expression of recombinant protein, CML, SDS-PAGE was used. In SDS-PAGE, 64 kDa of recombinant protein band and 30 kDa of polyhedrin band were exhibited in infected cell lysate. 64 kDa of recombinant protein is consistent with the expected CML size (Fig. 12). Moreover, the soluble form of recombinant protein was shown in SDS-PAGE and enzymatic activity was detected by *p*-nitrophenyl palmiate assay.

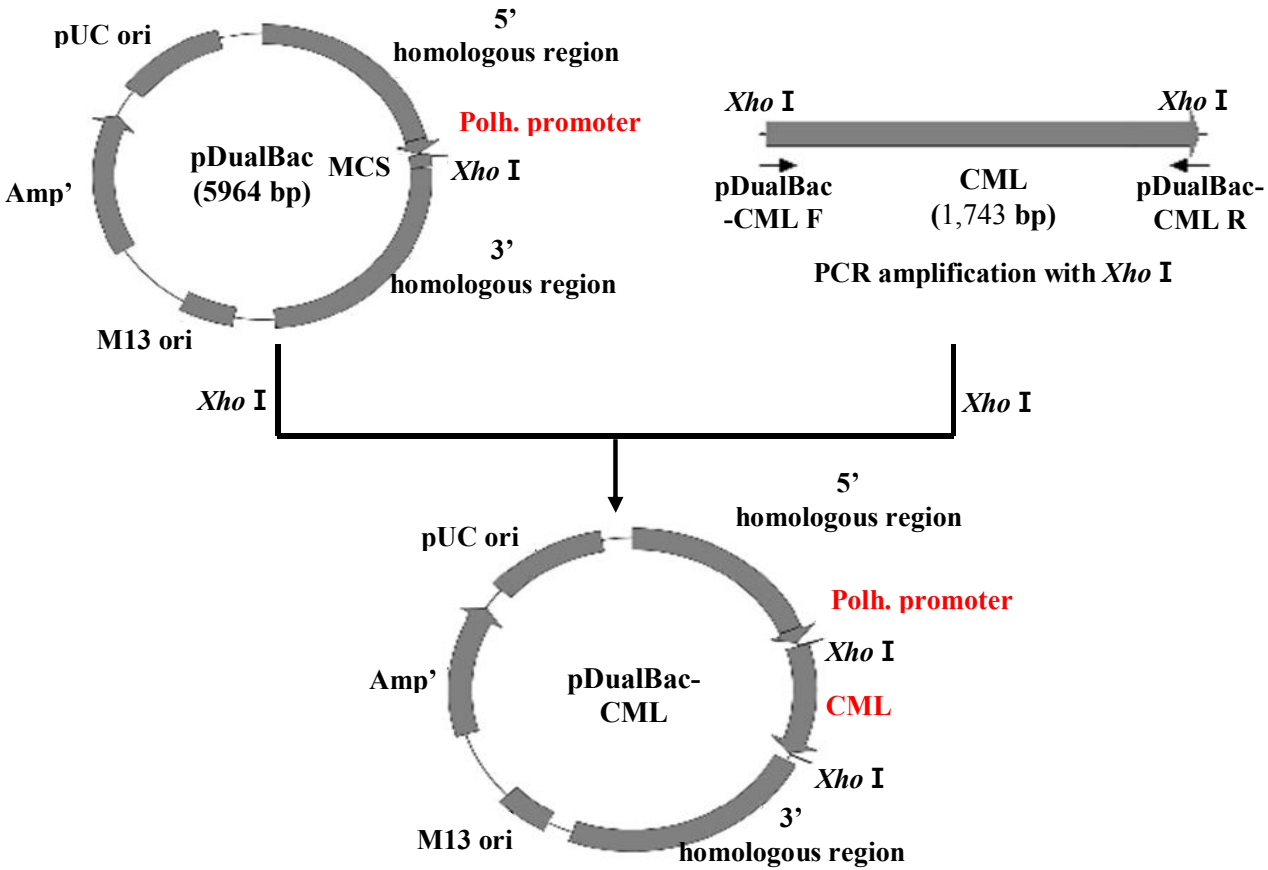

Fig. 10. Construction map of the transfer vector expressing CML gene under the control of polyhedrin promoter. The 1.7 kb of amplified CML gene was inserted into pDualBac to obtain the vector pDualBac-CML.

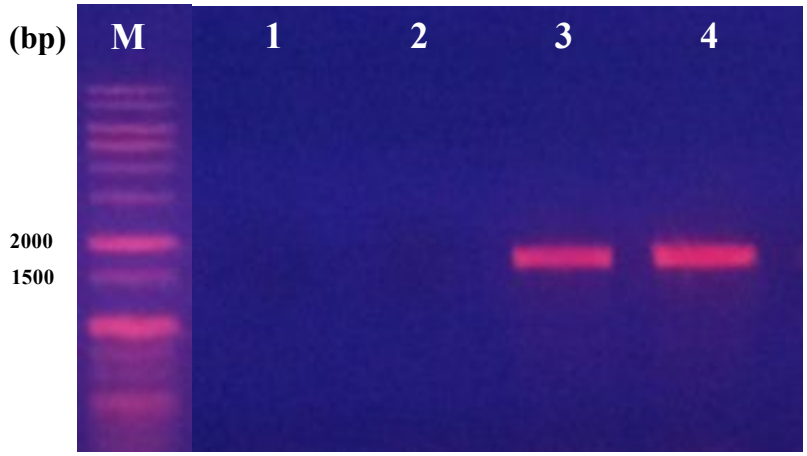

Fig. 11. Verification of the genomic structure of the recombinant baculovirus by PCR using specific primer. Lanes: M, SiZer^{TM}-1000 bp DNA marker (Intron); 1, mock-infected Sf9 cells; 2, bApGOZA-infected Sf9 cells; 3, recombinant baculovirus containing CML-infected Sf9 cells; 4, purified recombinant baculovirus by plaque assay.

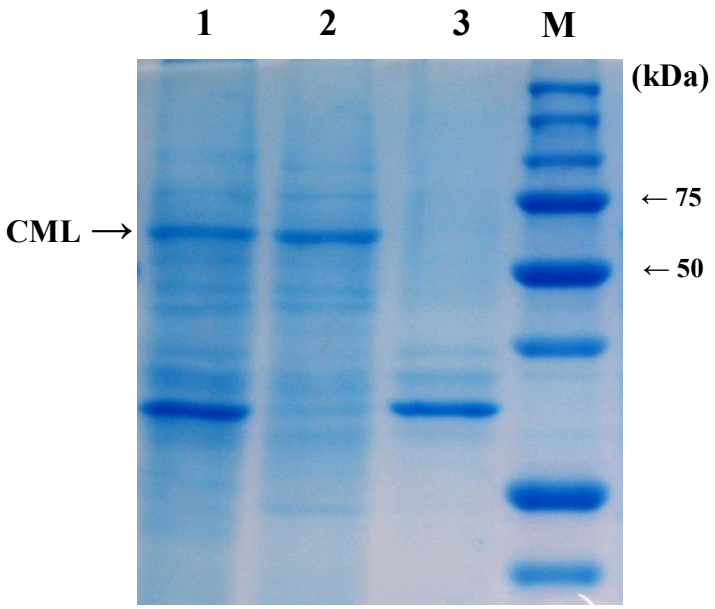

Fig. 12. SDS-PAGE analysis of recombinant protein expressed in baculovirus system after cell lysis. Lanes: M, protein molecular weight marker; 1, cell lysate; 2, lysate supernatant; 3, lysate pellet.

5. Characterization

5.1 Determination of kinetic parameters

The values of V_{max}, K_m, k_{cat}, and k_{cat}/K_m were determined from Hanes-Woolf plot under the optimum conditions of purified lipase from *Cordyceps militaris*. Lipase assay was carried out with concentrations of *p*-nitrophenyl palmitate from 0.1 mM to 0.4 mM. The values of V_{max}, K_m and k_{cat} of the recombinant CML lipase expressed in the baculovirus were 0.934 μmol·min^{-1}·mg^{-1}, 0.162 mM, and 0.79 min^{-1}, respectively. (Figure 13). The catalytic efficiency (k_{cat}/K_m) was calculated at the value of 4.87 min^{-1}·mM^{-1}. These results showed that K_m value of the recombinant CML in baculovirus was lower than those of the lipases from *Bacillus cereus* (2.0 mM) (Otnaess, Little, Sletten, Wallin, Johnsen, Flengsrud, et al., 1977) and *Brassica napus* (1.0 mM) (Novotná, Káš, Daussant, Sajdok, & Valentová, 1999). The catalytic efficiency (k_{cat}/K_m) value of the recombinant CML in baculovirus was higher than those of lipases from Staphylococcus xylosus (0.141 min^{-1}·mM^{-1}, 0.164 min^{-1}·mM^{-1}, 0.247 min^{-1}·mM^{-1}) (Kolling, Bertoldo, Brod, Vernal, Terenzi, & Arisi, 2010) and Homo sapiens (1.5 min^{-1}·mM^{-1}) (Schalk-Hihi, Schubert, Alexander, Bayoumy, Clemente, Deckman, et al., 2011).

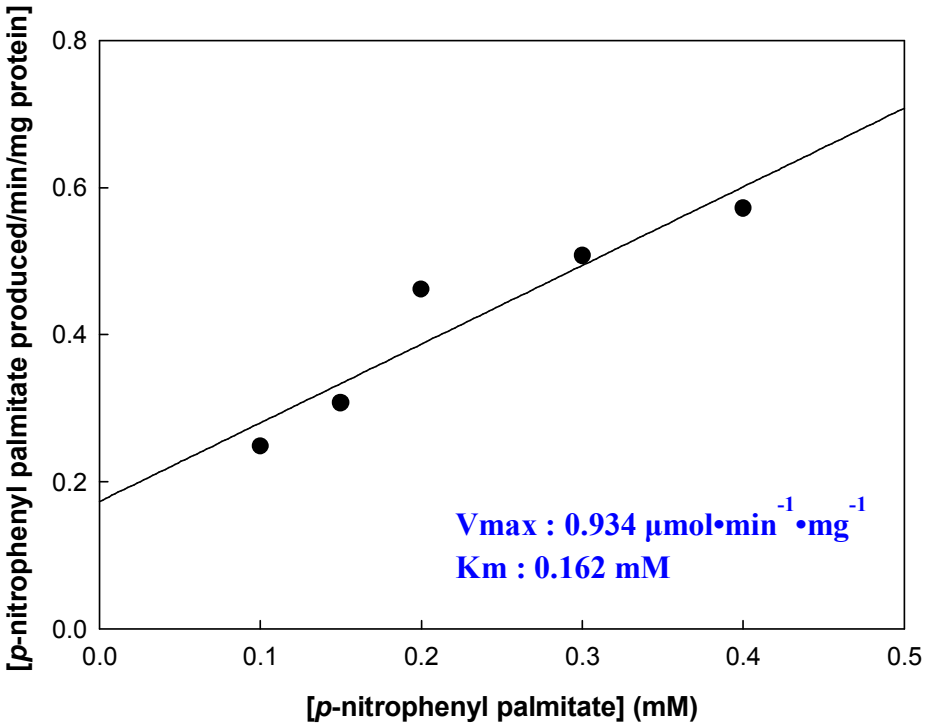

Fig. 13. Hanes-Woolf plot for the determination of kinetic parameters at the *p*-nitrophenol formation catalyzed by the recombinant CML in baculovirus system.

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kirilovaYoo, H.-Y., Simkhada, J. R., Cho, S. S., Park, D. H., Kim, S. W., Seong, C. N., & Yoo, J. C. (2011). A novel alkaline lipase from *Ralstonia* with potential application in biodiesel production. *Bioresource Technology, 102*(10), 6104-6111.

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국문초록

Lipase(triacylglycerol acylhydrolase, EC 3.1.1.3)는 triglyceride의 ester 결합의 가수분해 및 합성을 촉매하는 효소로서 생물 산업에서 주목 받고 있는 효소 중 하나이다.

선행연구로부터 동충하초 유래 lipase를 분리 및 정제하여 특성을 규명한 결과 triacylglycerol의 *sn*-1 위치에 특이적으로 작용하는 위치특이적 효소임을 규명하였다. 자연계에 거의 발견되지 않은 *sn*-1 specific lipase를 산업적으로 적용 시 다양한 생리활성을 지닌 재조합 유지를 고순도로 개발할 수 있을 것이며, 유지를 구성하는 지방산을 규명하는 등 학문적으로 중요한 가치를 지닌다고 할 수 있다. 그러나 분리·정제된 lipase의 수율이 2% 대로 매우 낮아 본 연구에서는 cDNA 클로닝을 통한 *E. coli* 및 베큘로바이러스 발현 시스템을 이용하여 동충하초 유래 lipase를 발현하고자 하였다.

정제된 효소의 단백질을 ESI-MS/MS를 통하여 동정한 결과, 동충하초 유래 extracellular lipase의 genome sequence와 높은 상동성을 가지는 것으로 확인하였으며, 이를 통해 동충하초 내 lipase gene을 예상하였다. Total RNA추출을 통해 cDNA를 합성을 진행하였으며, T-vector에 subcloning을 진행함으로써 동충하초 유래 extracellular lipase의 cDNA 서열을 확보할 수 있었다. PCR 결과 1,743bp 부근에서 lipase의 유전자가 증폭됨을 확인할 수 있었다. 증폭된 lipase 유전자를 3가지 벡터 (1) pET-29b(+) (2) pET-26b(+) (3) pColdⅢ 에 삽입하였으며, 단백질 발현을 위해 다음과 같은 4종의 Strains (1) BL21(DE3) (2) C41(DE3) (3) C43(DE3) (4) Origami(DE3)을 사용하였다. 그 결과 단백질 발현이 된 *E.*

coli system의 경우 상당수가 insoluble 형태로 존재하였으며, Western blot을 통해 soluble 형태로 일부 발현이 되는 것을 확인하였다. 그러나 활성 측정 결과, 활성을 나타내지 않는 것으로 판명되었다. 동충하초 유래 lipase의 경우, eukaryotic lipase로서 *E. coli* 발현시스템에서 활성형으로 발현되기에는 많은 제약점을 가지고 있는 것으로 판단된다.

따라서 eukaryotic lipase의 발현에 보다 적합한 진핵 생물 발현계인 베큘로바이러스 발현시스템을 이용하여 단백질을 발현시키고자 하였다. 전이벡터로는 pDualBac을 사용하였으며 동충하초 lipase유전자를 삽입함으로써 재조합 전이벡터를 구축하였다. 재조합 바이러스를 Sf9 세포에 접종시킨 후 발현 양상을 확인하였으며, *p*-NPP assay를 통해 wild type의 AcMNPV ((-)control)와 비교 시 활성을 갖는 것으로 확인하였다. 발현된 단백질의 특성을 분석하고자 kinetic parameter를 측정하였으며, 그 결과 V_{max}, K_m, k_{cat} 값은 각각 0.934 μmol·min^{-1}·mg^{-1}, 0.162 mM, 0.79 min^{-1} 로 나타났다. 촉매효율상수 (k_{cat}/K_m)는 4.87 min^{-1}·mM^{-1}로 확인되었다.

주요어 : *sn*-1 specific triacylglycerol lipase; 동충하초; 단백질 동정; cDNA 클로닝; 단백질 발현 시스템; *Escherichia coli*; 베큘로바이러스; bApGOZA; 곤충세포

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