A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Comparative study on the expression and characteristics of lipase genes from entomopathogenic fungi

곤충병원성 곰팡이 유래 지방질 효소 유전자의 발현과 특성 구명에 대한 비교 연구

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Major in Entomology

Department of Agricultural Biotechnology

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UNDER THE DIRECTION OF ADVISER YEON HO JE
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ABSTRACT

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. It is widely distributed throughout the living world, including animals, plants, and microorganisms. Among many industrially applied enzymes, the role of lipase that converts fats into fatty acids and monoglycerides is very important, and it is one of the enzymes attracting attention in the rapidly growing biological industry. Lipase derived from an

entomopathogenic fungi, *Cordyceps militaris*, was found to act specifically at position 1(3) of triacylglycerol. In this study, characteristics of lipases isolated from entomopathogenic fungi were investigated and conditions for mass expression using the baculovirus expression system were established.

Among the entomopathogenic fungi isolated in Korea, *Beauveria bassiana* JEF-351 strain, which showed the highest enzyme activity, was selected. Lipase genes of the selected strain (BBL351) and *Cordyceps militaris* (CML), which is known to have stereospecificity, were introduced into the genome of *Autographa californica* nucleopolyhedrovirus (AcMNPV), respectively, to express corresponding genes using the baculovirus expression system. Recombinant lipases were expressed as non-secreted protein and secreted protein, respectively. In both BBL351 and CML, their enzyme activity was higher when they expressed as secreted protein form, demonstrating that post-translational modification such as glycosylation is crucial for their activity. In addition, the enzyme activity of BBL351 was higher than that of the CML, suggesting that the lipase derived from the *B. bassiana* JEF-351 strain could be useful as biocatalyst in the biotechnological applications.

Key words: Lipase, entomopathogenic fungi, *Beauveria bassiana*, baculovirus expression system, secreted protein

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INTRODUCTION

Today, of the approximately 4,000 known enzymes, 200 enzymes are used commercially. Until the 1960s, total annual sales of enzymes were only several million dollars, but since then, the market has grown spectacularly (D. Wilke, 1999; T. Godfrey, S. West, 1996). An increasing number of enzymes are produced affordably as a result of a better understanding of production bio-chemistry, bio-process technologies, and protein engineering. Depending on environmental factors such as pH and temperature, one particular enzyme can catalyze different transformations, and as a result, the commercial use of enzymes has continued to increase (R. Sharma, Y. Chisti, U.C. Banerjee, 2001). Among many industrially applied enzymes, the lipase are ubiquitous enzymes, which are found in animals, plants, fungi and bacteria, and are of considerable physiological significance and industrial potential (R. Aravindan*, P. Anbumathi and T. Viruthagiri, 2007).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases of considerable physiological significance and industrial potential, which catalyze the hydrolysis and the synthesis of esters bonds formed from glycerol and long-chain fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (M. Ueda, S. Takahashi, M. Washida, S. Shiraga, A. Tanaka, 2002). A number of available industrial lipases has increased considerably since the 1980s in response to an increasing demands for these biocatalysts (U.T. Bornscheuer, C. Bessler, R. Srivinas, S.H. Krishna, 2002). Lipases that catalyze the hydrolysis of triacylglycerols have been widely applied for lipid modifications,

synthesis of bioactive esters, and production of food additatives and biodisel. Lipases have significant selectivity for the sn position of glycerol backbone (i.e., regioselectivity) (M. Kapoor, M. Nath Gupta, 2012). 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) (Jung Ha Park, Kyung-Min Park, Yoonjee Chang, Jun-Young Park, Jaejoon Han, Pahn-Shick Chang, 2018).

Entomopathogenic fungi could be useful natural sources for novel lipases because they produce a variety of lipases during the process of host invasion and proliferation. Recently, lipase with distinctive positional specificity has been reported from entomopathogenic fungi, *Cordyceps militaris* (Jung Ha Park, Kyung-Min Park, Yoonjung Yoo, Hyunjong Yu, Chang Joo Lee, Ho-Sup Jung, Keesung Kim, Pahn-Shick Chang, 2018). In this study, it was aimed to clone and mass express the sn-1(3) regioselective lipase from entomopathogenic fungi.

LITERATURE REVIEW

1. Lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of lipids in biological systems. This is popular and efficient catalysts in industrial potential, which detergent industry, food industry and fine chemical preparations (Schmid and Verger, 1998). Lipase is serine hydrolases of considerable physiological significance and industrial potential, which catalyze the hydrolysis and the synthesis of esters bonds formed from glycerol and long-chain fatty acids. For decades, many studies related to enzymatic characteristics, immobilization of lipase, and optimization for reaction conditions have been extensively investigated to enhance the yield of the lipase catalyzed bioconversion (Lee, Park, Choi, Shim, & Chang, 2013; Moniruzzaman, Hayashi, Talukder, & Kawanishi, 2007; Ognjanovic, Bezbradica, & Knezevic-Jugovic, 2009).

It's structure has been resolved in 1994 (Uppenberg et al., 1994). Lipase 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 (M. Kapoor, M.N. Gupta, 2012). One type of lipase act 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) (Xuebing Xu, 2000).

2. Entomopathogenic fungi

Entomopathogenic fungi (EPFs) control the density of host insects in nature by fungal disease for using ecological nutrient (Brownbridge, Humber, Parker, & Skinner, 1993; Roy et al., 2010). Fungi that cause disease in insects are all recognized as entomopathogenic fungi of the teleomorph or the anamorph and accepted that there is an interrelationship (Rehener & Buckley, 2005; Sung et al., 2007; Sung, Poinar Jr, & Spatafora, 2008; Frenando E Vega et al., 2012). Entomopathogenic fungi are known to more than 700 species of 100 genera by continuous separation and reporting (Spatafora, Sung, & Kepler, 2010; Sung et al., 2007). Entomopathogenic fungi have been widely studied, they are easy to handle, multiply, and formulate.

Entomopathogenic fungi can infect insects not only through the gut, but also through spiracles and particularly through the surface of the integument (Wang & Leger, 2007). The spores on the epidemics, they secrete various enzymes, penetrate the cuticle and reach in hemocoel. At this time, over 100 different genes are found to be used to penetrate each cuticle layer (Fang, Azimzadeh, & Leger, 2012). The fungus that reaches the hemocoel is rapidly proliferated using abundant nutrients in the hemolymph, it eventually obliterates the host. They will continue to live with other insects (Frenando E Vega et al., 2012).

Entomopathogenic fungi are increasingly recognized as important natural resources that can be used in many other studies by utilizing the various enzymes they secrete. (de Souza Santos et al., 2013; Ownley, Gwinn, & Vega, 2010; Schmidt et al., 2003; Sowjanya Sree, Padmaja, & Murthy, 2008; Fernando E Vega et al., 2009; Zhu, Halpern, & Jones, 1998).

3. Baculovirus expression vector system

The Baculoviridae are a family of occluded DNA viruses pathogenic pre-dominantly for holometabolous insects. Baculovirus is enveloped viruses having a circular doubled-stranded DNA genome of between 88 and 200 kb. Baculovirus is generally classified into the following two groups, nucleopolyhedrovirus (NPV) and granulovirus (GV). The NPVs also classified into multiple NPV (MNPVs) and single NPV (SNPV) with the number of nucleocapsid enveloped (O'Reilly, 1992).

Baculovirses have recently been used as expression vectors for medical research and biotechnology of protein (Jasny, B. R. 1988). This has led to the widespread use of baculoviruses in many laboratories and an increased interests in their biology. Baculovirus has been used in several fields including general biological overview (Granados, R. R. 1980), molecular characteristics of Baculoviridae and practical applications as pesticides (Granados, R. R., Federici, B. A. 1986), molecular biology (Doerfler, W., Bohm, P. 1986), and application as expression vectors (Luckow, V. A., Summers, M. D. 1988; Maeda, S. 1989).

Baculoviruses have been applied as expression systems in the mid-1980s (Smith, Summers, & Fraser, 1983a). Since then, the baculovirus expression vector system (BEVS) has been used to express thousands of recombinant targets genes. BEVS has proven highly suitable for recombinant protein production due to its high level of gene expression driven by the polyhedrin promoter in virus-infected insect cells. The majority of vectors used are based on the replacement of the polyhedrin gene with the foreign gene of interest. However, due to the large size of the baculovirus genome, it is difficult to insert genes directly into the virus genome using molecular cloning techniques. There is commercial system that

achieves direct transfer of the foreign gene into the virus genome, albeit via initial cloning into a Gateway® entry vector (BaculoDirect™, Thermo Fisher Scientific Inc.). Most baculovirus expression systems involve the use of an intermediate plasmid vector into which the foreign gene is cloned (King, L. A., Hitchman, R., & Possee, R. D. 2007).

METERIAL AND METHODS

1. Entomopathogenic fungi (EPF)

Seven strains of entomopathogenic fungi belong to *Beauveria bassiana* and *Isaria fumosorosea* were kindly provided from Prof. Jae Su Kim (Chonbuk National University). One strain belongs to *Cordyceps militaris* were obtained from the Korean Agricultural Culture Collection (KACC). These fungal strains were primary cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) and potato dextrose broth (PDB; Difco, Detroit, MI, USA) for 7 days at 25°C in darkness. Entomopathogenic fungi used in this study was listed in Table 1.

2. Preparation of lipase from culture broth of entomopathogenic fungi

For liquid culture, suspension of conidia (500 μl) from a primary culture was inoculated into 200 ml of PDB medium in 1 L flask and cultured at 25°C on a rotatory shaker at 150 rpm for 6 days. The mycelia of *C. militaris* were transferred to the seed culture medium by punching out about 6 mm diameter agar disc from culture grown on PDA plates; seed culture medium is PDB. The seed culture was incubated in a 1 L Erlenmeyer flask at 25 °C on a rotary shaker incubator at 150 rpm for 6 days. Only the supernatant was taken and concentrated using Amicon® Ultra-15 Centrifugal Filter Unit (Merck, Germany) (Fig. 1).

Table 1. List of entomopathogenic fungi strains used in this study

Fungal species	Isolate No.
Isaria fumosorosea	JEF-248
	JEF-250
Beauveria bassiana	JEF-344
	JEF-351
	JEF-354
	JEF-363
Cordyceps militaris	KACC No. 43996

Fig. 1. Liquid and solid culture conditions of selected entomopathogenic fungi.

3. Bacterial strains and transformation

Escherichia coli TOP10 strain was used for transformation and amplification of the plasmid DNA. Transformation was carried out by bacmid using chemically treated competent cells according to the manufacturer's instruction. Competent cells (200 μl) were mixed with 1 μg bacmid DNA after 30 min incubation in ice, is placed at 42°C for 45 seconds (heat shock) and then placed back in ice. Luria–Bertani (LB) media, which contains 10 g L^{-1} NaCl, 10 g L^{-1} tryptone, and 5 g L^{-1} yeast extract is added and the transformed cells are incubated at 37°C for 1 hour with agitation. For isolating colonies, transformed *E. coli* is plated on selective medium.

4. Insect cells and baculoviruses

Spodoptera frugiferda cell line Sf9 was continuously maintained in the TC-100 medium (WelGene, Korea) that was supplemented with 10% FBS (WelGene, Korea). High-Five cells originated from *Trichoplusia ni* was maintained in Express Five SFM (Invitrogen, Waltham, Massachusetts, USA) with L-glutamine, respectively. All insect cell lines were incubated at 27 °C and sub-cultured every 3–4 days. Wild-type AcMNPV and all of the recombinant baculoviruses used in this study were propagated in Sf9 cells maintained in TC-100 medium that was supplemented with 10% FBS.

5. RNA and reverse transcription PCR (RT-PCR)

Total RNAs of entomopathogenic fungi and Sf9 cells infected with recombinant baculoviruses were extracted using the QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA)

according to the manufacturer's instructions. After the RNA samples were treated with RNase-free DNase I (Takara, Japan), RT-PCR was carried out using ReverTra Ace-α-™ Kit (Toyobo, Japan) according to the manufacturer's instructions. To amplify the BBL351 gene, oligonucleotides BBL-RealTime-Fw (5'-ATGAGATTCACCGTTGTGG-3') and BBL-RealTime-Re (5'-TCAAAAATAGAGCGCTTTCGTA-3') were used. The amplified lipase genes were cloned into the pGEM T-Easy plasmid vector (Promega, Madison, WI, USA) (Fig. 2).

6. DNA synthesis

Lipase gene of *I. fumosorosea* was artificially synthesized by GenScript (Piscataway, New Jersey, USA) and cloned into pUC57 vector (Fig. 3).

7. Construction of baculovirus donor vectors

To construct a baculovirus donor vectors expressing non-secretory form of lipase genes without signal peptides under the control of the polyhedrin promoter, approximately 1.7 kb of the lipase genes were amplified using specific primers (Table 2). The PCR-amplified lipase gene fragments were inserted into pDualBac donor vector digested with *Bam*HI using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswitch, MA, USA) (Fig. 4). The pET29-CML used as template for amplification of CML gene was kindly provided from Prof. Pahn-Shick Chang (Center for Food and Bioconvergence, Seoul National University).

In order to fuse His-tag at N-terminus or C-terminus of the non-secretory form of

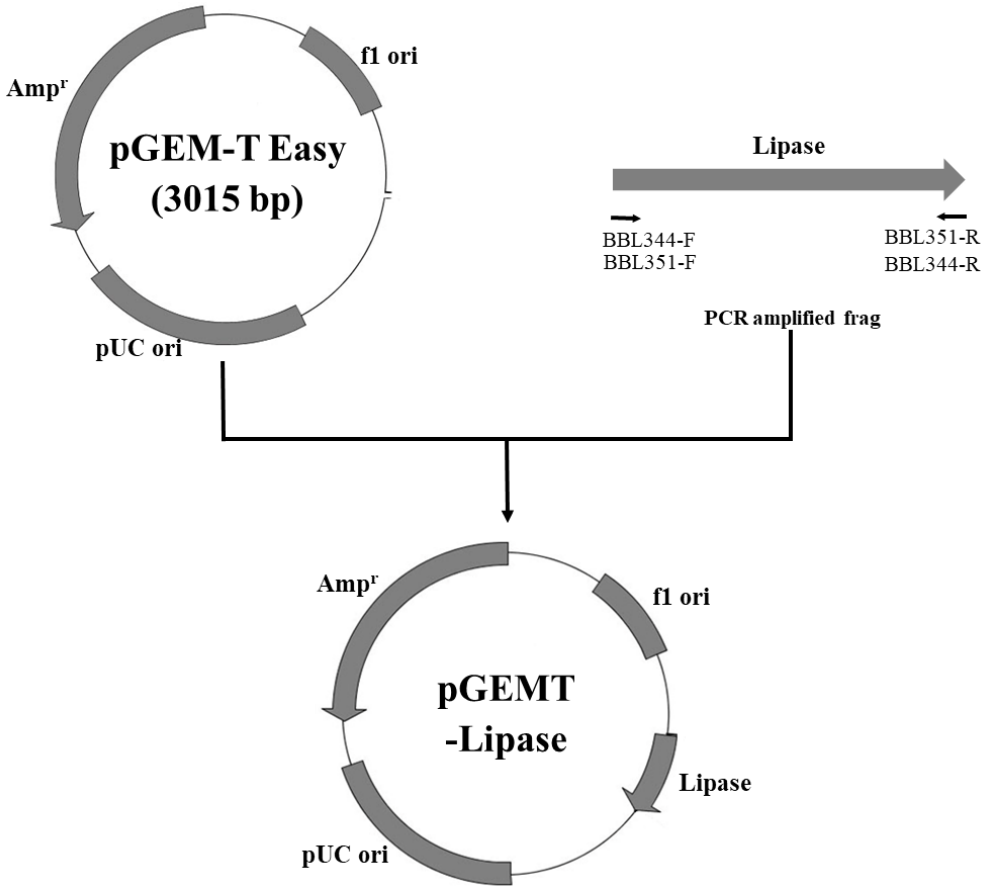


Fig. 2. Construction map of the vectors harboring BBL344 and BBL351 genes. The PCR-amplified (1.72 kb) and BBL351 (1.73 kb) lipase genes were inserted into pGEM-T Easy to obtain pGEMT-BBL344 and pGEMT-BBL351, respectively. Solid arrows indicate primer position used in PCR amplification.

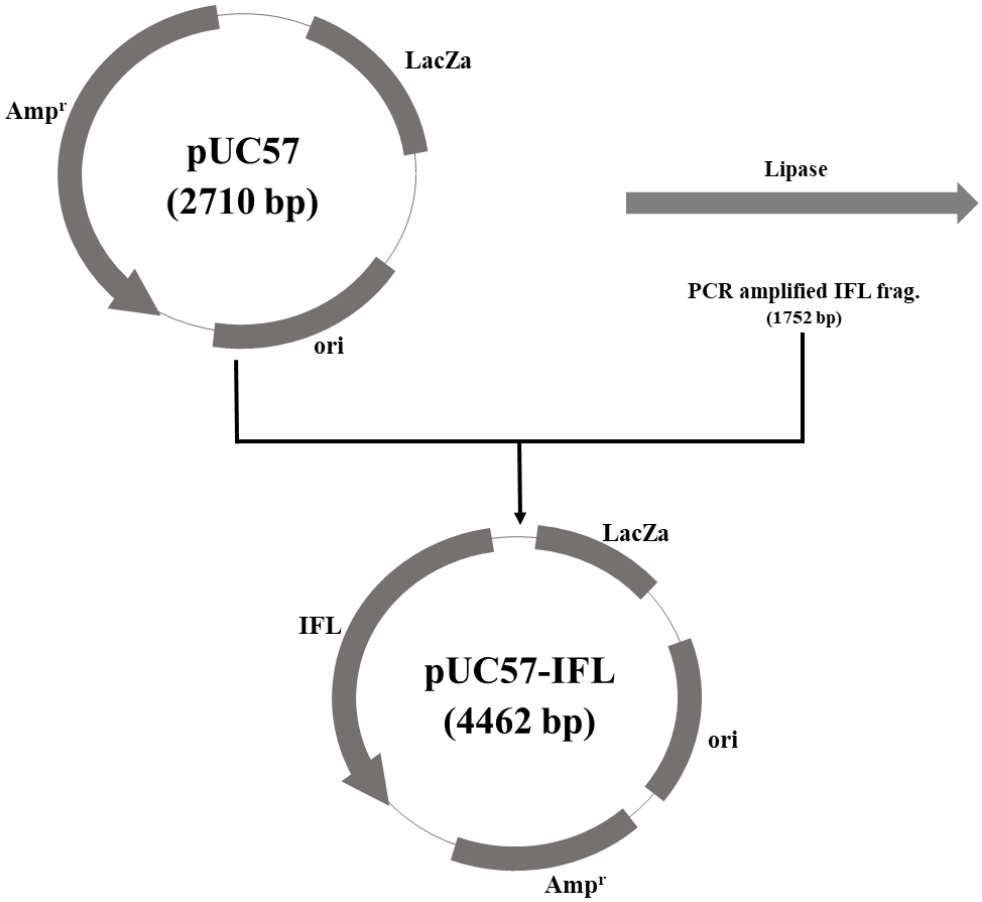


Fig. 3. Construction map of the vector harboring IFL gene. The 1.75 kb of PCR-amplified IFL gene was inserted into pUC57 to obtain pUC57-IFL. Solid arrows indicate primer position used in PCR amplification.

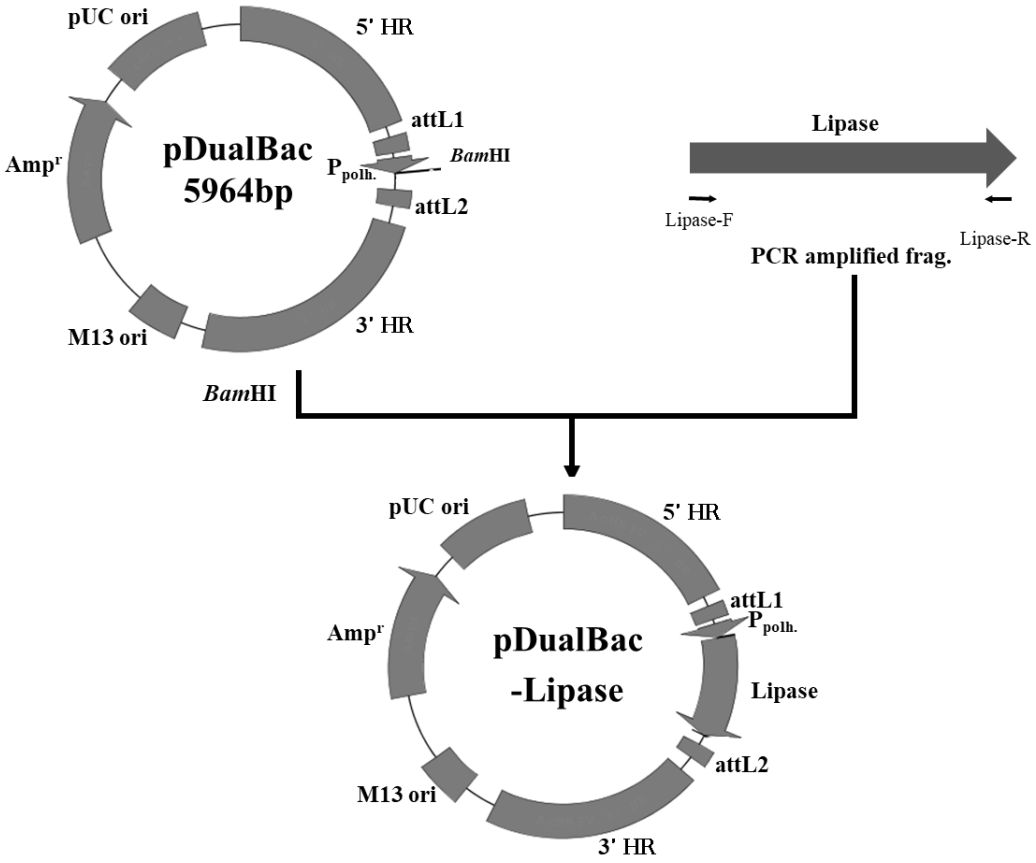


Fig. 4. Construction map of baculovirus donor vectors expressing non-secretory form of BBL344 and BBL351 genes under the control of polyhedron promoter. The PCR-amplified BBL344 and BBL351 lipase genes without signal peptides were inserted into pDualBac to obtain pDualBac-BBL344 and pDualBac-BBL351, respectively. Solid arrows indicate primer position used in PCR amplification.

Table 2. Primers used for construction of baculovirus donor vectors containing non-secretory lipase genes

Amplified target	Primer name	Primer sequence
CML	CML-F	5'- AAAAAAACCTATAAATACGATGGCGCCCCATTGCCCG -3'
	CML-R	5'- TAGATTCGAACTCGAGGCCTGCAGG TTAGAAGTAGAGTACCTCCGTATTCTC -3'
BBL344	BBL344-F	5'-AAAAAAACCTATAAATACGATGGCACCTCAAGCTACGGGAG -3'
	BBL344-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
BBL351	BBL351-F	5'- AAAAAAACCTATAAATACGATGGCACCTCAAGCTCAAGCTA-3'
	BBL351-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
IFL	IFL-F	5'- AAAAAAACCTATAAATACGATGGCACCCCGTTCCACGCCA -3'
	IFL-R	5'- CTCGAGGCCTGCAGGCTAGAACGCAAAGTCCCTCGT-3'

Table 3. Primers used for construction of baculovirus donor vectors containing His-tagged form of lipase genes from entomopathogenic fungi

Amplified target	Primer name	Primer sequence
HisCML	HisCML-F1	5'- ATGCATCATCACCATCACCACGCGCCCCATTGCCCGGGACAA-3'
	HisCML-F2	5'- AAAAAAACCTATAAATACGATGCATCATCACCATCACCACGCG-3'
	CML-R	5'- TAGATTCGAACTCGAGGCCTGCAGGT TAGAAGTAGAGTACCTCCGTATTCTC -3'
HisBBL344	HisBBL344-F1	5'- ATGCATCATCACCATCACCACGCACCTCAAGCTACGGGAGAT-3'
	HisBBL344-F2	5'- AAAAAAACCTATAAATACGATGCATCATCACCATCACCACGCAC-3'
	BBL344-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
HisBBL351	HisBBL351-F1	5'- ATGCATCATCACCATCACCACGCACCTCAAGCTCAAGCTACT-3'
	HisBBL351-F2	5'- AAAAAAACCTATAAATACGATGCATCATCACCATCACCACGCA-3'
	BBL351-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
HisIFL	HisIFL-F1	5'- ATGCATCATCACCATCACCACGC ACCCCGTTCCACGCCACCG-3'
	HisIFL-F2	5'- AAAAAAACCTATAAATACGATGCA TCATCACCATCACCACGCACCC-3'
	IFL-R	5'- CTCGAGGCCTGCAGGCTAGAACGCAAAGTCCCTCGT-3'
CMLHis	CML-F	5'- AAAAAAACCTATAAATACGATGGCGCCCCATTGCCCG -3'
	HisCML-R1	5'- TTAGTGGTGATGGTGATGATGGAA GTAGAGTACCTCCGTATTCTCC-3'
	HisCML-R2	5'- AACTCGAGGCCTGCAGGTTAGTGGTGATGGTGATGATGGAAG-3'

BBL344His	BBL344-F	5’-AAAAAAACCTATAAATACGATGGCACCTCAAGCTACGGGAG -3’
	HisBBL344-R1	5’- TCAGTGGTGATGGTGATGATGAA AATAGAGCGCTTTCGTATTGTTC-3’
	HisBBL344-R2	5’- AACTCGAGGCCTGCAGGTCAGTGGTGATGGTGATGATGAAAA-3’
BBL351His	BBL351-F	5’- AAAAAAACCTATAAATACGATGGCACCTCAAGCTCAAGCTA-3’
	HisBBL351-R1	5’- TCAGTGGTGATGGTGATGATGAA AATAGAGCGCTTTCGTATTGTTC-3’
	HisBBL351-R2	5’- AACTCGAGGCCTGCAGGTCAGTGGTGATGGTGATGATGAAAA-3’
IFLHis	IFL-F	5’- AAAAAAACCTATAAATACGA TGGCACCCCGTTCCACGCCA -3’
	HisIFL-R1	5’- CTAGTGGTGATGGTGATGATGGA ACGCAAAGTCCCTCGTCTTGTCC-3’
	HisIFL-R2	5’- AACTCGAGGCCTGCAGGCTAGTGGTGATGGTGATGATGGAAC-3’

lipase, nucleotide sequences coding 6×histidine were introduced into the lipase genes without signal peptides by sequencial PCR reaction using specific primers (Table 3). The PCR-amplified lipase gene fragments were inserted into pDualBac donor vector digested with *Bam*HI using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswitch, MA, USA) (Fig. 5 and 6).

For the construction of baculovirus transfer vectors expressing secretory form of lipase genes under the control of the polyhedrin promoter, approximately 1.7 kb of the lipase genes with native signal peptides were amplified using specific primers (Table 4). In addition, to replace the native signal peptides with insect-specific melittin signal peptides, nucleotide sequences coding melittin signal peptides were introduced into the lipase genes by sequencial PCR reaction using specific primers (Table 4). The PCR-amplified lipase gene fragments were inserted into pDualBac donor vector digested with *Bam*HI using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, USA) (Fig. 7 and 8).

8. *in vitro* transposition

The transposition reaction was carried out using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Waltham, Massachusetts, USA). In the transposition reaction, 150 ng of donor vector was mixed with 150 ng of bEasyBac bacmid DNA. In total, 2 μl of LR Clonase™ II Enzyme Mix (Invitrogen, Waltham, Massachusetts, USA) was added to the transposition reaction, and the mixture was mixed well by vortexing briefly twice. The reaction was centrifuged briefly and incubated at 25 °C overnight (approximately 12–16 h). A total of 1 μl of proteinase K solution was added to the reaction to terminate

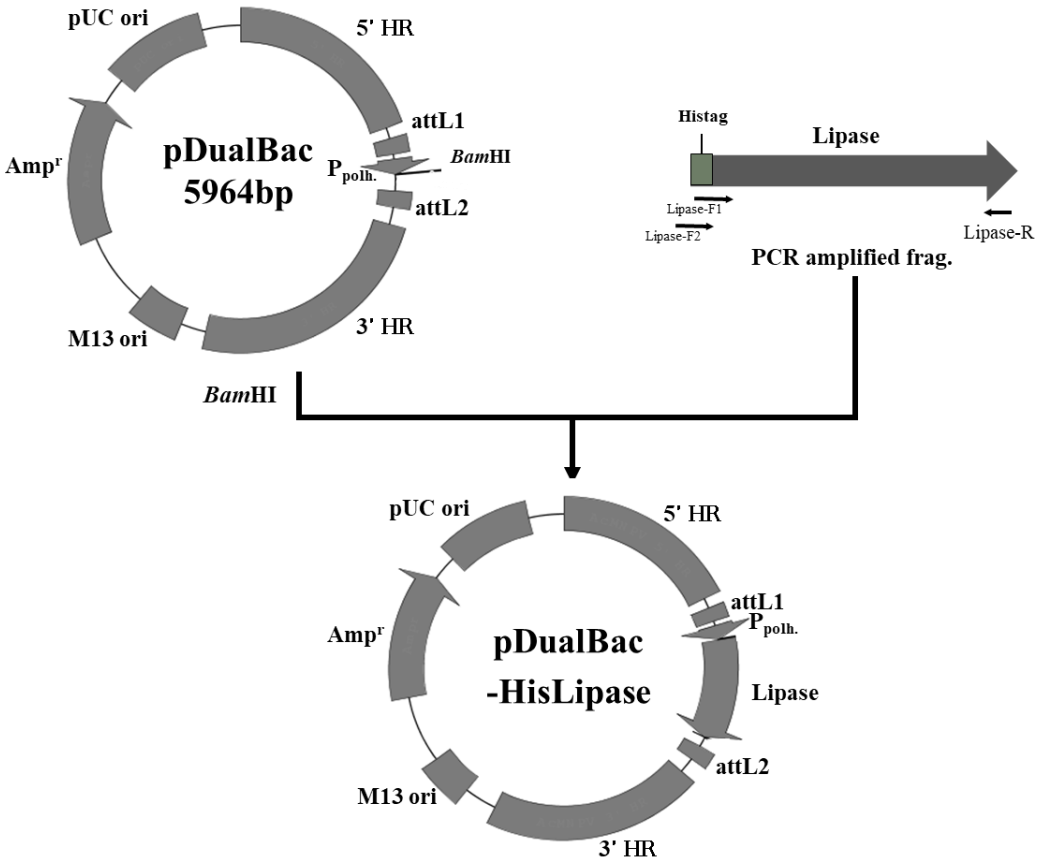


Fig. 5. Construction map of baculovirus donor vectors expressing N-terminally His-tagged lipase genes under the control of polyhedron promoter. The PCR-amplified lipase genes fused with 6×histidine codons at 5' were inserted into pDualBac to obtain pDualBac-HisCML, pDualBac-HisBBL344, pDualBac-HisBBL351, and pDualBac-HisIFL, respectively. Solid arrows indicate primer position used in PCR amplification.

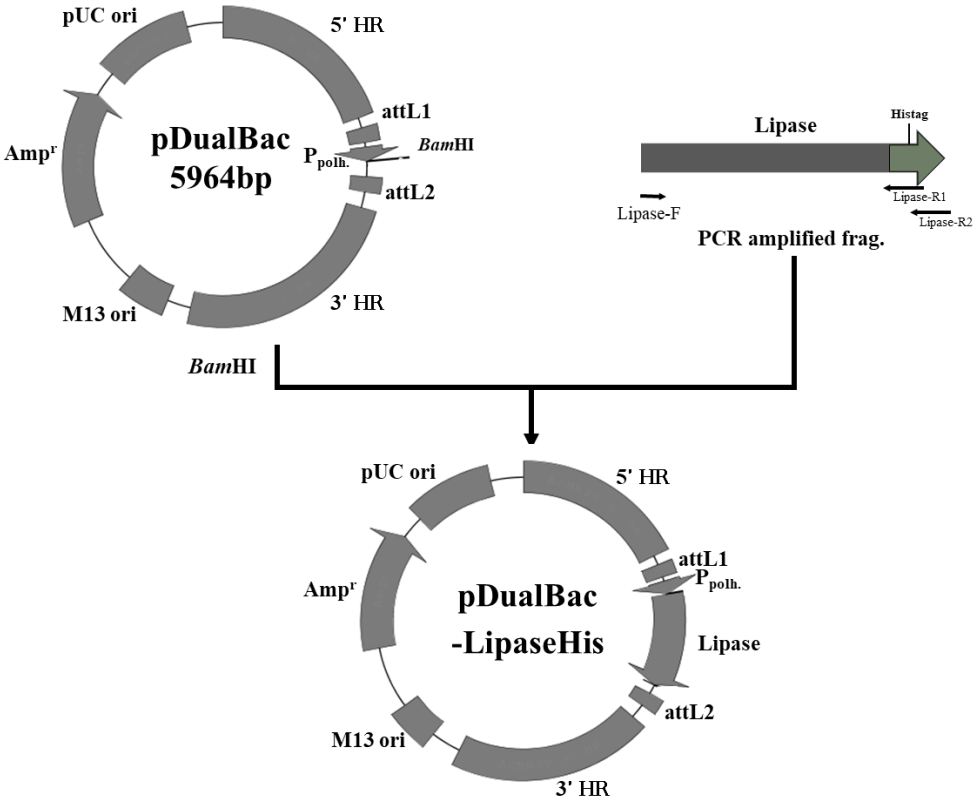


Fig. 6. Construction map of baculovirus donor vectors expressing C-terminally His-tagged lipase genes under the control of polyhedron promoter. The PCR-amplified lipase genes fused with 6×histidine codons at 3’ were inserted into pDualBac to obtain pDualBac-CMLHis, pDualBac-BBL344His, pDualBac-BBL351His, and pDualBac-IFLHis, respectively. Solid arrows indicate primer position used in PCR amplification.

Table 4. Oligonucleotides used for construction of baculovirus donor vectors containing His-tagged form of lipase genes from entomopathogenic fungi

Amplified target	Primer name	Primer sequence
MelCML	MelCML-F1	5'- TACATCTATGCGGCGCCCCATTGCCCG-3'
	MelCML-F2	5'- GGTCGTATACATTTCTTACATCTATGCGGCGCCCCA-3'
	MelCML-F3	5'- TTGCCCTTGTTTTTATGGTCGTA TACATTTCTTACATCTATGCG-3'
	MelCML-F4	5'- ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGT-3'
	MelCML-F5	5'- CCGATTCATTAATGCAGATGAAATTCTTAGTCAACGTTGCCC-3'
	MelCML-R	5'- TAGATTCGAACTCGAGGCCTGCAGGTT AGAAGTAGAGTACCTCCGTATTCTC -3'
MelBBL351	MelBBL351-F1	5'- TACATCTATGCGGCACCTCAAGCTCAAGCTACTG-3'
	MelBBL351-F2	5'- GGTCGTATACATTTCTTACATCTATGCGGCACCTCAAG-3'
	MelBBL351-F3	5'- TTGCCCTTGTTTTTATGGTCGTA TACATTTCTTACATCTATGCG-3'
	MelBBL351-F4	5'- ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGT-3'
	MelBBL351-F5	5'- CCGATTCATTAATGCAGATGAAATTCTTAGTCAACGTTGCCC-3'
	MelBBL351-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
NativeCML	NativeCML-F	5'- TGTAATAAAAAAACCTATAAATACGATG AAATTCTCACTTGTGGCTCTGGC-3'
	NativeCML-R	5'- CTCGAGGCCTGCAGGTCAAAAATAGAGCGCTTTCGTA-3'
NativeBBL351	NativeBBL351-F	5'- TGTAATAAAAAAACCTATAAATACGAT GAGATTCACCGTTGTGGCTCTCGC-3'
	NativeBBL351-R	5'- CTCGAGGCCTGCAGGCTAGAACGCAAAGTCCCTCGT-3'

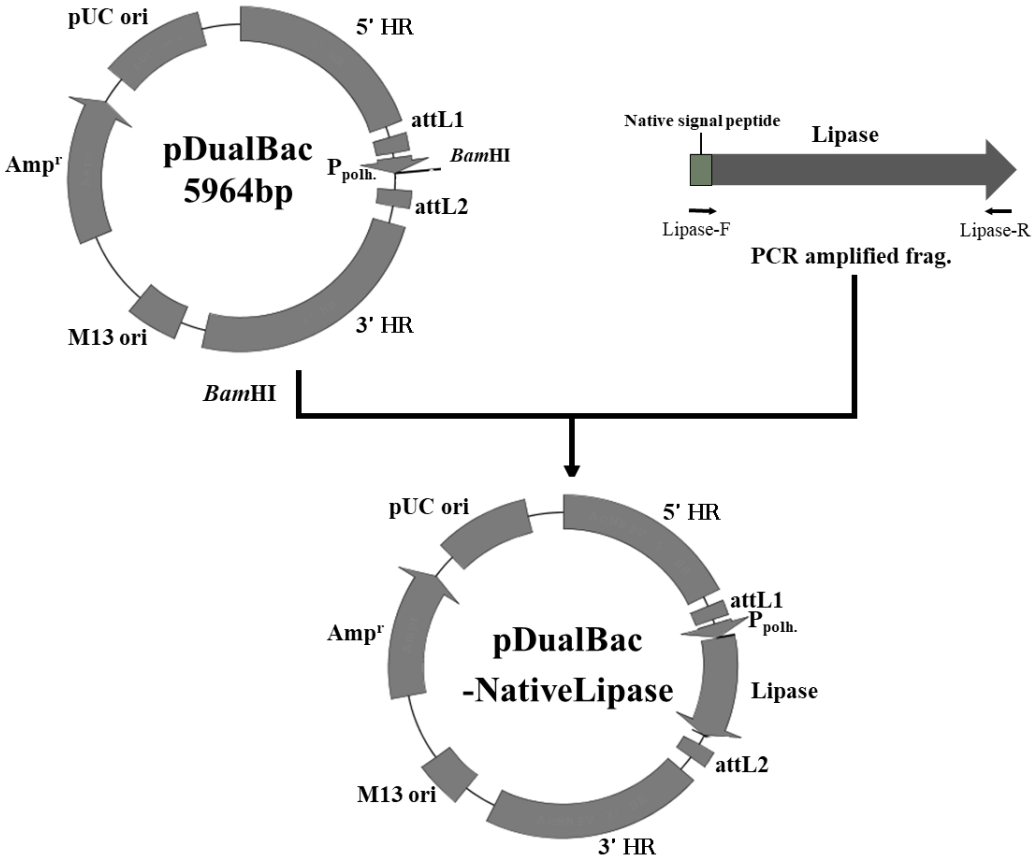


Fig. 7. Construction map of baculovirus donor vectors expressing secretory form of CML and BBL351 genes with native signal peptides under the control of polyhedron promoter. The PCR-amplified CML and BBL351 lipase genes with native signal peptides were inserted into pDualBac to obtain pDualBac-NativeCML and pDualBac-NativeBBL351, respectively. Solid arrows indicate primer position used in PCR amplification.

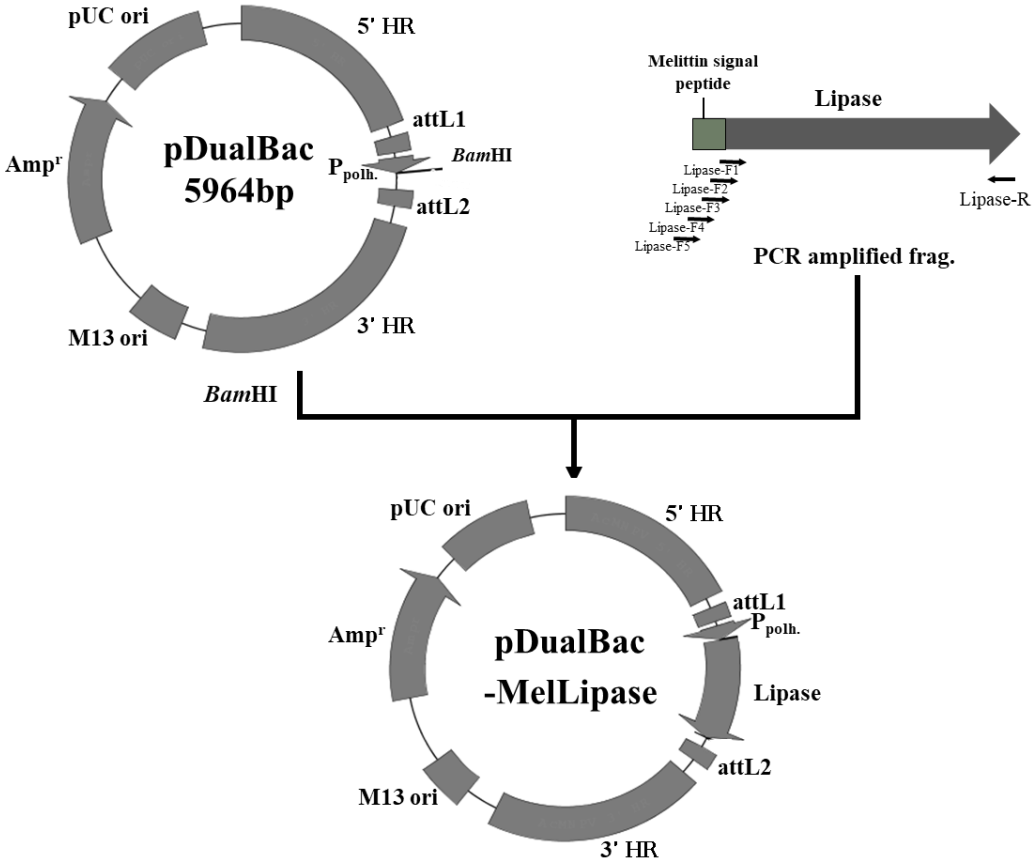


Fig. 8. Construction map of baculovirus donor vectors expressing secretory form of CML and BBL351 genes with melittin signal peptides under the control of polyhedron promoter. The PCR-amplified CML and BBL351 lipase genes with melittin signal peptides were inserted into pDualBac to obtain pDualBac-MelCML and pDualBac-MelBBL351, respectively. Solid arrows indicate primer position used in PCR amplification.

transposition and incubated at 37 °C for 10 min

9. Transfection

Approximately 5×10^4 Sf9 cells per well were seeded in a 24-well tissue culture plate and incubated at 27°C for 30 min to allow the cells attach. In total, 11 µl of LR recombination reaction was added to 100 µl of incomplete TC-100 medium in a polystyrene tube. In another polystyrene tube, 10 µl of Cellfectin™ (Invitrogen, Waltham, Massachusetts, USA) was mixed with 100 µl of incomplete TC-100 medium. The two solutions were gently mixed, and the matrix was incubated at room temperature for 45 min. The attached cells were washed once with 1 ml of incomplete TC-100 medium and refreshed with 0.5 ml of the same medium. The 1/5 or 1/10 fractions of Cellfectin-DNA complexes were added drop-wise per well to the medium covering the cells while the plate was gently swirled. After incubation at 27°C for 5 h, each well was refreshed with 2 ml of TC-100 medium supplemented with 10 % FBS and the transfected cells were incubated at 27°C. At 5 days the transfection supernatant was harvested by centrifugation at 500×g for 5 min and stored at 4°C.

10. Infection of cells with baculoviruses

Each insect cells were seeded into 100-mm diameter tissue culture dishes at a density of 5×10^5 cells per well and inoculated with each recombinant virus at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cell. After incubation at 27°C for 3 days infected cells were harvested by centrifugation at 500×g for 5 min, and stored at 4°C. The recombinant baculoviruses were purified by end-point assay on Sf9 cells according to the

method of O'Reilly *et al*. (1992)

11. Extraction of viral DNA

A total of 250 µl of each sample was added to 250 µl of buffer-saturated phenol/chloroform/isoamyl alcohol (pH 7.5-7.8) and 25 µl of 3M sodium acetate pH 5, vortexed for 1 min, incubated in ice for 15 min, and centrifuged for 10 min at 12,000 rpm at room temperature. The aqueous layer was transferred to a 1.5 ml screw-cap plastic tube containing 250 µl of isopropanol and incubated at -20°C for 5 min. The sample was centrifuged (5 min, 12,000 rpm at 4°C) in order to precipitate DNA. The supernatant was removed, and the pellet resuspended in 500 µl of 70% ethanol, vortexed and centrifuged for 2 min at 13,000 rpm at room temperature. The supernatant was removed again and the remaining ethanol eliminated by a quick centrifuge spin. The pellet was air-dried in a half-open tube for at least 30 min, suspended in 20 µl of TE (10 mM Tris-HCl, 1 mM EDTA pH 8) by vortexing, and stored at -20°C prior to use.

12. Purification of non-secretory recombinant protein

The recombinant protein was purified using HisPur™ Ni-NTA Spin Columns (Thermo Scientific™, Carlsbad, CA, USA) according to the manufacturer's instructions, and target protein was eluted from the beads using PBS with 6 M guanidine·HCl and 250 mM imidazole; pH 7.4. The eluted recombinant lipase protein from peak fractions were analyzed on 12% SDS-PAGE (Laemmli, 1970).

13. Precipitation of secretory recombinant protein using ammonium sulfate

Ammonium sulfate precipitation of the supernatant was performed at 45–80% saturation. Precipitated proteins were collected by centrifugation at 10,000g for 15 min, and the resulting pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.0). The suspension was dialyzed overnight against the same buffer using dialysis tubing (molecular weight cut-off [MWCO] 20 kDa, Thermo Scientific™, Carlsbad, CA, USA). The protein concentration of the enzyme solution was determined by the Bradford protein assay.

14. Lipase activity assay

Lipase Assay of the Crude Enzyme Extracts from Entomopathogenic Fungi. Lipase activity was measured using a spectrophotometric method with slight modifications. The substrate solution was prepared by dissolving 0.0227 g *p*-nitrophenyl palmitate (*p*-NPP) in 30 mL distilled water with 0.3 g Triton X-100 and 0.0051 g sodium dodecyl sulfate (SDS). The substrate solution (95 µl) was added to an equal volume of Tris–HCl buffer (pH 8.0), and the mixture was preincubated in a water bath at 37°C for 10 min. Lipase-catalyzed hydrolysis was initiated by adding 10 µl of the crude enzyme solution to the substrate solution, followed by magnetic stirring at 400 rpm. During enzymatic hydrolysis, the absorbance was measured at 400 nm to analysis 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 min.

RESULTS

1. Lipase genes from entomopathogenic fungi

To find a gene similar to *Cordyceps militaris* lipase, which is known to be stereospecificity, searched NCBI BLAST. A total of 402 lipase genes were obtained and 68 of them were full-length lipase genes (Fig. 9). A phylogenetic analysis was performed with 68 full-length lipase genes. The strains most similar to *Cordyceps militaris* lipase were analyzed as *Beauveria bassiana* and *Isaria fumosorosea* (Fig. 10).

Lipase assay was performed with the fungal cultures of each strain. The lipase activity of *Beauveria bassiana* JEF-351 strain was about 8 times higher than that of *Cordyceps militaris* (Fig. 11).

2. Cloning and sequencing of lipase genes from EPF strains

To obtain the DNA required for cloning, total RNA was extracted from the fungal culture and lipase gene specific RT-PCR was performed. mRNA was obtained from *Beauveria bassiana* JEF-344 strain and *Beauveria bassiana* JEF-351 strain. In *Isaria fumosorosea* strains, mRNA was not detected, so the DNA of the *I. fumosorosea* lipase was obtained through gene synthesis (Fig. 12). *B. bassiana* JEF-344 lipase (BBL344) and *B. bassiana* JEF-351 (BBL351) were cloned into the pGEM T-Easy vector. Cloned 1728 bp fragment and 1734 bp fragment of the *B. bassiana* genome. The internal structure of the vector was confirmed by restriction enzyme digestion and nucleotide sequence analysis (Fig. 13).

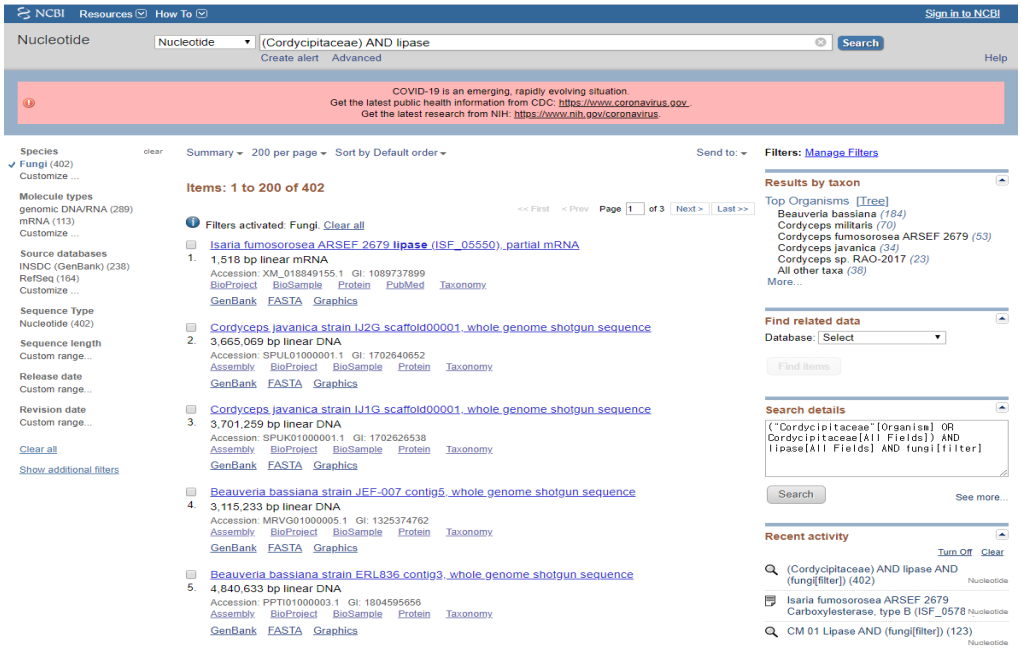


Fig. 9. Detection of lipase genes derived from insect pathogenic fungi in NCBI.

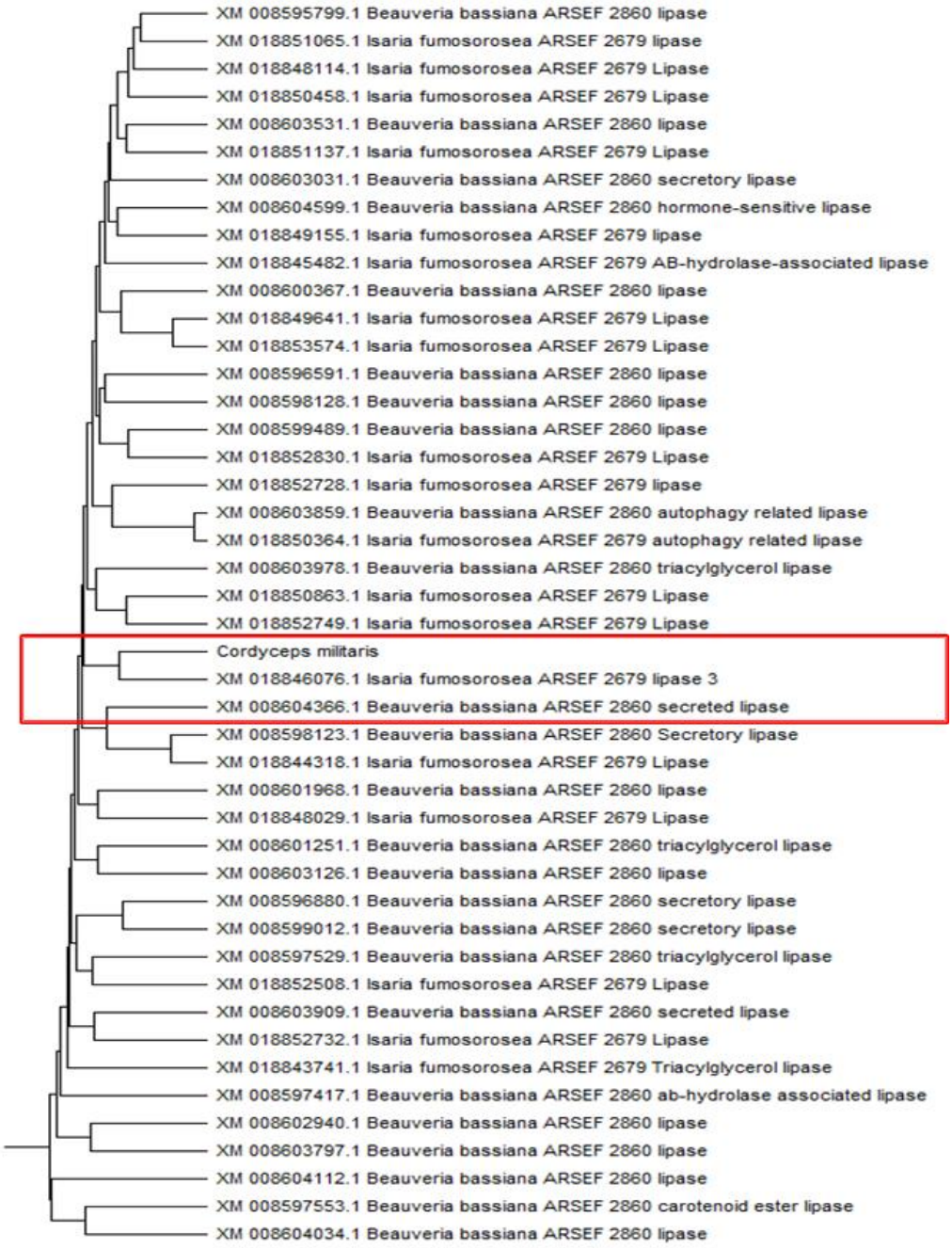


Fig. 10. A phylogenetic tree displaying the relationship of lipase genes.

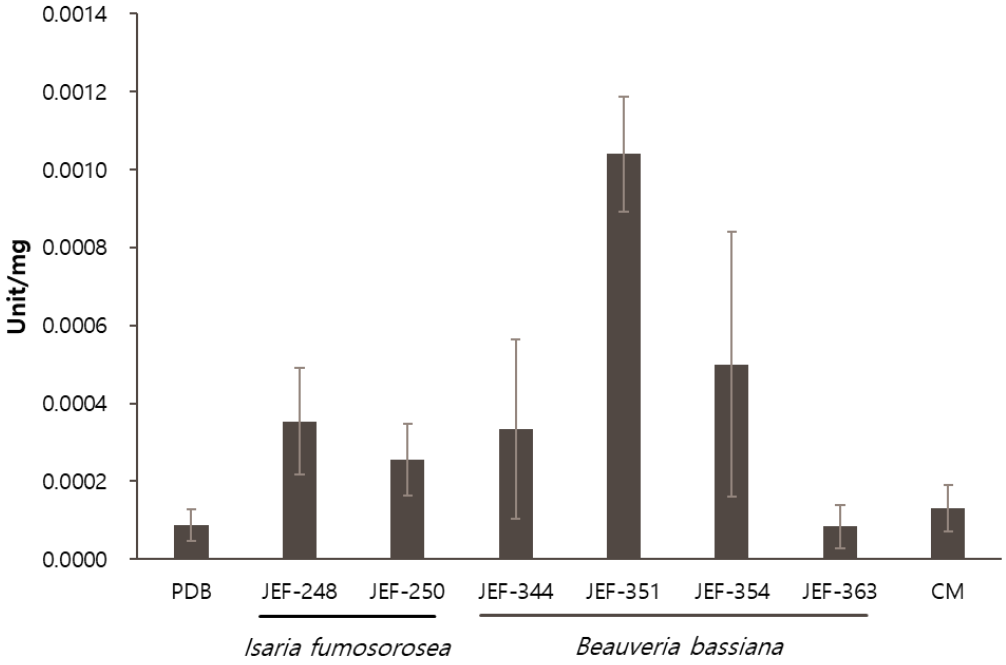


Fig. 11. Lipase assay of EPF strains.

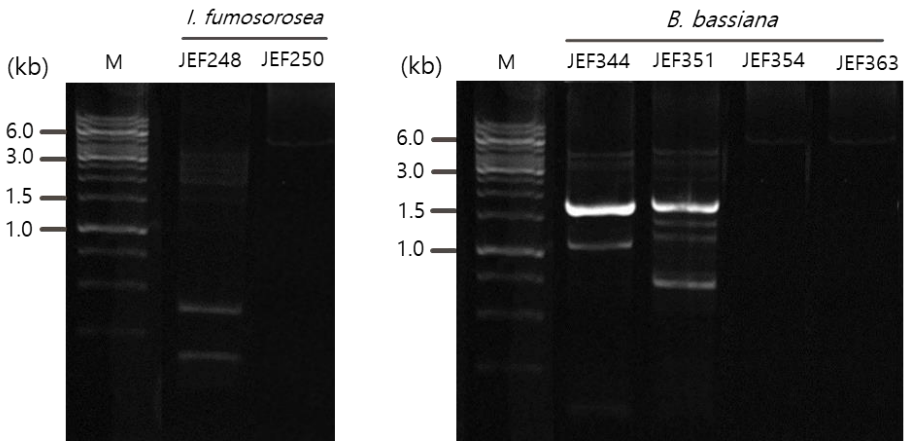


Fig. 12. RT-PCR of lipase. Lane: M, 1 Kb DNA Ladder.

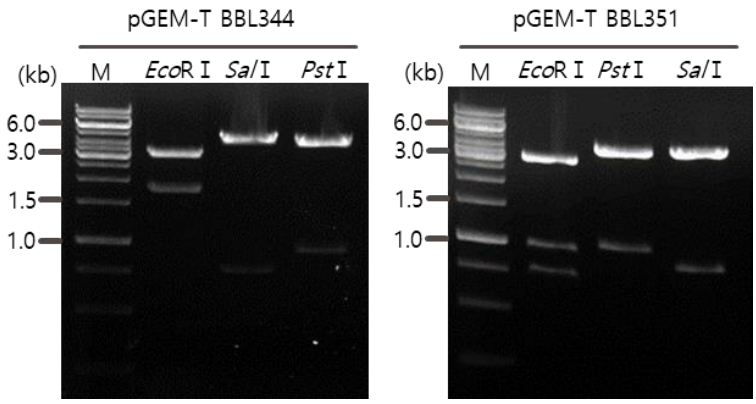


Fig. 13. Confirmation of the internal structure of the transfer vector, pGEM-T BBL344 and pGEM-T BBL351 by restriction endonuclease digestion pattern. Lane: M, 1 Kb DNA Ladder.

Amino acid sequences were compared with the obtained four strains, BBL344 and BBL351, *Isaria fumosorosea*, lipase (IFL) and *Cordyceps militaris* lipase (CML) (Fig. 14). The four strains had a signal peptide in common and a domain called the α/β hydrolase superfamily.

3. Expression of lipase genes in the baculovirus expression system

3.1 Construction of the recombinant bacmid bEasyBac

To generate a recombinant baculovirus, the transfer vector pDualBac-lipase were constructed and confirmed by restriction enzyme digestion and nucleotide sequence analysis (Fig. 15). In this transfer vector, the lipase genes were fused under the control of the polyhedrin promoter. The recombinant baculovirus were generated by the co-transfection of bEasyBac DNA and pDualBac-lipase in Sf9cells. The Sf9 cells was infected with recombinant baculovirus and observed at 3 days (Fig. 16).

RT-PCR (Fig, 17), viral PCR (Fig. 18), and SDS-PAGE (Fig. 19) present in the infected cell lysate were performed to confirm recombinant lipase gene expression. The resulting approximately 1.7 kb of PCR amplified recombinant lipase genes and approximately 68-kDa recombinant protein are consistent with the expected size. I was able to collect the non-secretory soluble protein from the pellet of cell and secretory soluble protein from the supernatant of cell.

3.2 Purification of the non-secretory recombinant baculoviruses

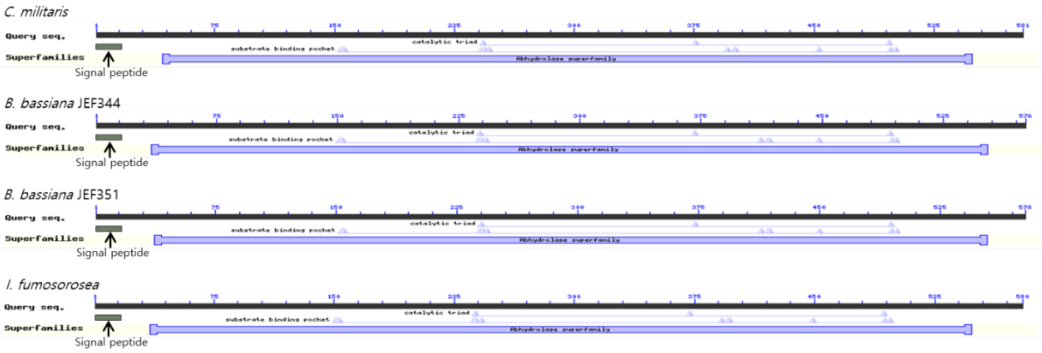
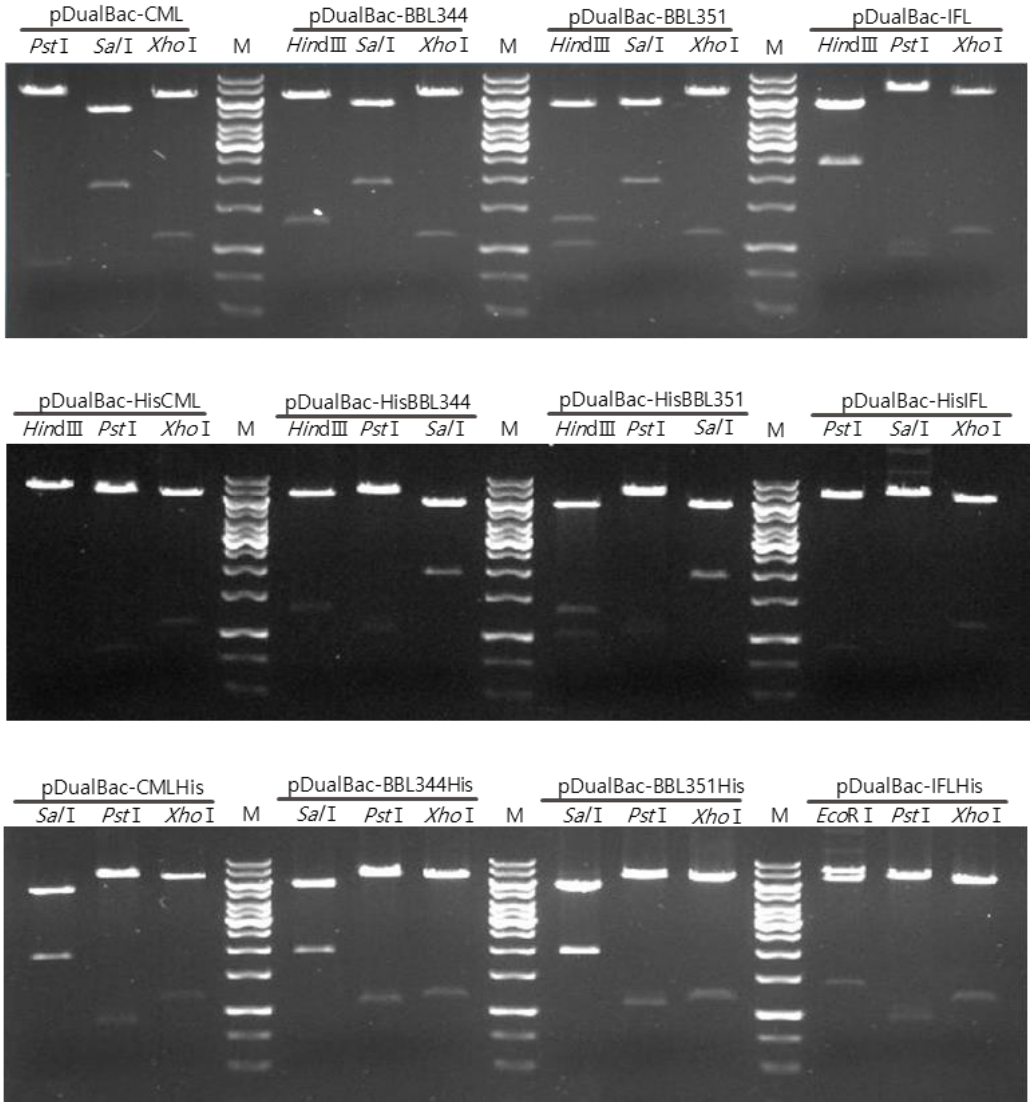


Fig. 14. Amino acid sequence analysis of lipase from EPF strains.

(A)


pDualBac-CML
PstⅠ SalⅠ XhoⅠ M
pDualBac-BBL344
HindⅢ SalⅠ XhoⅠ M
pDualBac-BBL351
HindⅢ SalⅠ XhoⅠ M
pDualBac-IFL
HindⅢ PstⅠ XhoⅠ
pDualBac-HisCML
HindⅢ PstⅠ XhoⅠ M
pDualBac-HisBBL344
HindⅢ PstⅠ SalⅠ M
pDualBac-HisBBL351
HindⅢ PstⅠ SalⅠ M
pDualBac-HisIFL
PstⅠ SalⅠ XhoⅠ
pDualBac-CMLHis
SalⅠ PstⅠ XhoⅠ M
pDualBac-BBL344His
SalⅠ PstⅠ XhoⅠ M
pDualBac-BBL351His
SalⅠ PstⅠ XhoⅠ M
pDualBac-IFLHis
EcoRⅠ PstⅠ XhoⅠ

(B)

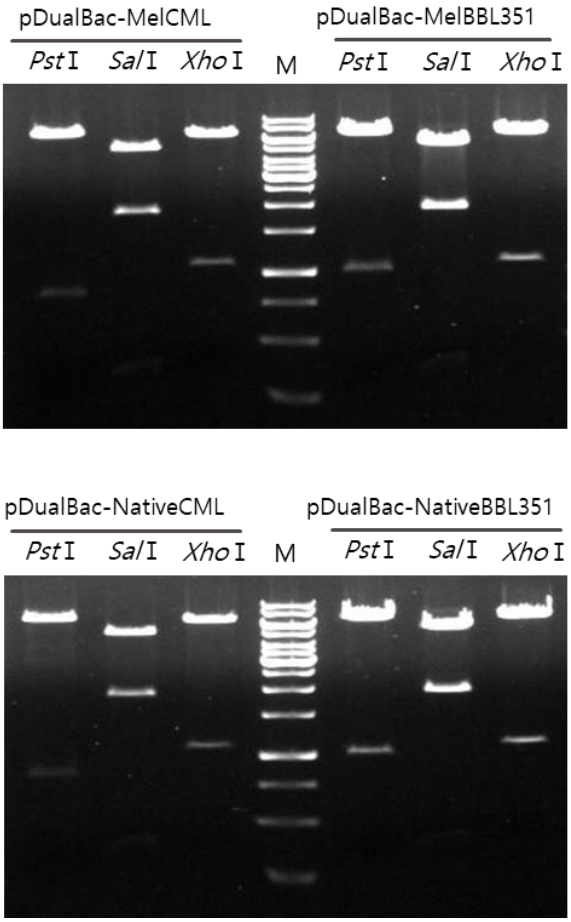
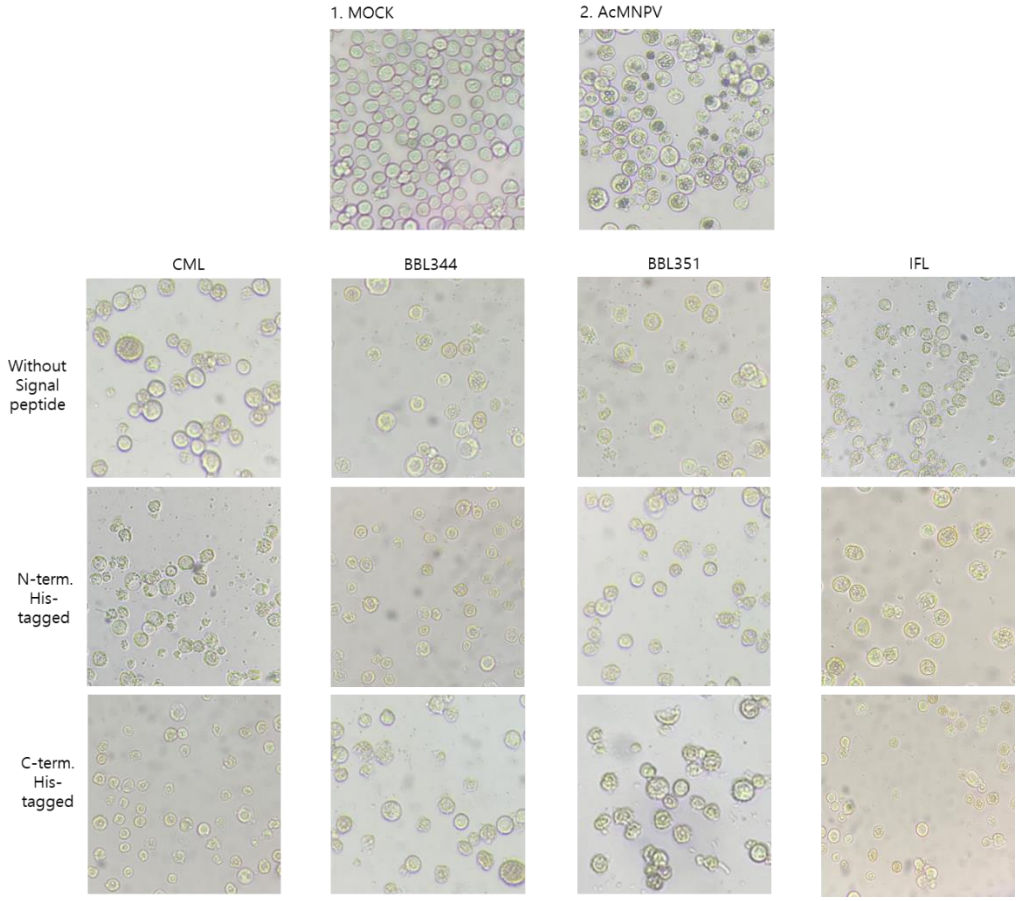


Fig. 15. Confirmation of EPF lipase genes into baculovirus donor vector by restriction endonuclease digestion pattern. (A) non-secretory lipase genes and (B) secretory lipase genes. Lane: M, 1Kb DNA Ladder.

(A)


1. MOCK
2. AcMNPV
CML
BBL344
BBL351
IFL
Without Signal peptide
N-term. His-tagged
C-term. His-tagged

(B)

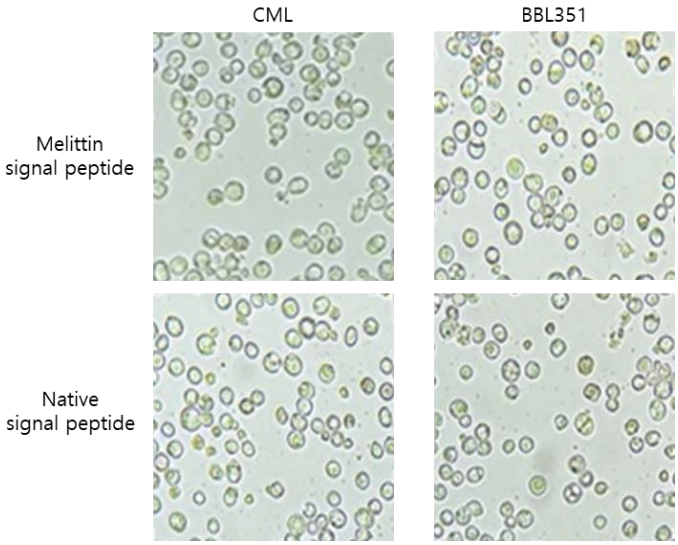
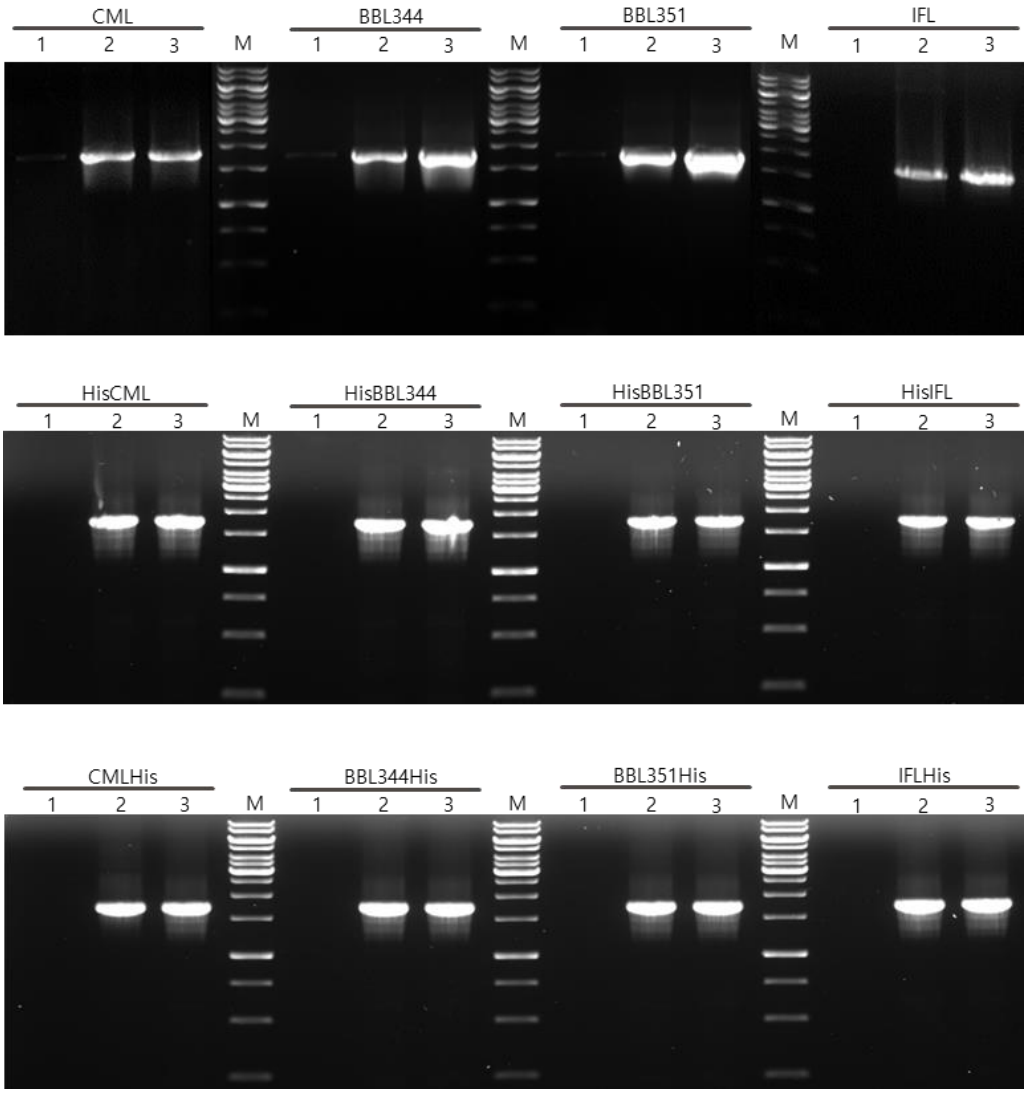


Fig. 16. Sf9 cells were infected with recombinant baculovirus. (A) Non-secretory recombinant baculoviruses 1, Mock cell; 2, Sf9 cells infected AcMNPV. (B) Secretory recombinant baculoviruses.

(A)


CML
1 2 3 M
BBL344
1 2 3 M
BBL351
1 2 3 M
IFL
1 2 3
HisCML
1 2 3 M
HisBBL344
1 2 3 M
HisBBL351
1 2 3 M
HisIFL
1 2 3
CMLHis
1 2 3 M
BBL344His
1 2 3 M
BBL351His
1 2 3 M
IFLHis
1 2 3

(B)

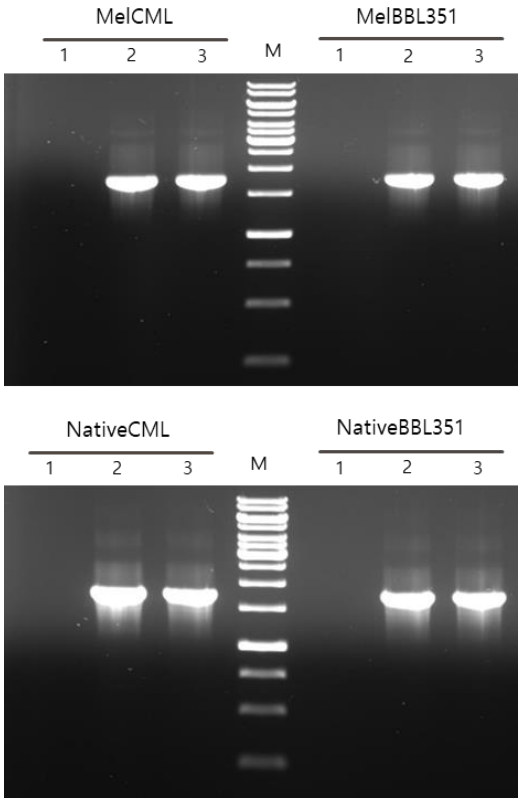
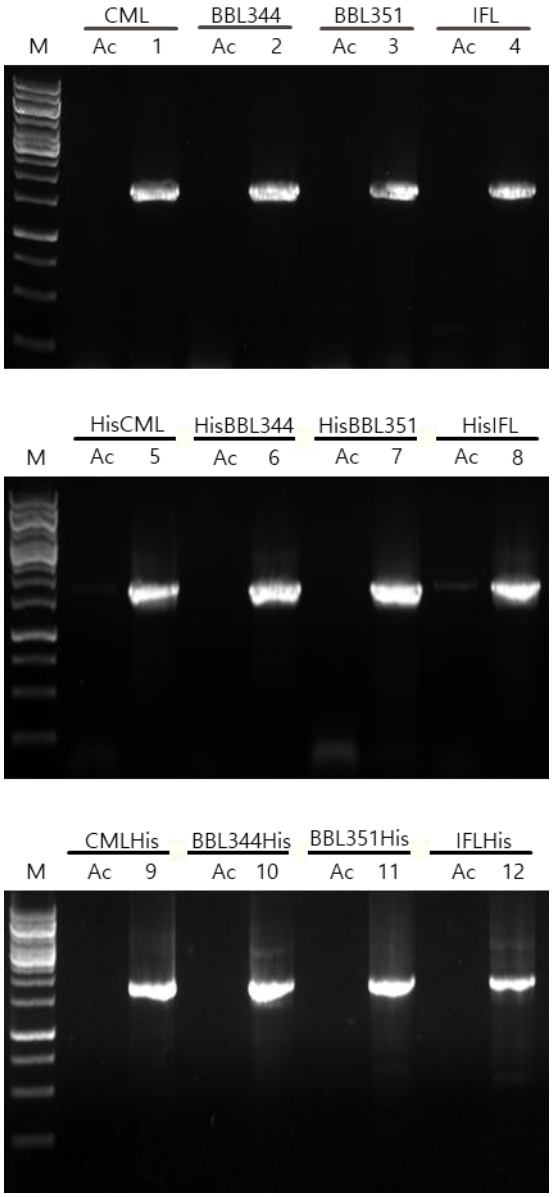


Fig. 17. Confirmation genome structure of recombinant baculoviruses expressing lipase gene under the control of polyhedrin promoters by RT-PCR using specific primer sets. (A) Recombinant baculoviruses expressing non-secretory lipase gene. (B) Recombinant baculoviruses expressing secretory lipase gene. Land: M, 1Kb DNA Ladder; 1, Mock-infected; 2, pDualBac-lipase; 3, AcEasy-lipase

(A)


CML
BBL344
BBL351
IFL
M
Ac 1
Ac 2
Ac 3
Ac 4
HisCML
HisBBL344
HisBBL351
HisIFL
M
Ac 5
Ac 6
Ac 7
Ac 8
CMLHis
BBL344His
BBL351His
IFLHis
M
Ac 9
Ac 10
Ac 11
Ac 12

(B)

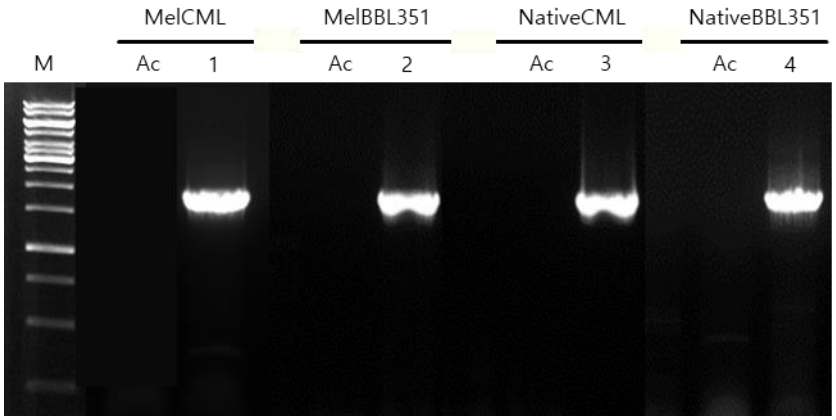
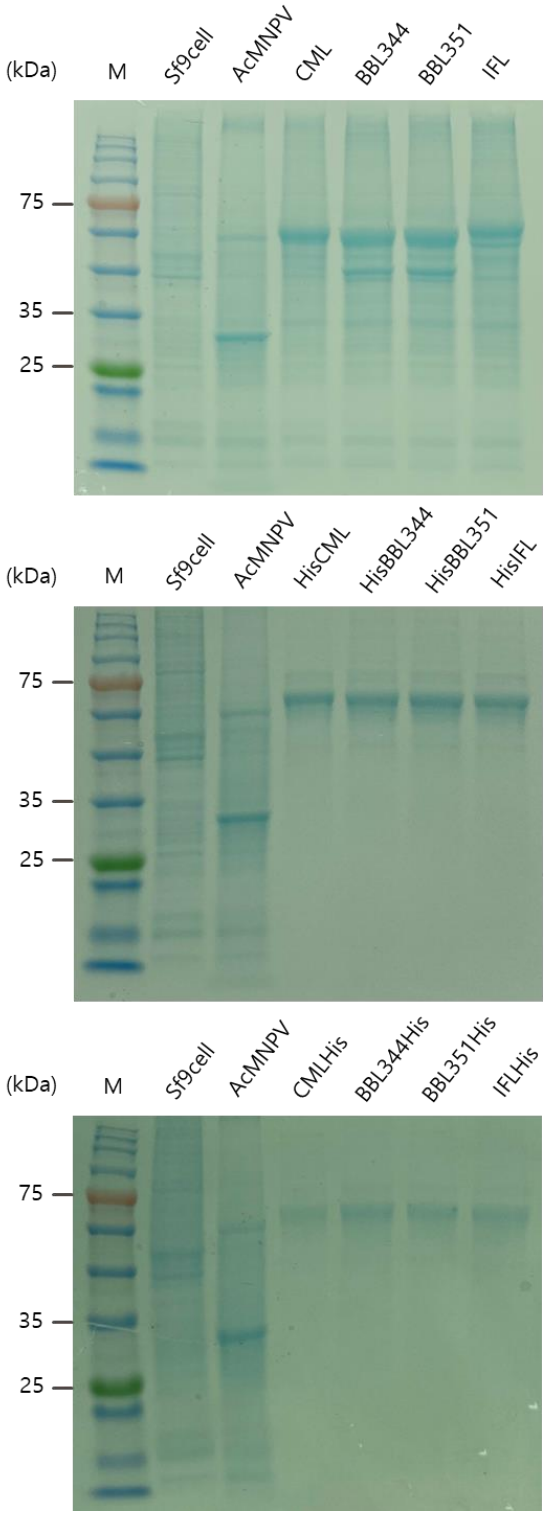


Fig. 18. Transcription of lipase gene from recombinant baculoviruses expressing lipase gene under the control of polyhedrin promoters by PCR using specific primer sets. (A) Recombinant baculoviruses expressing non-secretory lipase gene. Land: M, 1Kb DNA Ladder; Ac, AcMNPV; 1, AcEasy-CML; 2, AcEasy-BBL344; 3, AcEasy-BBL351; 4, AcEasy-IFL; 5, AcEasy-HisCML; 6, AcEasy-HisBBL344; 7, AcEasy-HisBBL351; 8, AcEasy-HisIFL; 9, AcEasy-CMLHis; 10, AcEasy-BBL344His; 11, AcEasy-BBL351His; 12, AcEasy-IFLHis. (B) Recombinant baculoviruses expressing secretory lipase. Land: M, 1Kb DNA Ladder; Ac, AcMNPV; 1, AcEasy-MelCML; 2, AcEasy-MelBBL351; 3, AcEasy-NativeCML; 4, AcEasy-NativeBBL351.

(A)


(kDa)
M
Sf9cell
AcMNPV
CML
BBL344
BBL351
IFL
75
35
25
(kDa)
M
Sf9cell
AcMNPV
HisCML
HisBBL344
HisBBL351
HisIFL
75
35
25
(kDa)
M
Sf9cell
AcMNPV
CMLHis
BBL344His
BBL351His
IFLHis
75
35
25

(B)

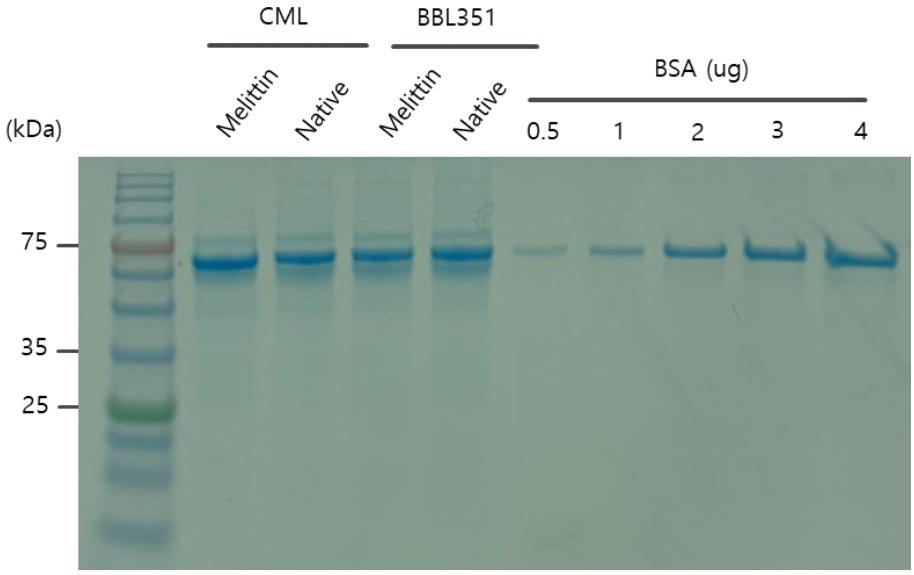


Fig. 19. Expression of lipase protein by recombinant baculoviruses. Land: M, 3-color prestained protein size marker. (A) non-secretory proteins and (B) secretory proteins.

For purification, a histag was attached to the N-terminal of the lipase gene without a signal peptide. I tried to obtain only pure lipase gene using Ni-NTA spin columns. Each step was observed through SDS-PAGE (Fig. 20). Histag failed to bind to resin in the columns.

4. Confirmation of enzyme activity by lipase assay

4.1 Non-secretory protein

Expression of non-secretory proteins from the Sf9 and High-Five cells were investigated with enzyme assay by infecting 5×10^5 of these cells with each recombinant baculoviruses at 10 MOI. The recombinant lipase displayed enzyme activity towards *p*-NPP (Fig. 21). Among the recombinant baculoviruses infected with Sf9 cells, BBL351 showed the highest activity, and the activity was about twice that of CML which is known to be stereospecificity of lipase. When the assay was performed after infection with Hi5 cells, the overall activity was high, and BBL351 was about 8 times higher than that of CML. When a histag was attached to the genes, it was found that the enzyme activity disappeared.

4.2 Secretory protein

Recombinant bacluoviruses infected Sf9 cells and the enzyme activity was measured in extra cellular and intra cellular to confirm whether signal peptide was normally expressed (Fig. 22). For the estimation of enzyme activities, 5×10^5 cells were infected with each recombinant baculoviruses at 10 MOI. The infected cells were harvested at 3 days and

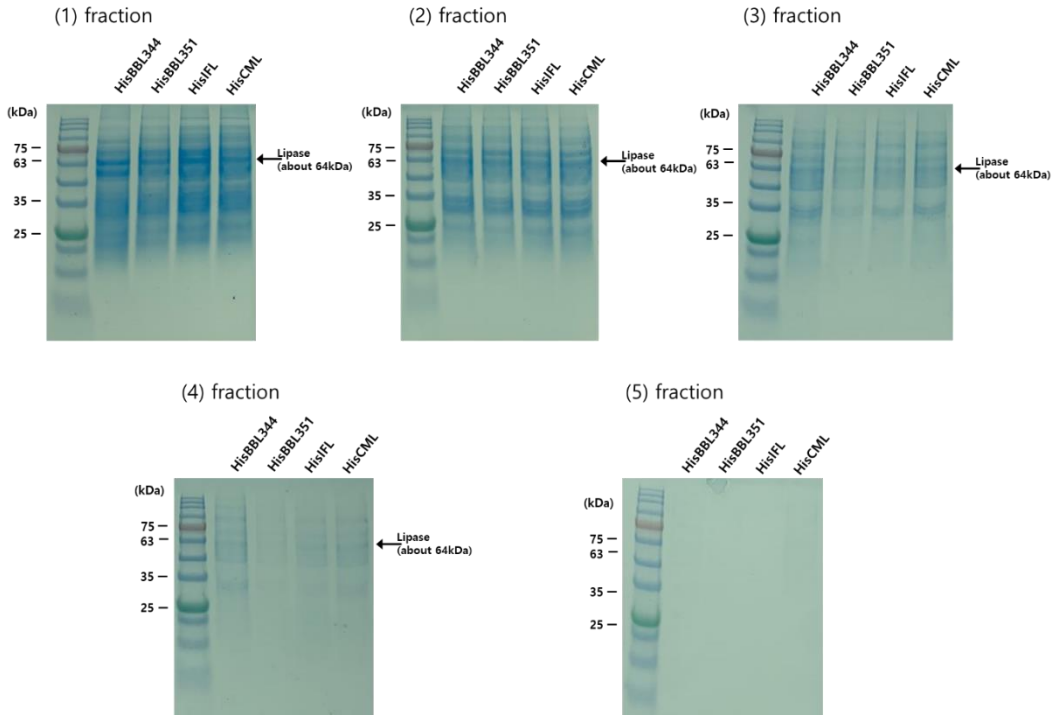


Fig. 20. Gel electrophoresis of HisPur^{TM} Ni-NTA Spin Column purification of Hislipase gene. (1), flow-through; (2), (3), wash resin of wash buffer and collect fraction; (4), (5), elute His-tagged protein from the resin of elution buffer and collect fraction.

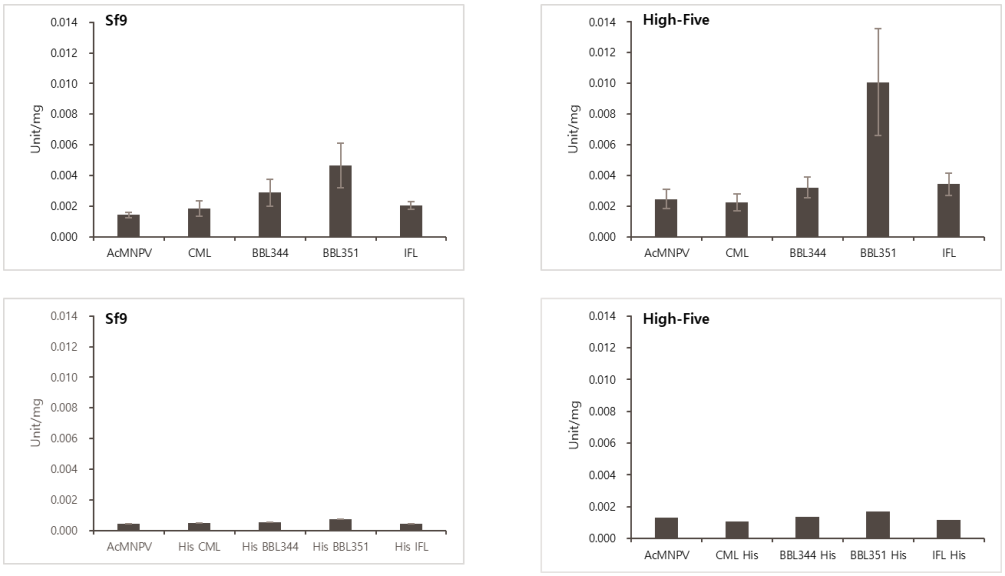


Fig. 21. Expression of non-secretory protein in insect cells infected with recombinant baculoviruses. Cells were seeded at a density of 5×10^5 cells/dish. The infected cells were harvested at 3 days.

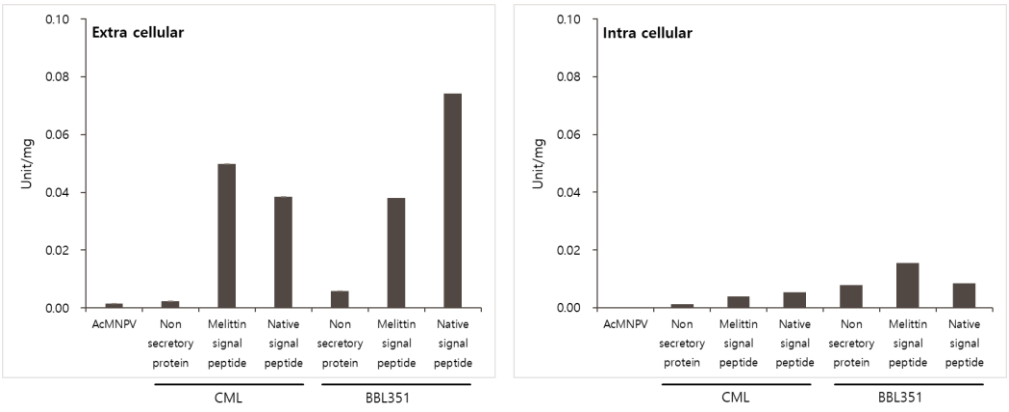


Fig. 22. Expression of secretory protein in insect cells infected with recombinant baculoviruses. Cells were seeded at a density of 5×10^5 cells/dish. The infected cells were harvested at 3 days.

using supernatant to confirm enzyme activity. The supernatant was concentrated using ammonium sulfate precipitation. Signal peptide was expressed and the enzyme activity in extra cellular was higher than that in intra cellular. Also, the enzyme activity of BBL351 was higher than that of CML. BBL351 had higher enzyme activity when the native fungal signal peptide was used than the melittin signal peptide.

DISCUSSION

Fungal virulence of different entomopathogenic fungi, like *Cordyceps militaris*, *Beauveria bassiana* and *Isaria fumosorosea* has been mostly associated with cuticle-degrading enzymes that can be regulated depending on nutrient conditions. These enzymes are pointed out as important in the infection process, since they hydrolyze polymer protein, chitin and lipid complexes, the major components of the insect's cuticle (Cristina Petrisor, Gheorghe Stoian, 2017). Lipases are responsible for the hydrolysis of ester bonds of lipoproteins, fats and waxes found at the interior part of the insect integument (Ali et al., 2009; Mondal et al., 2016). The *Cordyceps militaris* lipase (CML) derived from an entomopathogenic fungi 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 back bone, facilitating positional analysis for various lipid derivatives (Jung Ha Park, Kyung-Min Park, Yoonjee Chang, Jun-Young Parka, Jaejoon Han, Pahn-Shick Chang, 2018). A strain similar to the lipase gene CML derived from an entomopathogenic fungi was searched and obtained which are *B. bassiana* JEF-344, *B. bassiana* JEF-351 and *I. fumosorosea*. As a result of measuring the enzyme activity with these strains, the activity of the *B. bassiana* JEF-351 strain was the highest.

Lipase genes of *Cordyceps militaris*, *B. bassiana* JEF-344, *B. bassiana* JEF-351 and *I. fumosorosea* were mass expressed using a baculovirus expression vector system (BEVS). Baculovirus-insect cell expression systems have been widely used to express proteins as it

reliably results in high-yield and natively folded recombinant proteins (P. Stolt-Bergner *et al*., 2018). Using insect cells as the host, recombinant lipase proteins were expressed. Enzyme activity assay was performed with recombinant baculoviruses expressed with BEVS, and BBL351 showed the highest activity as when the activity was measured with the strain. For purification, the enzyme activity was measured by histagging the liapse genes, but the activity disappeared. The lack of N-glycosylation in the expression system could be one of the reasons why protein of triacylglycerol hydrolase with a six-His tag at the C-terminal is not active (Mustafa Alam, Dennis E. Vance, Richard Lehner, 2002). Therefore, in order to purify the lipase genes, the purification conditions must be set by a method other than histagging.

To find baculovirus with higher activity, the baculovirus was expressed in the form of a secreted protein. The baculovirus with the entomopathogenic fungi signal peptide of the original lipase and the baculovirus with the melittin signal peptide of honey bee were expressed using BEVS, respectively. Since there is a possibility that the native signal peptide, which is the original signal peptide of the entomopathogenic fungi, could not be expressed in insect cells, a gene substituted with the melittin signal peptide of honey bee, which is mainly used for the expression of insect cells, was produced (Daniel C. Tessier *et al*., 1991). As a result of measuring enzyme activity with baculovirus made in the form of secreted protein, as in the previous experiment, the activity of BBL351 was higher than that of CML, and all baculoviruses had higher activity than baculovirus of the form of non-secreted protein.

In this paper, which is describe the cloning, sequencing, and functional characterization of the gene encoding the lipase of an entomopathogenic fungi. Using the cloned lipase

genes as templates, baculoviruses were prepared with BEVS. As a result of measuring enzyme activity by preparing samples of entomopathogenic fungi, non-secreted protein, and secreted protein, the activity of BBL351 was the highest. Therefore, it can be seen that the use value of the BBL351 enzyme is high.

In conclusion, this is a report on the stereospecificity lipase gene of baculoviruses. In order to find a gene with higher activity, we will perform a comparative experiment by making several types of baculoviruses by mutation of the gene. We will purify the lipase genes through structural analysis and elucidate the stereospecificity of the lipase of recombinant baculoviruses using HPLC.

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ABSTRACT IN KOREAN

곤충병원성 곰팡이 유래 지방질 유전자의 발현과 특성 구명에 대한 비교 연구

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초 록

Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3)의 ester결합을 가수분해하여 glycerol과 fatty acid의 생성을 촉진하는 효소로서, 동물과 식물, 미생물 등 생물계 전반에 널리 분포되어 있다. 산업적으로 응용되고 있는 많은 효소들 가운데 지방질을 지방산과 monoglyceride 로 분해시키는 lipase가 차지하는 역할은 매우 중요하며, 급속히 성장하고 있는 생물 산업에서 주목 받고 있는 효소 중 하나이다. 곤충병원성 곰팡이인 동충하초 유래 lipase에서

lipase의 가장 큰 특징 중 하나인 위치 특이성이 triacylglycerol의 1(3)번 위치에 특이적으로 작용하는 것으로 판명되면서 많은 연구가 요구되었다. 따라서 본 연구를 통하여 곤충병원성 곰팡이로부터 lipase를 분리하여 그 특성을 규명하고, baculovirus expression system을 이용하여 대량발현 할 수 있는 조건을 구축하고자 하였다.

국내에서 분리한 곤충 병원성 곰팡이 중 lipase 활성이 가장 높은 균주를 선발하였다. 선발한 균주의 lipase와 위치특이성이 있다고 알려진 동충하초 유래 lipase를 베큘로바이러스 발현 벡터계 (baculovirus expression vector system)를 이용하여 재조합 베큘로바이러스를 만들었다. 비분비단백질과 분비단백질로 각각 베큘로바이러스를 발현시켰다. lipase활성을 비교한 결과 분비단백질 형태일 때 활성이 더 높았다. 곰팡이 배양액, 비분비단백질 그리고 분비단백질 3가지의 조건으로 lipase 활성을 측정을 해보았는데 모든 결과값에서 동충하초 유래 lipase보다 *Beauveria bassiana* JEF-351 균주 유래 lipase의 활성이 높게 측정되는 것으로 보아 *Beauveria bassiana* JEF-351 균주 유래 lipase가 매우 유용하게 이용이 될 수 있는 효소인 것으로 판단되었다.

검색어: Lipase, 곤충병원성 곰팡이, 동충하초, *Beauveria bassiana*, 베큘로바이러스 발현계, 분비단백질

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