A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Studies on the biological characteristics and mass expression conditions for lipases from entomopathogenic fungi

By

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Major in Entomology

Department of Agricultural Biotechnology

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곤충병원성 곰팡이 유래 지방질 효소의 생물학적 특성 및 대량 발현 조건 설정 연구

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ABSTRACT

Lipase is one of the most important enzymes which catalyze the hydrolysis/esterification of ester bonds in triacylglycerol between fatty acids and glycerol. Recently, lipase derived from entomopathogenic fungi *Cordyceps militaris* was found to act specifically at position *sn*-1,3 of triacylglycerol. In this study, activities of lipases isolated from entomopathogenic fungi were comparatively investigated, and conditions for mass expression using the baculovirus expression system were established. Lipase genes isolated from *C. militaris* (CML) and *Beauveria bassiana* JEF-351 strain (BBL351) were introduced into the

baculovirus genome of *Autographa californica* nucleopolyhedrovirus (AcMNPV), and recombinant lipases were expressed in insect cells. When the hydrolysis efficiency of these lipases was investigated by substrate length, both CML and BBL351 showed the highest activity against *p*-nitrophenyl butyrate (C4). Although, both CML and BBL351 showed higher expression efficiency in High-Five cells, the lipase activity of BBL351 was approximately 2-fold higher than that of CML.

Additionally, improvement of lipase activity through CML mutagenesis and secretion optimization with signal peptides were performed. The mutants of CML with higher lipase activity were selected through mutagenesis based on the protein tertiary structure and amino acid comparison of CML and BBL351. The 13 sequence-based mutants and 6 structure-based mutants were constructed through multi-site-directed mutagenesis. Among the 19 mutants, Mut-3 and Mut-4 from structure-based mutants showed higher lipase activity than wild-type CML.

These results suggested that lipases from entomopathogenic fungi could be useful in various industries with their unique properties and potentially be a novel natural source of biocatalysts.

Keywords: lipase, entomopathogenic fungi, *Cordyceps militaris*, *Beauveria bassiana,* baculovirus expression system

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INTRODUCTION

Lipase is one of the most crucial hydrolase enzymes, which mainly catalyzes hydrolysis and esterification of ester bonds in triacylglycerols between fatty acids and glycerol. Hydrolysis generally refers to the cleavage of fat/ester bonds into acid and glycerol/alcohol in the presence of water (Gandhi, 1997). This reaction is currently applied in various industries, especially food, medical, chemical, detergent, and cosmetic. Lipase applications in the food industry have great potential because effective lipid metabolism is important for human health and food production. In the past, lipase was usually applied in dairy products such as milk, cheese, butter, and margarine for flavor development that accelerate the maturation of dairy products (Arnold, Shahani, & Dwivedi, 1975; Kosikowski, 1976; Nelson, 1972; Posorske, 1984). Recently, modification of lipase to make low-fat foods and low-calorie lipids were in the spotlight with growing concerns about health and balancing diet (Rodríguez-García, Sahi, & Hernando, 2014; Speranza & Macedo, 2012). Also, in the medical clinic, lipases can be used as a diagnostic indicator against pancreatic diseases such as pancreatitis, and it could be an effective treatment for malignant tumors (Ismail & Bhayana, 2017; Rompianesi et al., 2017; Semb, Peterson, Tavernier, & Olivecrona, 1987; Takasu, Mutoh, Takahashi, & Nakagama, 2012; Treacy et al., 2001; Ye et al., 2011).

Esterification, called ester synthesis, catalyzes the reverse hydrolysis reaction between acid and alcohol, synthesizing novel ester bonds (Gandhi, 1997). With this characteristic, lipases were used as food emulsifiers that work as biosurfactants in salad dressing and bread softening agents (Huang et al., 2013). The cosmetic industry helps produce volatile chemicals and perfumes (Choudhury & Bhunia, 2015; Gamayurova, Zinov'eva, Shnaider,

& Davletshina, 2021). Lipase also has been bio-catalyzed to produce biodegradable polyesters. Trimethylolpropane esters were synthesized as lubricants by direct esterification in organic solvent systems, and aromatic polyesters can be synthesized by lipase biocatalysts (Choudhury & Bhunia, 2015; Linko et al., 1998).

According to versatile lipase industrial applications, it has been continuously known as one of the most important enzymes in nature. Its usage in the enzyme market is gradually increasing (Chandra, Singh, & Arora, 2020). Since lipase was first found in the animal pancreas, it has been widely found throughout plants, fungi, bacteria, and other biological organisms. Among them, microbial lipase gained attention with its inexpensive production cost and ability to high-yield mass production (Ray, 2012). Also, their genetic manipulation is relatively simple and generally not interrupted by seasonal fluctuation. Additionally, it is comparatively safer and more convenient to handle than animal/plant-derived lipase (Chandra et al., 2020; Reetz, 2013).

In the group of microbial lipase, fungal lipase was considered the best source for its substrate specificity and stability under various chemical and physical conditions (Facchini, Vici, Pereira, Jorge, & de Moraes, 2016; Mehta, Bodh, & Gupta, 2017). Among the various fungal lipases, lipase derived from *Cordyceps militaris*, entomopathogenic fungi, was reported to exhibit *sn*-1(3) regioselectivity (J. H. Park et al., 2018; J. Y. Park et al., 2019). The regioselectivity of lipase is a distinctive property that distinguishes lipase from other hydrolase enzymes. Lipase regioselectivity generally refers to the selective cleave some fatty acids from the glycerol backbone of a lipid (forming one positional isomer over another) (Choi, Park, & Chang, 2021; Ray, 2012). The *sn*-1(3) regioselectivity is crucial for positional analysis in triacylglycerol that helps to identify esterified fatty acid at the *sn*-

1(3) position of the glycerol backbone (Aranda, Gomez-Alonso, Del Álamo, Salvador, & Fregapane, 2004; J. H. Park et al., 2018). Moreover, as mentioned previously, this characteristic could be effectively applied commercially with hydrolysis and esterification reactions (Lu, Chen, & Liu, 2012). Also, lipase regioselectivity could manufacture animal fats or waste oils to eco-friendly alternative energy such as bio-diesel (Yoo et al., 2011). Additionally, in the chemical reaction, high purity of regioisomer could be obtained from various products. This reaction is essential to separate one desired isomer, which shows high efficacy among the different products (José, Toledo, & Briand, 2016; Sarney, Barnard, MacManus, & Vulfson, 1996). These applications suggested that lipase regioselectivity could be useful in various industries and has prominent potential.

In the previous study, among the various entomopathogenic fungi, *Beauveria bassiana* JEF-351 strain which was reported as phylogenetically close to *C. militaris* and showed high lipase activity was selected for comparative study with *C. militaris* lipase (Kim, 2022). Therefore, in this study, the lipase activity of CML (*C. militaris* lipase), which is known to show *sn*-1(3) regioselectivity, and BBL351 (*B. bassiana* JEF-351 lipase), which exhibits high lipase activity, were compared. Also, the mass expression conditions against two enzymes using insect cells were established, and each lipase's biological characteristics were investigated by constructing CML mutants to improve lipase activity. These entomopathogenic fungi-derived lipases could be effective natural sources for novel biocatalysts.

LITERATURE REVIEW

1. Lipase

Enzymes, as a biocatalyst, catalyze various bio-reactions enriching human life. All living organisms perform biological processes like chemical reactions, and most are regulated by enzymes (Kirk, Borchert, & Fuglsang, 2002). Without enzymes, many of these reactions would not occur at a perceptible rate and catalyze various aspects of metabolism. The digestion of food necessary for metabolism in the body, mainly catalyzed by digestive enzymes, helps nutrient macromolecules break down into several molecule units and transform into chemical energy. Also, many inherited human diseases, such as albinism and phenylketonuria, occur by a deficiency of particular enzymes (Britannica, 2022).

Among the various enzymes, lipase is an essential biocatalyst for biotechnological applications (Choudhury & Bhunia, 2015). Since lipase was first identified in the pancreas by Eberle in 1834 and Bernard in 1856, associated research has been actively carried out (Hou, 2002). Lipases mediate many reactions, not only the hydrolysis but also the reaction of synthesis, esterification, interesterification, alcoholysis, and acidolysis. These reactions have been widely applied to the food, chemical, medical, and detergent industry (Gandhi, 1997). Also, it could synthesize novel structured triglycerides such as bio-diesel and food additives (Tan, Lu, Nie, Deng, & Wang, 2010).

In various lipase properties, the regioselectivity, which selectively cleaves some fatty acids of the triacylglycerol backbone, has gained a high value (Akanbi, Adcock, & Barrow, 2013). It is the most crucial property distinguishing lipase from other enzymes (Choi et al.,

2021). With positional selectivity of lipase, they could be classified into two groups which are *sn*-1,3 regiospecific and non-regiospecific/random. The *sn*-1,3 regiospecific lipases react at the glycerol backbone's outer position, including lipases derived from *Aspergillus niger* and *Rhizopus delemar* (Lanser, Manthey, & Hou, 2002). Also, the non-regiospecific lipases randomly act on all three positions of the glycerol backbone. The lipases from *Geotrichum candidum* and *Penicillium cyclopium* are represented in non-regiospecific lipases (Lanser et al., 2002; Rogalska, Ransac, & Verger, 1990). Additionally, few *sn*-2 regiospecific lipases were reported that react center position of the glycerol backbone, including *Geotrichum candidum* lipase and *Candida antarctica* A lipase (Rogalska, Cudrey, Ferrato, & Verger, 1993; Sugihara, Shimada, & Tominaga, 1991).

Recently, with regioselectivity of lipase, several attempts have been made to study it industrially. The long-chain omega-3 fatty acids cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), which are used as the treatment of cardiovascular, Alzheimer's and Parkinson's diseases were obtained in a low yield, maximum of 30% in fish oil (Akanbi et al., 2013; Mazza, Pomponi, Janiri, Bria, & Mazza, 2007). They could be artificially synthesized into more stable lipids with high yields using *sn*-2 regiospecific *Thermomyces lanuginosus* lipase (Akanbi et al., 2013). Also, in the alternative energy industry, bio-fuel such as bio-diesel could be produced with regioselective lipase (Nanssou Kouteu, Baréa, Barouh, Blin, & Villeneuve, 2016; Šinkūnienė & Adlercreutz, 2014). Besides these examples, its property was applied in producing fat-derived nutritional supplements and novel reconstructed food additives (Gandhi et al., 2000).

2. Entomopathogenic Fungi (EPF)

Entomopathogenic fungi (EPF), an insect-specific fatal microorganism of more than 750 species, are known to infect insects (Ramanujam, Rangeshwaran, Sivakmar, Mohan, & Yandigeri, 2014). They had been regulating insect populations with their insecticidal activity in nature. Representatively, the genus of *Beauveria* (*B. bassiana* and *B. brongniartii*), *Isaria* (*I. fumosorosea*, *I. farinosa,* and *I. tennuipes*), *Ophiocordyceps* (*O. sinensi*, *O. unilateralis*), and *Cordyceps* (*C. militaris*) were included (Castro et al., 2016; Jaihan, Sangdee, & Sangdee, 2016; Khan, Guo, Maimaiti, Mijit, & Qiu, 2012). In their infectious process, insects would not need to ingest it. They directly adhesion and penetrate the insect cuticle (Bilgo et al., 2018). Firstly, EPF spores were attached to the cuticle and germinated at the proper temperature (20°C-30°C) to form appressoria. The appressoria are described as a pressure organ of fungal mycelium that causes strong mechanical pressure on the cuticle to penetrate host insects. Also, the various lytic fungal enzymes, such as lipase and protease, were secreted (Bilgo et al., 2018; Keyhani, 2018; Lacey, 1998; Litwin, Nowak, & Różalska, 2020; Skinner, Parker, & Kim, 2014). After decomposing the cuticle layer, EPF penetrated the insect body cavity, and the fungal hyphae started to grow (Litwin et al., 2020). With this infectious process, EPF has been used as one of the efficient biopesticides.

Besides pesticides, some EPFs were known to play a role as endophytes of plants and used as clinical therapy in Korean medicine. Especially in the genus of *Metarhizium* and *Beauveria,* which are generally distributed in the soil, play an important ecological role as endophytes of plant roots, stems, and leaves (Jaber & Enkerli, 2017). These EPFs support plant growth by providing nutrients such as nitrogen that were assimilated during the

parasitization of insects (Behie & Bidochka, 2014; Ríos-Moreno et al., 2016). Also, the colonized endophytes protect the plant from other microbial pathogens and get more insect resistance by shooting in addition to plant roots (Klieber & Reineke, 2016; Ramakuwela et al., 2020). Additionally, in the genus of *Cordyceps*, EPF-derived active substances such as cordycepin were used as an efficient medicine against various diseases. The pharmacological effects of these substances are being actively investigated, and some effective reports of anti-cancer, anti-inflammatory, and immune-enhancing activities have been revealed (Lee, Debnath, Kim, & Lim, 2013; Tuli, Kashyap, & Sharma, 2015; Zhang, Hao, Zhao, Zhang, & Zhang, 2017).

3. Baculovirus expression vector system (BEVS)

Baculovirus is an entomopathogenic virus that infects more than 600 host species in different orders (Chambers et al., 2018; Herniou, Olszewski, Cory, & O'Reilly, 2003; Possee et al., 2010). In nature, it was usually used as a pesticide with its insecticidal activity to host insects, especially *Lepidoptera*. Also, industrially, it was used to express foreign target genes due to their host specificity and safety for the environment and human health (van Beek & Davis, 2016). Baculoviruses exhibited large rod-shaped virions containing supercoiled 88 to over 160 kbp size of dsDNA genomes. The outstanding feature is that the pathogenic virions are embedded in the crystalline protein matrix, the occlusion body called polyhedrin, and infect the insect cells. This polyhedrin size was estimated to be approximately 0.5 μm to 15 μm. Also, it exhibited high protein expression efficiency at the late stage of viral infection, which synthesized about 30%-40% of polyhedrin protein among the total infected cells (Rohrmann, 1992). The high efficiency of polyhedrin gene

expression was caused by its powerful polyhedrin gene (*polh.*) promoter in virus-infected insect cells. While the polyhedrin gene is known to be not essential for viral replication in virus-infected cells, the system that replaces the polyhedrin gene with a foreign target gene was developed and called as baculovirus expression vector systems (BEVS) (Rohrmann, Leisy, Chow, Pearson, & Beaudreau, 1982; Smith, Fraser, & Summers, 1983; Smith, Vlak, & Summers, 1983; Van Iddekinge, Smith, & Summers, 1983).

In BEVS, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which contains approximately 130 kbp size dsDNA and harbors 154 ORF, was commonly used. With AcMNPV, the BEVS showed a high gene expression level of recombinant protein production, and it is easy to scale up in shaking cultures or bioreactors using insect cells (He et al., 2021; Hitchman, Possee, & King, 2009). Because this system uses highly eukaryotic insect cells, the expressed recombinant protein showed high biological and immunological activity. Compared with bacteria and yeast expression systems, the baculovirus expression system showed highly close post-translational modifications such as glycosylation and folding of proteins to mammals. That's why it has been used in various industries with the wide field of molecular design (Grabherr & Ernst, 2001).

As baculoviruses are not pathogenic to humans, it usually applied to the medical industry. Baculoviruses have been used as vaccine vectors to prepare antigen or subunit vaccines (Lu et al., 2012). Also, it has been widely used to produce recombinant proteins in insect cells and larvae (Kost, Condreay, & Jarvis, 2005). Additionally, it is applied in producing infectious viruses such as adeno-associated virus (AAV), used as a potential gene therapy vector, and interfering and microRNA delivery (Kost & Kemp, 2016).

MATERIALS AND METHODS

1. Bacterial strains and transformation

The *Escherichia coli* TOP10 strain was used to transform the plasmid DNA. The transformation was performed using chemically treated competent cells according to the manufacturer's instructions. The competent cells (200 µl) were mixed with 50 ng plasmid DNA. After 30 min incubation in ice, the mixture was placed in a 42°C water bath for 45 seconds (heat shock) and then back in ice for 3 min. Luria–Bertani (LB) media (10 g NaCl, 10 g tryptone, and 5 g yeast extract per 1 liter of distilled water) 1 ml was added and incubated at 37°C, 220 rpm for 1 hr. Transformed *E. coli* was spread on a selective medium for one colony selection.

2. Insect cells and baculoviruses

The Sf9 cells in two different media and High-Five cells were used in this study. The Sf9 cells (Thermo Fisher Scientific, USA) were derived from *Spodoptera frugiferda* that were continuously maintained in the TC-100 medium (WelGene, Korea) supplemented with 10% fetal bovine serum (WelGene, Korea) and Sf-900™ III SFM (Thermo Fisher Scientific, USA), respectively. The High-Five cells (Thermo Fisher Scientific, USA), which originated from *Trichoplusia ni,* were maintained in Express Five SFM (Thermo Fisher Scientific, USA) with L-glutamine (200mM) (Thermo Fisher Scientific, USA). All insect cell lines were incubated at 27°C and sub-cultured every 3-4 days. All the recombinant baculoviruses and wild-type *Autographa californica* multiple nucleopolyhedrovirus

(AcMNPV) used in this study were propagated in Sf9 cells and High-Five cells.

3. Construction of donor vectors harboring lipase genes

The lipase gene derived from *B. bassiana* JEF-351 (BBL351, GenBank accession number; MW 773727) was amplified using specific primers (Table 1). The *C. militaris* lipase (CML) gene was provided by Prof. Pahn-Shick Chang (Center for Food and Bioconvergence, Seoul National University) as inserted into the pET29 vector (pET29_CML).

CML and BBL351 were amplified using specific primers and inserted into the pDualBac vector (Fig. 1). The melittin signal peptide was replaced with the native signal peptide by PCR with specific primers to express lipases as secretory proteins under the control of the polyhedrin promoter. Approximately 1.7 kb of lipase genes were amplified as NativeCML, NativeBBL351, MelCML, and MelBBL351. They were inserted into the pDualBac donor vector digested with *Bam*HI using In-Fusion® HD Cloning Kit (Takara Biotech Co. Ltd., Dalian, China) (Fig. 2). A total of 19 mutant lipase genes were also inserted into the pDualBac donor vector digested with *Bam*HI and *Eco*RI using In-Fusion® HD Cloning Kit (Takara Biotech Co. Ltd., Dalian, China) (Fig. 3).

4. Generation of mutant lipase genes

Ten pairs of specific primers were designed to change 20 amino acids in MelCML lipase region (Table 2). All the mutagenic primers were designed based on *C. militaris* codon usage (Table 3). Primers were designed to be between 25 and 45 bases in length with a melting temperature (Tm) over 75°C, which was calculated using Enzynomics Tm

Table 1. Nucleotide sequence of primers used to amplify lipase genes from entomopathogenic fungi.

Amplified target	Primer name	Primer sequence
C. militaris lipase (CML)	NativeCML-F	5'-ATGAAATTCTCACTTGTGGCTC-3'
	MelCML-F	5'-ATGAAATTCTTAGTCAACGTT-3'
	CML-R	5'-TTAGAAGTAGAGTACCTCCG-3'
B. bassiana lipase (BBL351)	NativeBBL351-F	5'-ATGAGATTCACCGTTGTGG-3'
	MelBBL351-F	5'-ATGAAATTCTTAGTCAACGTTGCC-3'
	BBL351-R	5'-GGATCCTCAAAAATAGAGCGC-3'
AcMNPV *polh.* promoter	Bac 1	5'-ACCATCTCGCAAATAAATAAG-3'
	Bac 2	5'-ATTAAAATACGTACAACAATTGTC-3'

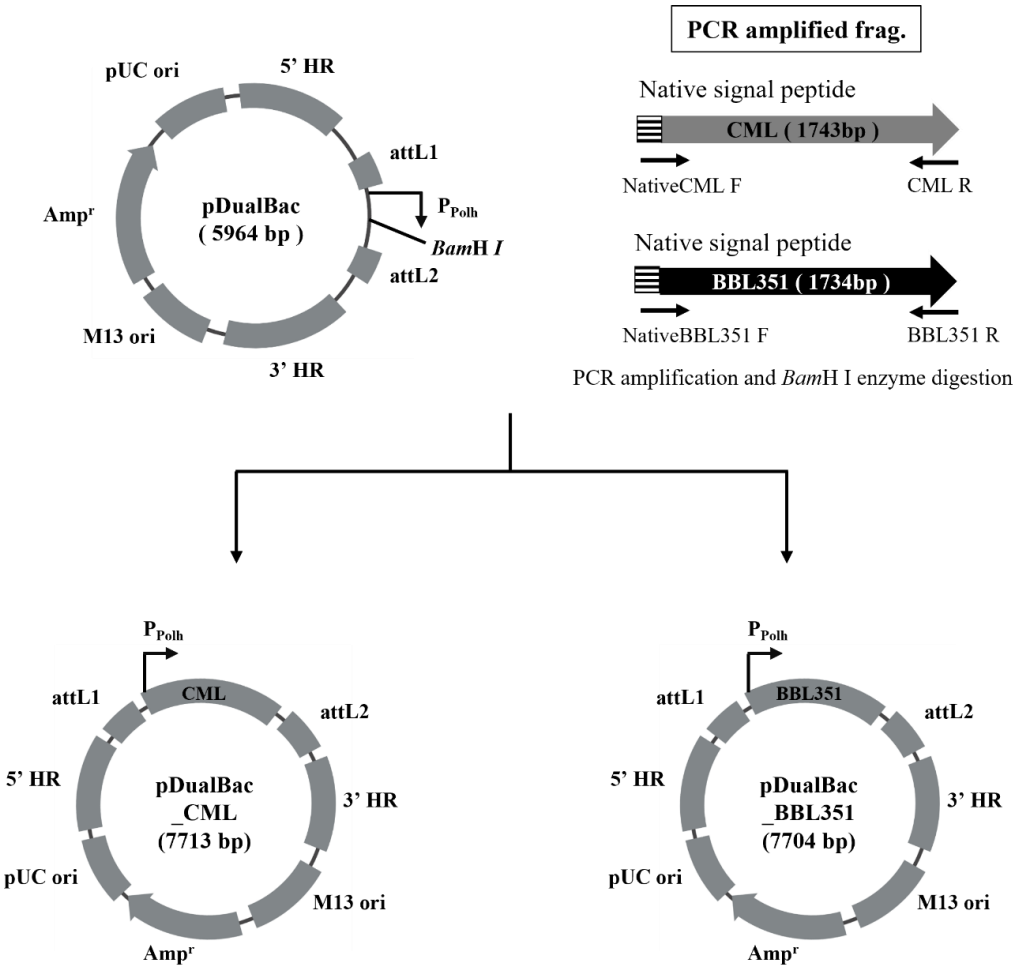


Fig. 1. Construction map of baculovirus donor vectors harboring CML and BBL351 genes. The PCR amplified fragment of CML (1.74 kb) and BBL351 (1.73 kb) genes were inserted into pDualBac to obtain pDualBac_CML and pDualBac_BBL351, respectively. Solid arrows indicate primers used in PCR amplification.

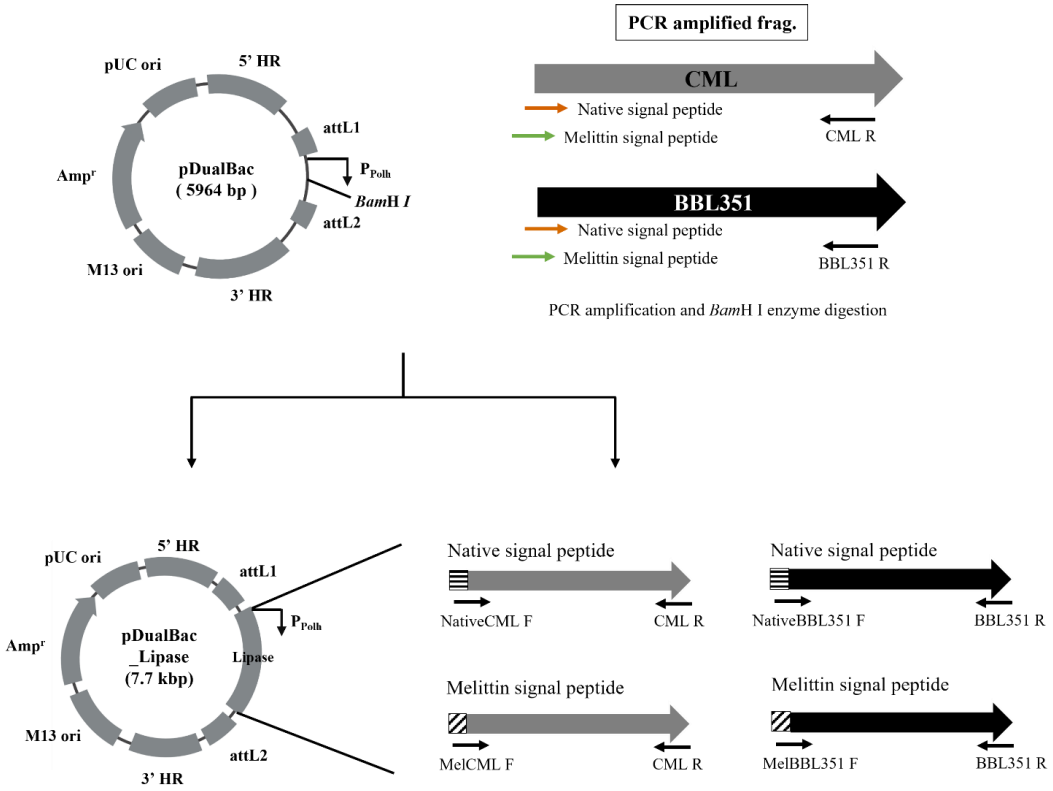


Fig. 2. Construction map of baculovirus donor vectors harboring lipase genes with signal peptide sequence. The PCR-amplified CML and BBL351 lipase genes with native signal peptides sequence or melittin signal peptide sequence were inserted into pDualBac to obtain pDualBac_NativeCML, pDualBac_MelCML, pDualBac_NativeBBL351, and pDualBac_MelBBL351, respectively. Solid arrows indicate primer position used in PCR amplification.

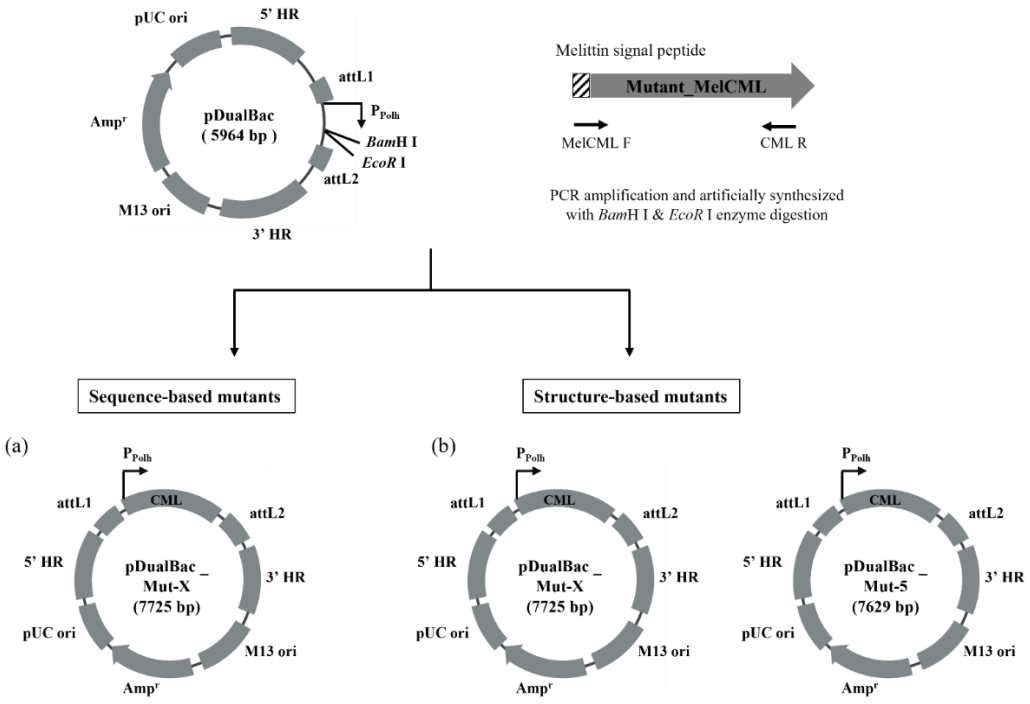


Fig. 3. Construction map of baculovirus donor vectors harboring mutant CML genes. (a) The sequence-based MelCML mutants were inserted into pDualBac to obtain sequence-based pDualBac_Mut-X, the capital letter 'X' represents 2318, 4171, 417, 31, 316, 4117, 413, 416, 411, 352, 46, 466, and 35. (b) The structure-based MelCML mutants were inserted into pDualBac to obtain structure-based pDualBac_Mut-X, the capital letter 'X' represents 1, 2, 3, 4, 5, and 6. Solid arrows indicate primer position used in PCR amplification.

Table 2. Nucleotide sequence of primers used to generate mutant CML genes.

Mutagenesis site	Primer name	Primer sequence
SE-Region I	SE-Mut1-FW	5'-CCAACTGCAGCTACTCCACCACCTCCAAGCTGGACTGTCTTCG-3'
	SE-Mut1-RE	5'-CTTGGAGGTGGTGGAGTAGCTGCAGTTGGCCGCTTCGA-3'
SE-Region II	SE-Mut2-FW	5'-GACTATCTGCTCGAGACGTTTTTCTCCAAGGCGACAAGGG-3'
	SE-Mut2-RE	5'-TTGTCGCCTTGGAGAAAAACGTCTCGAGCAGATAGTCGAC-3'
SE-Region III	SE-Mut3-FW	5'-CGGCCTCGTGGCCACATACCCAGAGGCCTCGGCCGATGG-3'
	SE-Mut3-RE	5'-CATCGGCCGAGGCCTCTGGGTATGTGGCCACGAGGCCGGCG-3'
SE-Region IV	SE-Mut4-FW	5'-GGTACGAGAACACCTACGGTACCGGCAACGGCTTCAAGAGGG-3'
	SE-Mut4-RE	5'-CTTGAAGCCGTTGCCGGTACCGTAGGTGTTCTCGTACCACTCG-3'
SE-Region V	SE-Mut5-FW	5'-CAACATGATTTTTAAGGGCATTGGCATACCTGCGCTGACTACC-3'
	SE-Mut5-RE	5'-GCAGGTATGCCAATGCCCTTAAAAATCATGTTGACATCTGC-3'
ST-Region I	ST-Mut1-FW	5'-CCCTGTTTGCCGTCGCCCAGAGGCACGTC-3'
	ST-Mut1-RE	5'-GCCTCTGGGCGACGGCAAACAGGGTTCC-3'
ST-Region II	ST-Mut2-FW	5'-CATGGCCCACGGGGTCGTGGGCTTCTACGGTACG-3'
	ST-Mut2-RE	5'-GTAGAAGCCCACGACCCCGTGGGCCATGGAGTTC-3'
ST-Region III	ST-Mut3-FW	5'-GGGATTGTGGGCTACTGGGGTACGGGACACGGTGC-3'
	ST-Mut3-RE	5'-GTGTCCCGTACCCCAGTAGCCCACAATCCCGTGG-3'
ST-Region IV	ST-Mut4-FW	5'-CGGTGCAGATCTCAACATGATTTTTGAAGGCATTGGC-3'
	ST-Mut4-RE	5'-CAAAAATCATGTTGAGATCTGCACCGTGTCCCGTACCG-3'
ST-Region VI	ST-Mut6-FW	5'GTCGTCTGGGGCGGCCTCGGGCGTGGCGTCCTTGCCGAGAAGCGGCG-3'
	ST-Mut6-RE	5'-GCAAGGACGCCACGCCCGAGGCCGCCCCAGACGACCTCATCCGGGGCCA-3'

Table 3. Codon usage in pooled sequences of *C. militaris* genes.

Amino acid	Codon	Preference,%	Amino acid	Codon	Preference,%
Glycine (G)	GGU	25	Threonine (T)	ACU	18
	GGC	54		ACC	39
	GGA	16		ACA	25
	GGG	6		ACG	18
Alanine (A)	GCU	24	Cysteine (C)	UGU	28
	GCC	40		UGC	72
	GCA	18			
	GCG	15			
Valine (V)	GUU	28	Tyrosine (Y)	UAU	36
	GUC	48		UAC	64
	GUA	8			
	GUG	15			
Leucine (L)	UUA	5	Asparagine (N)	AAU	29
	UUG	15		AAC	71
	CUU	18			
	CUC	29			
	CUA	6			
	CUG	28			
Isoleucine (I)	AUU	47	Glutamine (Q)	CAA	40
	AUC	45		CAG	60
	AUA	8			
Methionine (M)	AUG	100	Aspartate (D)	GAU	41
				GAC	59
Phenylalanine (F)	UUU	42	Glutamate (E)	GAA	46
	UUC	58		GAG	54
Tryptophan (W)	UGG	100	Lysine (K)	AAA	31
				AAG	69
Proline (P)	CCU	25	Arginine (R)	CGU	21
	CCC	27		CGC	33
	CCA	26		CGA	21
	CCG	21		CGG	11
				AGA	10
				AGG	4
Serine (S)	UCU	19	Histidine (H)	CAU	31
	UCC	22		CAC	69
	UCA	12			
	UCG	19	* TER	UAA	60
	AGU	8		UAG	20
	AGC	18		UGA	20

calculator (Tm: 81.5 +0.41(% GC) - (675/N (primer length)) - % mismatch).

A total of 18 mutants were generated from pDualBac_MelCML plasmid using EZchange™ Multi Site-directed Mutagenesis Kit (Enzynomics, Korea). Amplification of the template DNA in a mutagenesis reaction for mutant strand synthesis was performed according to the following cycle parameter; step 1, 95°C, 1 min, one cycle; step 2, 95°C, 1 min; step 3, 95°C, 1 min; step4, 65°C, 16 min (every successive cycle repeats step 2 to 4, 30 cycles); step 5, 12°C, unlimited. All the PCR amplifications were performed with the DNA Thermal Cycler (BIO-RAD, USA). After mutant strand synthesis, 1 μl of *Dpn*I (Enzynomics, Korea) was added to digest methylated template DNA. The mutated single-stranded DNA was transformed into DH5α competent cell (Enzynomics, Korea, Cat # CP010). The ST-Mut-5 lipase gene was artificially synthesized by GenScript (Piscataway, New Jersey, U.S.A.). Each mutant was confirmed by restriction enzyme digestion and DNA sequencing.

5. Transposition and transfection

The *in vitro* transposition was performed with bEasyBac bacmid DNA and donor vectors to construct recombinant baculoviruses. The transposition reaction was performed using Gateway® LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, USA) (Fig. 4, 5, and 6). Each 150 ng of bEasyBac bacmid DNA and donor vectors were mixed, and 2 μl of LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, USA) was added. The mixture was mixed well by vortexing briefly twice. They were centrifuged briefly and incubated at 25°C overnight (approximately 14-16 hrs). After incubation, 1 μl of proteinase K solution was added to the mixture and incubated at 37°C for 10 min.

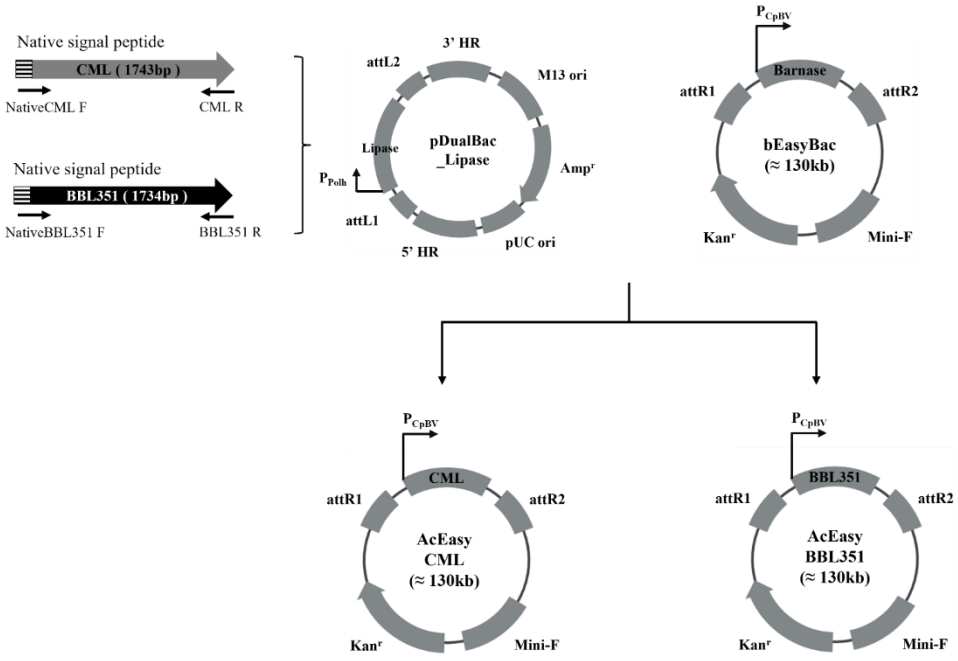


Fig. 4. Generation of recombinant baculoviruses expressing CML and BBL351. The CML and BBL351 genes were transposed into bEasyBac bacmid DNA to obtain AcEasy_CML and AcEasy_BBL351.

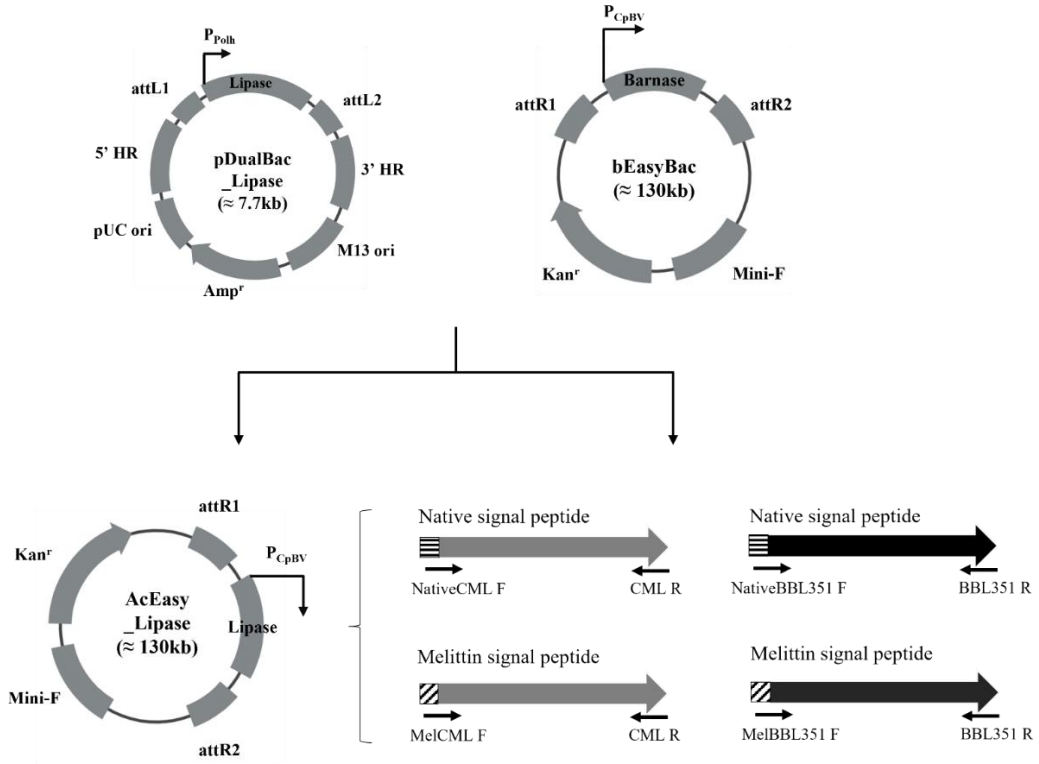


Fig. 5. Generation of recombinant baculoviruses expressing CML and BBL351 with signal peptide. The CML and BBL351 genes were transposed into bEasyBac bacmid DNA to obtain AcEasy_NativeCML, AcEasy_MelCML, AcEasy_NativeBBL351 and AcEasy_MelBBL351.

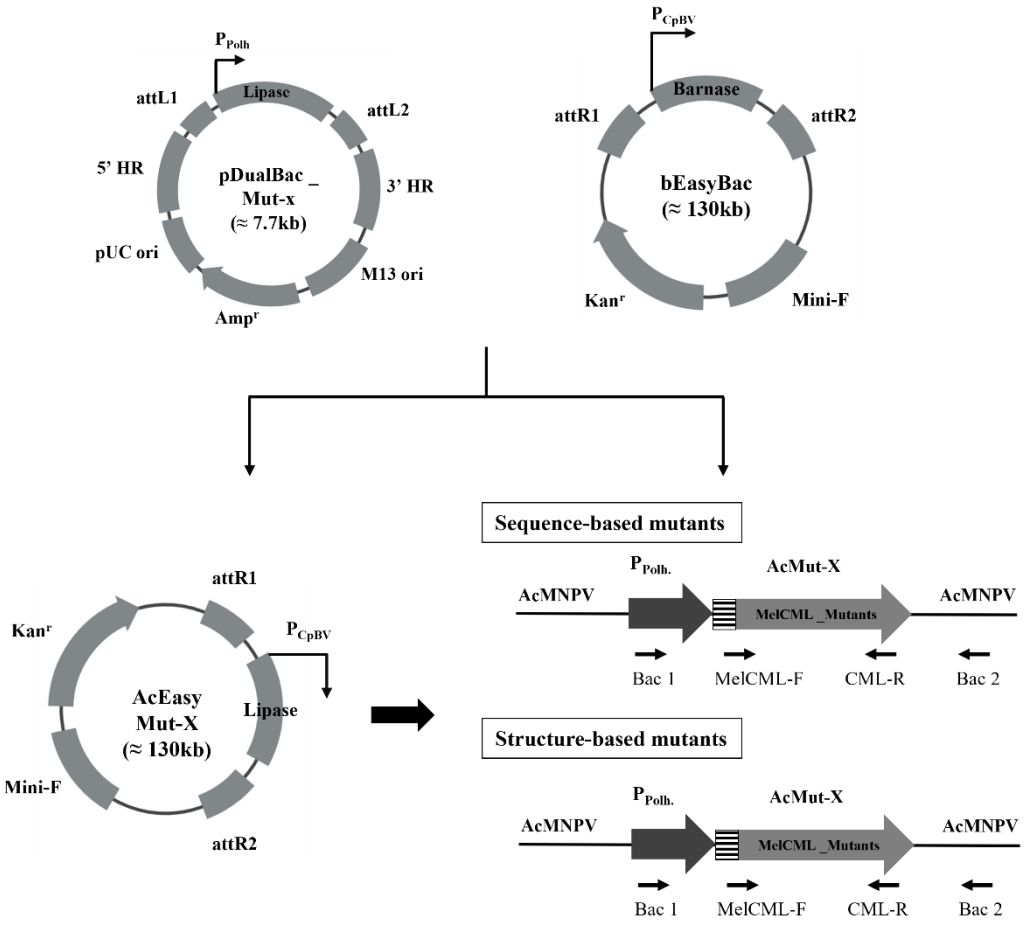


Fig. 6. Generation of recombinant baculoviruses expressing MelCML mutants. The mutant MelCML genes were transposed into bEasyBac bacmid DNA to obtain AcEasy_SE-Mut-X and AcEasy_ST-Mut-X, the capital letter 'X' in AcEasy_SE-Mut-X represents 2318, 4171, 417, 31, 316, 4117, 413, 416, 411, 352, 46, 466, and 35 and in AcEasy_ST-Mut-X, the capital letter 'X' represents 1, 2, 3, 4, 5, and 6. Solid arrows indicate primer position used in PCR amplification.

After *in vitro* transposition, approximately 1×10^5 Sf9 cells per well were seeded in a 24-well tissue culture plate and incubated at 27°C for 30 min for cell attaching. The transposition mixture was added to 100 µl of incomplete TC-100 medium in a 5 ml polystyrene round bottom tube (Falcon, Bedford, MA, USA). In another polystyrene tube, 8 µl of Cellfectin™ II Reagent (Thermo Fisher Scientific, USA) was mixed with 100 µl of incomplete TC-100 medium per sample. The two solutions were gently mixed, and the mixture was incubated at room temperature for 45 min. The attached cells were washed twice with 1 ml of incomplete TC-100 medium and refreshed with 0.5 ml of the same medium. The Cellfectin-DNA complex mixtures were divided into five amounts and added dropwise per well at 27°C for 5 hrs. Afterward, each well was refreshed with 2 ml of TC-100 medium supplemented with 10% FBS with antibiotic-antimycotic (Thermo Fisher Scientific, USA) and incubated at 27°C for 5 days. After 5 days, the supernatant was harvested and centrifuged at 500×g for 5 min stored at 4°C.

6. Infection of recombinant baculoviruses

For mass amplification, 5×10^6 cells were seeded into 100 mm diameter tissue culture dishes and inoculated with each recombinant virus at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) / cell. After incubation at 27°C for 3 days, the supernatant was 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 (O'Reilly, Miller, & Luckow, 1994). For mass expression of lipases using insect cells, High-Five cells were infected with 5 MOI of recombinant baculovirus and incubated at 27°C for 3 days.

7. Extraction of viral DNA

When virus symptoms were observed in infected cells, cell pellets were harvested by centrifugation at 500×g for 5 min. The 250 µl distilled water and 250 µl lysis buffer (50 mM Tris-HCl (pH 8.0), 0.4% SDS, 10 mM EDTA, 5% 2-Mercaptoethanol) were mixed with the pellet. The 5 µl of RNase A was added and incubated at room temperature for 10 min. The proteinase K (Sigma Aldrich, Inc. St. Louis, MO. USA) 15 µl was added to the mixture and incubated at 37°C overnight. After that, 600 µl of buffer-saturated phenol/chloroform/isoamyl alcohol (25: 24: 1; pH 7.5-7.8) were added, vortexed for 1 min, and centrifuged at 15,000 rpm 5 min at 4°C. The aqueous layer was transferred to a 1.5 ml tube, mixed with 500 µl of buffer-saturated chloroform / isoamyl alcohol (24: 1), vortexing for 1 min, and centrifuged for 7 min at 4°C. The supernatant 400 µl, 3M sodium acetate (pH 5.0) 40 µl, and 100% EtOH 800 µl were gently inverted and incubated at -70°C for 2 hrs. The mixture was centrifuged at 15,000 rpm for 30 min and discard the supernatant. The supernatant was removed, and the pellet resuspended in 1 ml of 70% ethanol, vortexed, and centrifuged for 2 min at 13,000 rpm. The supernatant was removed again, and the remaining ethanol was eliminated by air-dried. Finally, virus DNA was suspended with 50-70 µl of distilled water and performed PCR amplification with specific primers.

8. RNA extraction and reverse transcription PCR (RT-PCR)

According to the manufacturer's instructions, the total RNA of recombinant baculoviruses were extracted using the QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA). RT-PCR and cDNA synthesis were conducted with AccuPower® RT-PCR PreMix (Bioneer, Korea) with lipase-specific primers according to the manufacturer's instructions.

9. SDS-PAGE

The secretory protein expressed by recombinant baculovirus-infected cells was exhibited on the Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant of infected cells was mixed with the same volume of Sample buffer, Laemmli 2× Concentrate (Sigma Aldrich, Inc. St. Louis, MO. USA), and heated at 100°C for 15 min. After heating, samples were loaded into 12% Mini-PROTEAN® TGX™ Precast Gels (BIO-RAD, USA) and stained with Coomassie brilliant blue for 1 hr. The PAGE gel was destained with destaining solution (methanol, 45%; acetic acid, 10%; distilled water, 45%) for overnight (16-18 h).

10. *p*NP assay

Lipase activity assay was measured by spectrophotometric method with *p*-nitrophenyl substrates containing ester bonds. In the spectrophotometric analysis method, *p*-nitrophenol, the colored product, was measured as a result of the hydrolysis of *p*-nitrophenyl substrates by lipase. The *p*NPP assay, which uses *p*-nitrophenyl palmitate (*p*NPP) as a substrate, is the most common measure of lipase activity with 2mM *p*NPP solution (0.757 g *p*-nitrophenyl palmitate (*p*-NPP), 10 g Triton X-100 and 0.17 g sodium dodecyl sulfate (SDS) per 1 liter of distilled water). The solution was heated at 65°C, followed by magnetic stirring at 400 rpm until the *p*NPP was dissolved clearly. The 2mM *p*NPP solution (95 µl), 50mM Tris–HCl buffer (pH 8.0), and 10 µl of the recombinant baculoviruses infected cell supernatants (extra-cellular secretory lipase protein) were added to a 96-well plate. When measuring intra-cellular lipase activity, the cell pellets were sonicated for 120 sec and centrifuged at 15,000 rpm for 10 min. The 96-well plate with samples was incubated at 37°C for 3-5 min.

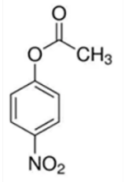
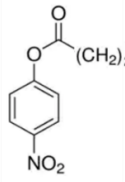
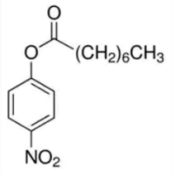
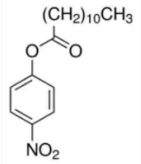
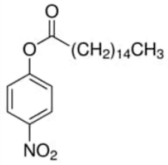
Afterward, the lipase activity was measured by entering the 96-well plate into the microplate reader at 400 nm, 37°C, and 5 min intervals for 25 min.

In order to measure the lipase activity along substrate carbon chain length, *p*-nitrophenyl substrates with various carbon lengths were used, which are *p*-nitrophenyl acetate (4-Nitrophenyl acetate), *p*-nitrophenyl butyrate (4-Nitrophenyl butyrate), *p*-nitrophenyl octanoate (4-Nitrophenyl octanoate), *p*-nitrophenyl dodecanoate (4-Nitrophenyl dodecanoate) (Table 4). The lipase activity was measured using the same previous method with each *p*-nitrophenyl substrate. One unit of activity was defined as the amount of enzyme liberating 1 μ mol *p*-nitrophenol per min.

11. Concentration of lipase proteins

Measure the volume of the infected cell supernatant and determine the amount of ammonium sulfate (YAKURI, Japan) by saturating 0% to 45% and 45% to 80% according to the ammonium sulfate calculator (https://www.encorbio.com/protocols/AM-SO4.htm). Dissolved the ammonium sulfate by stirring 300-400 rpm at 4°C for 1 hr 30 min for each saturation. The ammonium sulfate precipitated proteins were centrifuged at 10,000×g for 30 min at 4°C. The collected pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.0) and dialyzed for overnight (14-16 h) in the same buffer using Slide-A-Lyzer™ G2 Dialysis Cassettes, 20K MWCO (Thermo Fisher Scientific, USA). The concentrated protein was determined by the Bradford protein assay.

Table 4. The *p*-Nitrophenyl (*p*NP) substrates used for measuring lipase activity.

***p* – Nitrophenyl (*p*NP) substrates**				
				
***p* – Nitrophenyl acetate (C2)**	***p* – Nitrophenyl butyrate (C4)**	***p* – Nitrophenyl octanoate (C8)**	***p* – Nitrophenyl dodecanoate (C12)**	***p* – Nitrophenyl palmitate (C16)**

RESULTS

1. Biological characteristics of entomopathogenic fungi lipase

The CML and BBL351 belonged to the same α/β hydrolase superfamily lipase domain. Also, both lipases have signal peptides on the front side of the lipase sequence, expressed as secretory protein forms (Fig. 7).

1-1. Comparative study of lipase activity from BBL351 and CML

A. Construction of recombinant baculoviruses

The pDualBac_Lipase donor vectors were constructed and confirmed by restriction enzyme digestion and nucleotide sequencing to generate the recombinant baculoviruses (Fig. 8). Lipase genes were inserted under the control of the polyhedrin promoter. The *in vitro* transposition reaction between the baculovirus genome, bEasyBac, and pDualBac_Lipase donor vectors was performed for constructing the recombinant baculoviruses. They were transfected into Sf9 cells and observed post-infection 5 days (Fig. 9).

B. Verification of recombinant baculoviruses

After virus symptoms were observed in infected insect cells, RT-PCR and viral DNA PCR were performed to confirm the lipase genes correctly inserted into recombinant baculoviruses. To verify the internal genome structure of recombinant baculoviruses, each extracted virus DNA was amplified by PCR with specific primers at four different

- ***Cordyceps militaris*** **lipase (CML)**

- ***Beauveria bassiana*** **JEF351 lipase (BBL351)**

Fig. 7. Model of CML and BBL351 lipase domain.

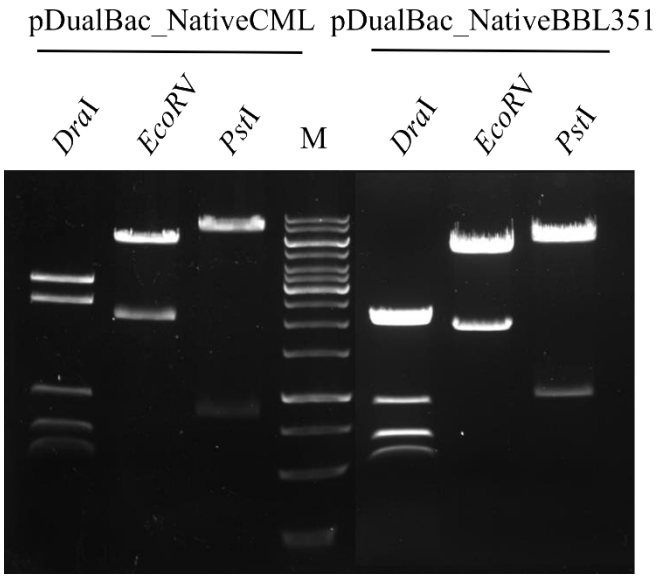


Fig. 8. The restriction endonuclease digestion pattern of lipase genes into baculovirus donor vector. Lane, M, 1Kb DNA Ladder; D, *Dra*I; E, *Eco*RV; P, *Pst*I

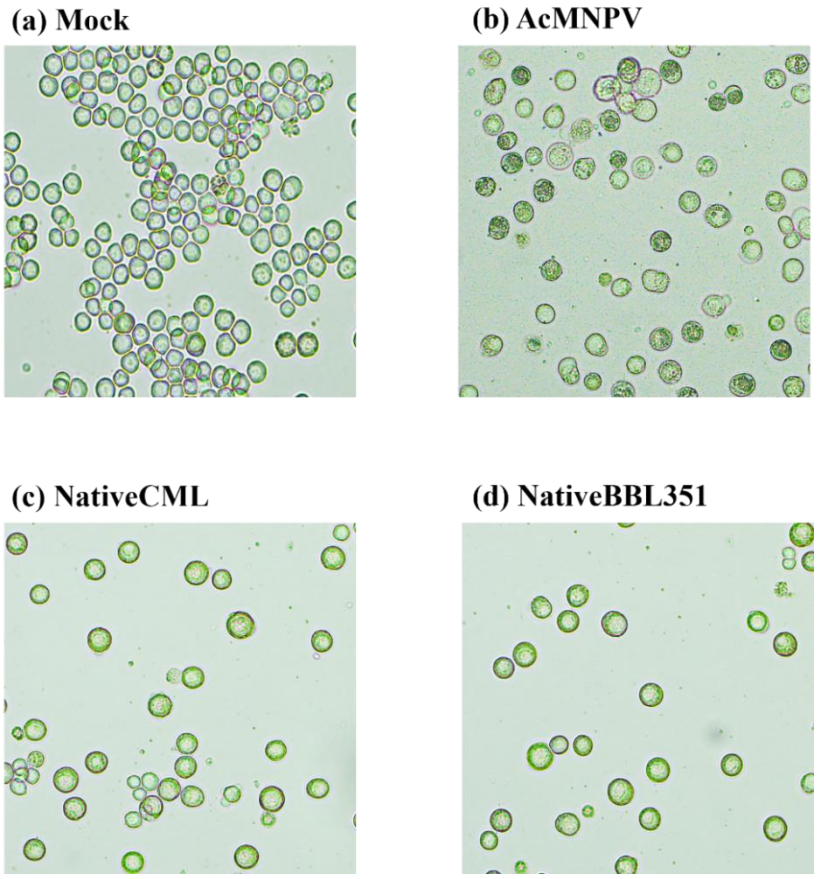


Fig. 9. The Sf9 cells were infected with AcEasy_NativeCML and AcEasy_NativeBBL351. (a) Mock, not-infected Sf9 cells; (b) AcMNPV, Sf9 cells infected AcMNPV; (c), Sf9 cells infected with AcEasy_NativeCML; (d), AcEasy_NativeBBL351.

sites. Approximately 1.7 kb of lipase genes were amplified with virus DNA. As a negative control, AcMNPV DNA without lipase genes was amplified at about 0.8 kb (Fig. 10). RT-PCR was conducted to confirm transcription, and approximately 1.7 kb of lipase genes were transcribed with cDNA in Lane 3. Lane 1, AcMNPV, negative control without lipase gene, was not amplified with lipase-specific primer. Lane 2, PCR amplified with pDualBac_Lipase, as a positive control with lipase genes (Fig. 11).

C. Confirmation of enzyme activity by lipase assay

In SDS-PAGE, approximately 64 kDa proteins were exhibited with the expected size of each lipase protein (Fig. 12). Lipase activity was measured with recombinant baculovirus-infected High-Five cells. Extra-cellular lipase activity was measured as a secretory protein, and intra-cellular lipase activity was measured as a non-secretory protein. As a result, BBL351 showed approximately 2-fold higher lipase activity than CML, and both lipase activities showed in extra-cellular (Fig. 13). Lipase activity was measured by the substrate carbon chain length to determine the biological properties. Consequently, both lipases show the highest activity against *p*-nitrophenyl butyrate (Fig. 14).

1-2. Establishment of mass expression conditions

To make a more efficient expressed form of lipase, the melittin signal peptide derived from the honeybee, which was widely used in insects, was replaced with its native signal peptides by PCR amplification. As a result, four lipase genes were synthesized and cloned into the pDualBac donor vector. They were confirmed by restriction enzyme digestion and nucleotide sequence analysis (Fig. 15).

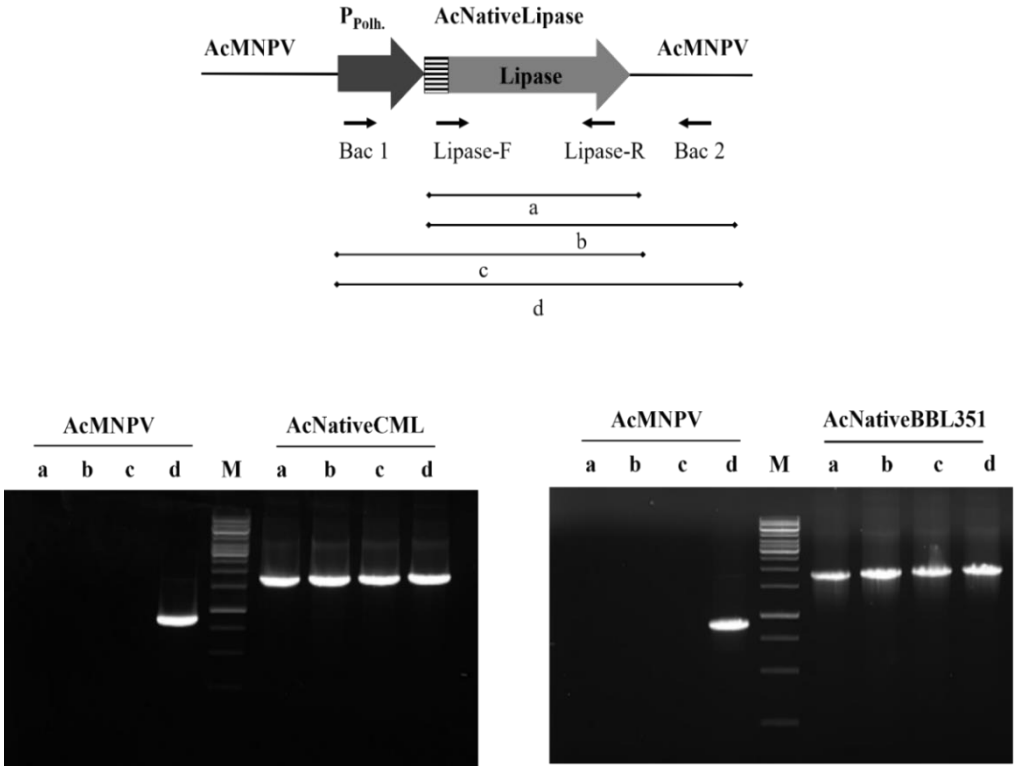


Fig. 10. Confirmation of internal genome structure of AcEasy_NativeCML and AcEasy_NativeBBL351 by viral DNA PCR. PCR amplification was performed at four positions using specific primer sets. Lane: M, 1Kb DNA Ladder.

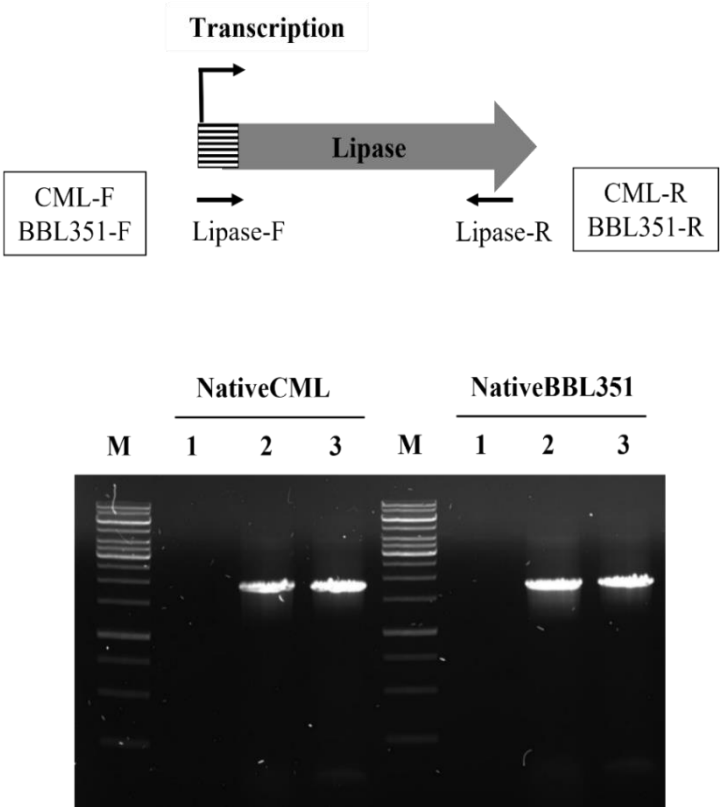


Fig. 11. Confirmation transcription of AcEasy_NativeCML and AcEasy_NativeBBL351 by RT-PCR. Lane: M, 1Kb DNA Ladder; 1, AcMNPV 2, pDualBac_NativeCML, and pDualBac_NativeBBL351; 3, AcEasy_NativeCML and AcEasy_NativeBBL351.

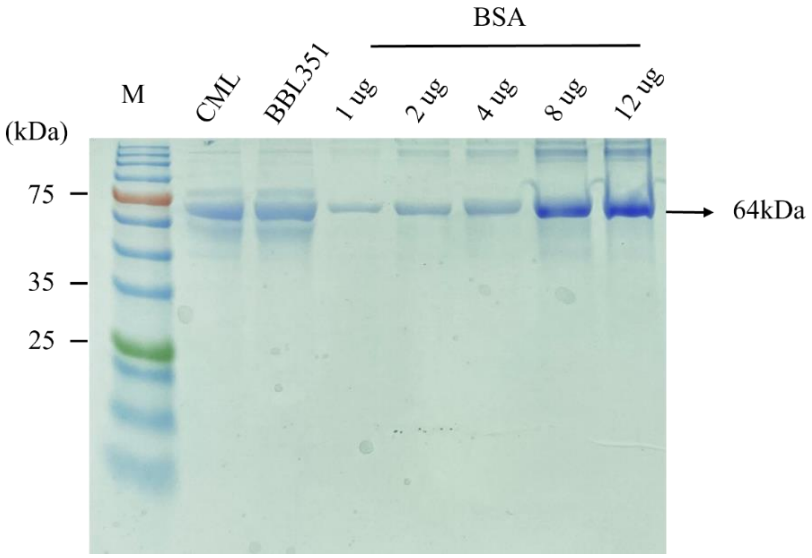


Fig. 12. Analysis of CML and BBL351 protein expression by SDS-PAGE. Lipases were expressed in High-Five cells. Lane: M, 3-color prestained protein size marker.

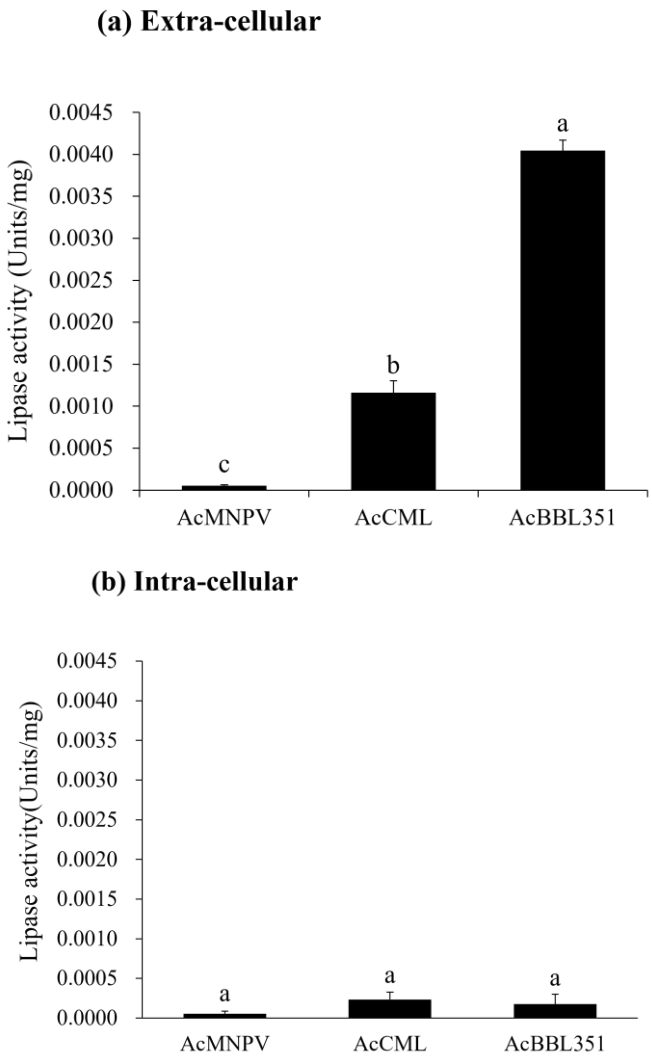


Fig. 13. Lipase activity of Ac_NativeCML and Ac_NativeBBL351 by *p*NPP assay. Expression of lipase proteins in High-Five cells infected with recombinant baculoviruses. (a) Lipase activity measurment at extra-cellular in infected cells. (b) Lipase activity measurment at intra-cellular in infected cells. High-Five cells were seeded at a density of 5×10^6 cells/ dish. The infected cells were harvested at post-infection 3 days.

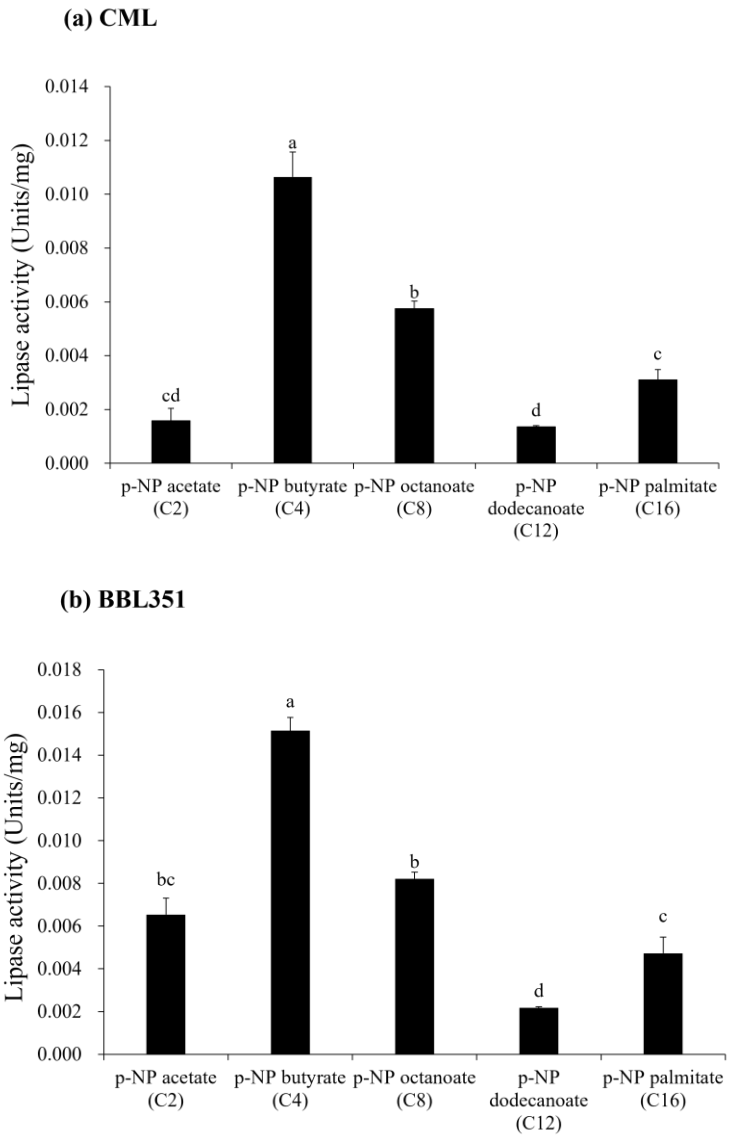


Fig. 14. Comparison of enzyme activities along substrate carbon chain length. (a) The lipase activity of CML against *p*-Nitrophenyl substrates, (b) The lipase activity of BBL351 against *p*-Nitrophenyl substrates.

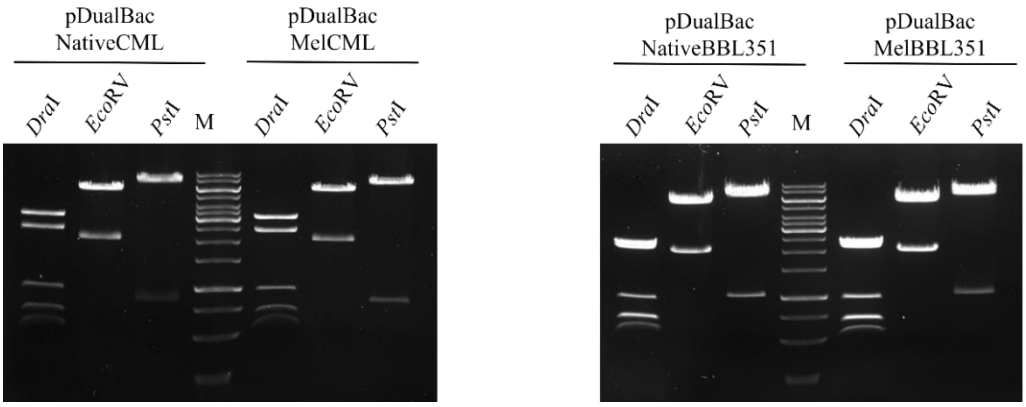


Fig. 15. The restriction endonuclease digestion pattern of lipase genes into baculovirus donor vector. Lane, M, 1Kb DNA Ladder; D, *Dra*I; E, *Eco*RV; P, *Pst*I

Recombinant baculoviruses were constructed with four lipase genes. They were confirmed by cell symptoms infected by recombinant baculoviruses (Fig. 16), viral DNA PCR (Fig. 17), RT-PCR (Fig. 18), and SDS-PAGE (Fig. 19). All the lipase genes were inserted into the virus genome properly, and lipase expression was confirmed through SDS-PAGE. Measuring the lipase activity of four types of lipases, BBL351 showed approximately 2-fold higher lipase activity than CML. Among the CML, lipase with melittin signal peptide (MelCML) showed higher activity than lipase with native signal peptide (NativeCML). The BBL351, lipase with native signal peptide (NativeBBL351) exhibited higher activity than lipase with melittin signal peptide (MelBBL351) (Fig. 20). Therefore, CML with melittin signal peptide and BBL351 with native signal peptide were selected for mass expression with their high activities.

A. Selection of insect cells for mass expression of lipases

The lipase activity was compared against insect cell types according to the inoculation concentrations and sample types to select efficient insect cells. The Sf9 and High-Five cells were inoculated with each recombinant virus at a multiplicity of infection (MOI) of 1, 5, and 10 plaque-forming units (PFU) / cell, respectively. As a result, regardless of the inoculation concentration and sample, the High-Five cells showed higher activity than the Sf9 cells (Fig. 21). When comparing the total amount of protein expressed by two kinds of cell types, the High-Five cells expressed slightly higher or similar amounts of protein than the Sf9 cells (Fig. 22). These results suggested that High-Five cells exhibited higher lipase activity and better lipase quality than Sf9 cells. Therefore, the High-Five cell was selected as an efficient insect cell for mass expression.

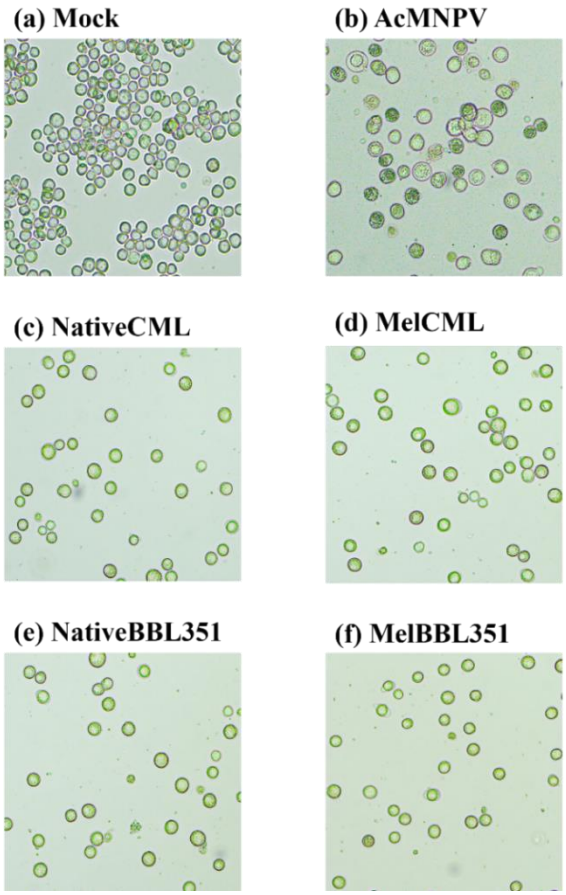


Fig. 16. The Sf9 cells were infected with AcEasy_NativeCML, AcEasy_MelCML, AcEasy_NativeBBL351 and AcEasy_MelBBL351. (a) Mock, not-infected Sf9 cells; (b) AcMNPV, Sf9 cells infected AcMNPV; (c) ~ (f), Sf9 cells infected with recombinant baculoviruses, AcEasy_NativeCML, AcEasy_MelCML, AcEasy_NativeBBL351 and AcEasy_MelBBL351.

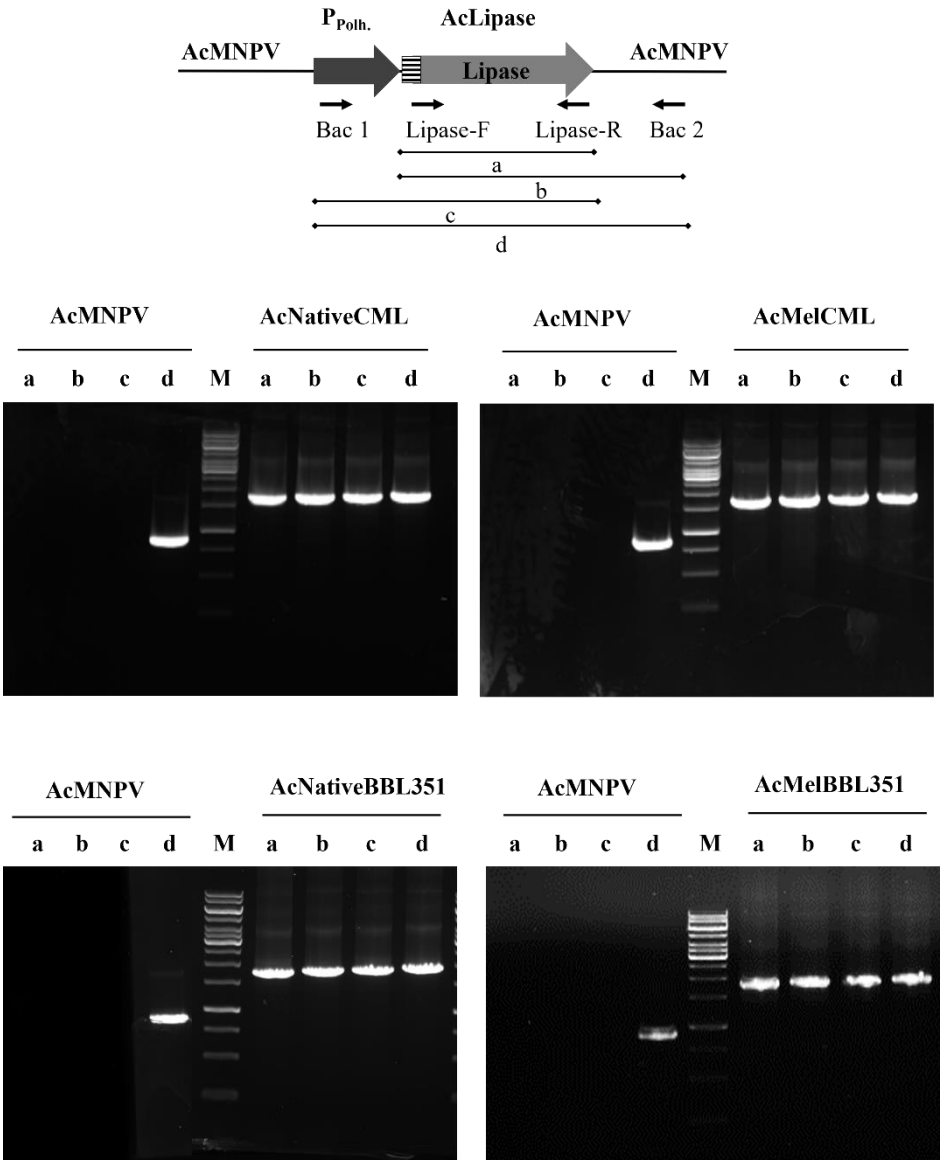


Fig. 17. Confirmation of internal genome structure of recombinant baculoviruses by viral DNA PCR. PCR amplification was performed at four positions using specific primer sets. Lane: M, 1Kb DNA Ladder.

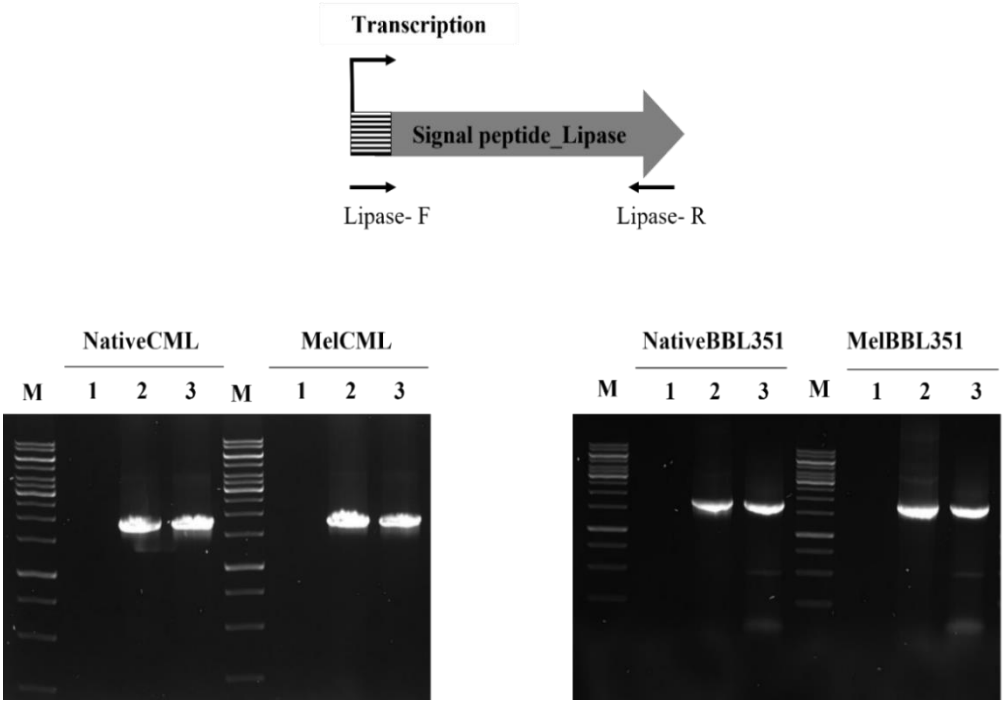


Fig. 18. Confirmation transcription of recombinant baculoviruses by RT-PCR. Lane: M, 1Kb DNA Ladder; 1, AcMNPV 2, pDualBac_NativeCML, pDualBac_MelCML, pDualBac_NativeBBL351, and pDualBac_MelBBL351; 3, AcEasy_NativeCML, AcEasy_MelCML, AcEasy_NativeBBL351 and AcEasy_MelBBL351.

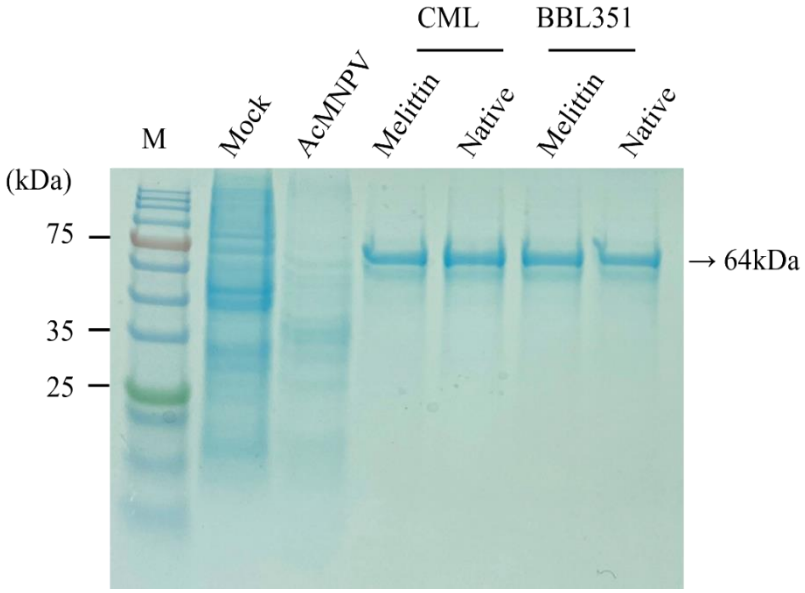


Fig. 19. Analysis of lipase proteins expression by SDS-PAGE. Proteins were expressed as the secretory form in High-Five cells. Lane: M, 3-color prestained protein size marker; Mock, not-infected High-Five cell.

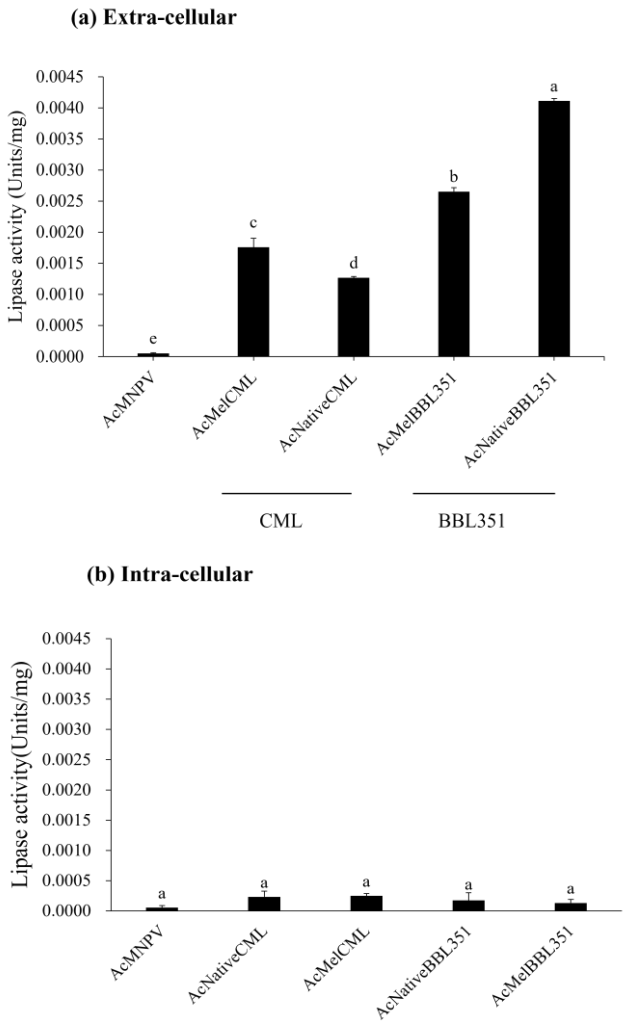


Fig. 20. Lipase activity of Ac_NativeCML, Ac_MelCML, Ac_NativeBBL351 and Ac_MelBBL351 by *p*NPP assay. Expression of lipase proteins in High-Five cells infected with recombinant baculoviruses. (a) Lipase activity measurement at extra-cellular in insect cells infected with recombinant baculoviruses. (b) Lipase activity measurement at intra-cellular in insect cells infected with recombinant baculoviruses. High-Five cells were seeded at a density of 5×10^6 cells/dish. The infected cells were harvested at post-infection 3 days.

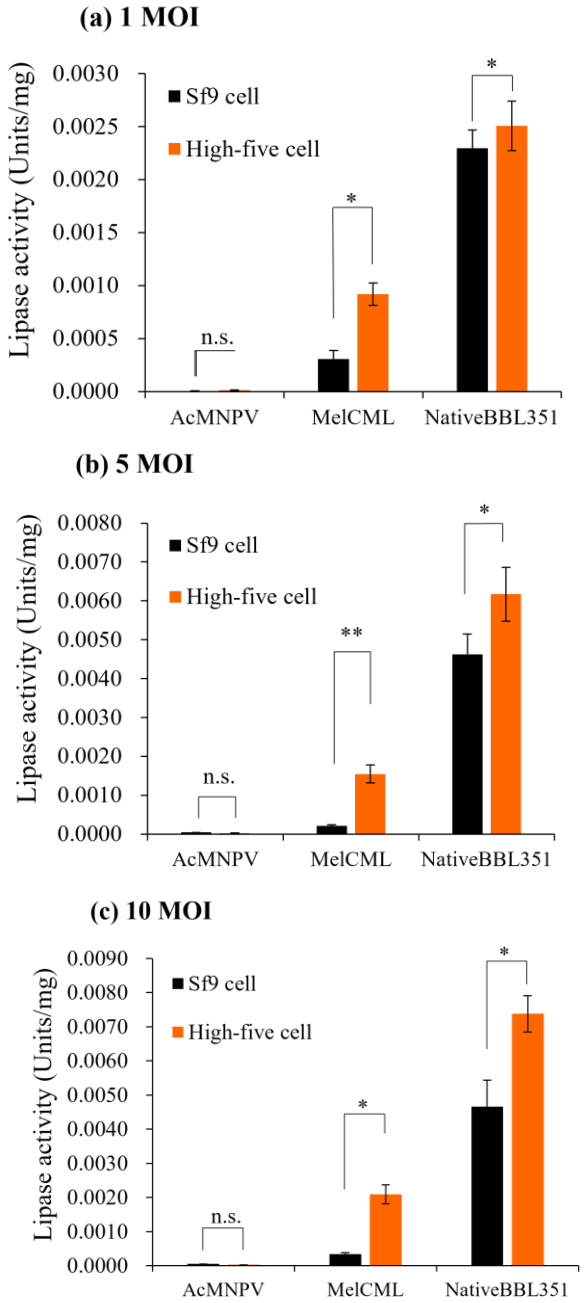


Fig. 21. Comparison of lipase activity of CML and BBL351 infected Sf9 cells and High-Five cells. (a) ~ (c), innoulum concentration; (a) 1 MOI; (b) 5 MOI; (c) 10 MOI

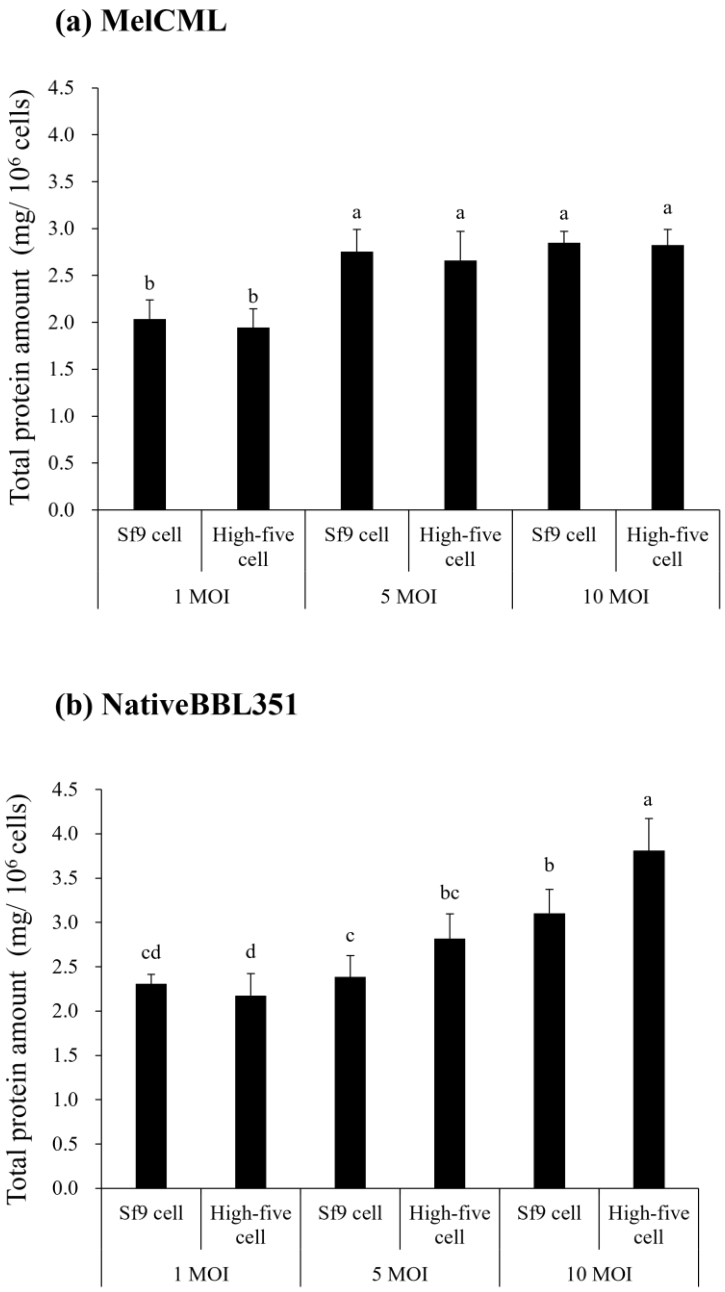


Fig. 22. Estimation of total protein amount with Sf9 cells and High-Five cells.

B. Time-course expression of lipases in High-Five cells

After selecting an efficient insect cell, to determine the appropriate inoculation concentration and harvest timing of the recombinant baculoviruses, the lipase activity was measured for 5 days infected at 1, 5, and 10 MOI by time-course. As a result, the final lipase activity reached almost the same for each inoculation concentration. However, it was confirmed that when cells were infected at 5 and 10 MOI, which are high MOI that over 90% of cells infected at once, the lipase activity reached the high level fast (Fig. 23). Therefore, 5 MOI inoculation concentration was selected for efficient expression which was using a small amount of inoculum and exhibited high activities. Harvest timing was chosen as post-infection 3 days before the cells were degraded after being infected with recombinant baculoviruses.

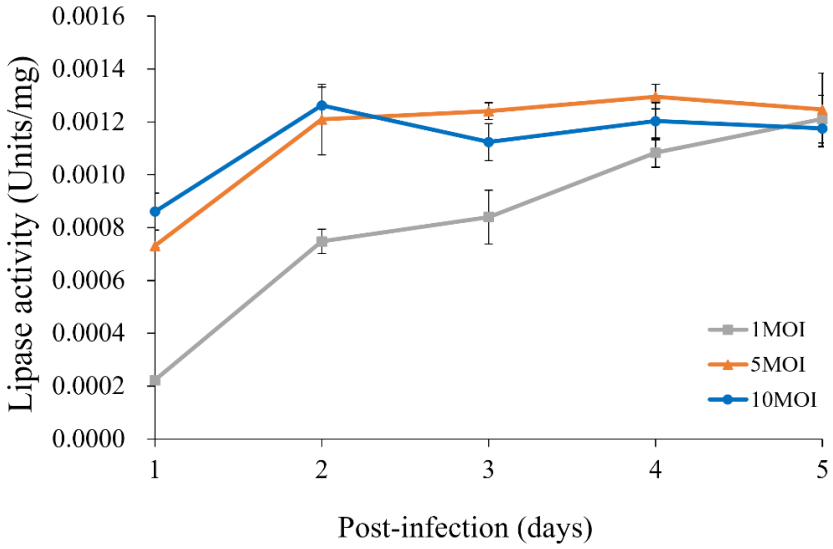
2. Improvement of lipase activity through CML mutagenesis

Based on results from the previous experiment, BBL351 exhibited higher lipase activity than CML. Therefore, to make the CML, which has regioselectivity, into a more active lipase like BBL351, mutants were constructed by comparing the amino acid sequences of the two lipases.

2-1. Construction of sequence-based CML mutants

The amino acid sequence of MelCML and NativeBBL351 which were selected as showing higher lipase activity than others was compared and both lipase sequences showed approximately 70% similarity. Also, the catalytic triad, which is the active site of the enzyme, well preserved in the two lipases in a red box, and the part marked with green

(a) MelCML



(b) NativeBBL351

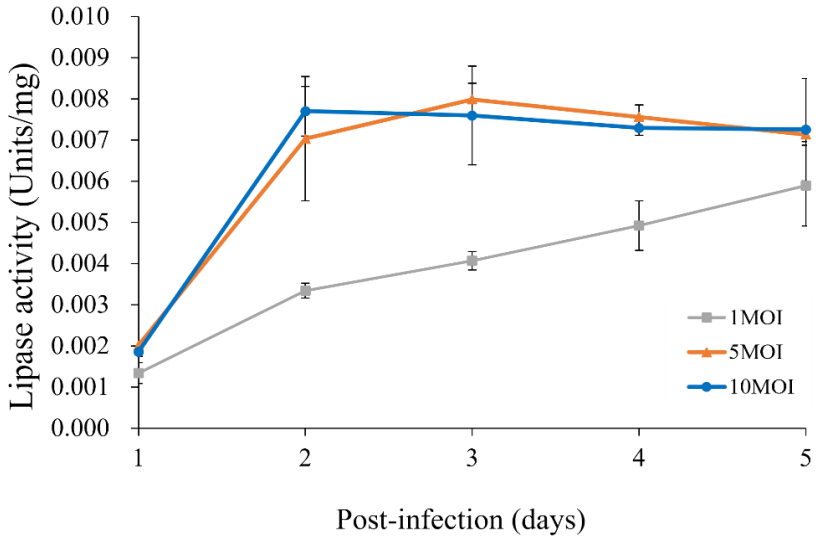


Fig. 23. Analysis of lipase activity at the time-course expression of CML and BBL351 in High-Five cells.

boxes indicate a substrate binding pocket where the substrates were attached to the lipase. Five parts with different amino acid properties were selected as mutation sites by analyzing the amino acid sequences of the two lipases. Then the multi-site-directed mutagenesis was performed with these five sites (Fig. 24). Total 13 types of mutants were constructed using 1 to 3 pairs of specific primers containing a mutant site through several repetitions (Fig. 25). Mutants were named Mut-X (the capital letter 'X' represents the clone number), respectively, and the mutated regions were confirmed by restriction enzyme digestion and DNA sequencing (Fig. 26). The constructed mutants were expressed using baculovirus expression systems. They were confirmed by observing virus symptoms in infected insect cells (Fig. 27), viral DNA PCR (Fig. 28), RT-PCR (Fig. 29), and SDS-PAGE (Fig. 30). According to the verification processes, all the mutant lipase genes were inserted to virus genome correctly, transcription, and translation was well performed. Approximately 1.7 kb amplified lipase genes, and 64 kDa of expressed lipase proteins were identified with recombinant baculoviruses. As a result of the lipase activity assay, it was confirmed that all 13 mutants had lost lipase activity like the wild-type virus, AcMNPV (Fig. 31).

2-2. Construction of structure-based CML mutants

The structure of CML was predicted based on the closest lipase in the sequence. As a result, the lid-like structure was present in the CML. The lid appeared upside of the catalytic triad in two states, opened and closed form (Fig. 32). By comparing amino acid sequences of CML and BBL351 based on the three-dimensional structure of CML, total 6 mutant sites were selected (Fig. 33). The four sites around the catalytic triad where substrates were binding were selected. Other mutants related to the lid and eliminated the lid structure were

selected. Five mutants were constructed through the multi-site-directed mutagenesis method as sequence-based CML mutants. Another mutant that deleted the lid structure was produced by requesting gene synthesis (Fig. 34). In order to identify six types of mutants, restriction enzyme digestion and nucleotide sequencing were performed, and recombinant baculoviruses were constructed with confirmed donor vectors (Fig. 35).

The virus symptoms were observed in infecting Sf9 cells with recombinant baculoviruses (Fig. 36). Approximately 1.7 kb of lipase genes were amplified with each viral DNA (Fig. 37) and transcribed by RT-PCR (Fig. 38). In addition, it was confirmed that 64 kDa of lipases were expressed through SDS-PAGE (Fig. 39). Meanwhile, Mut-3 and Mut-4 showed higher activity than the control of CML by measuring the lipase activity (Fig. 40).

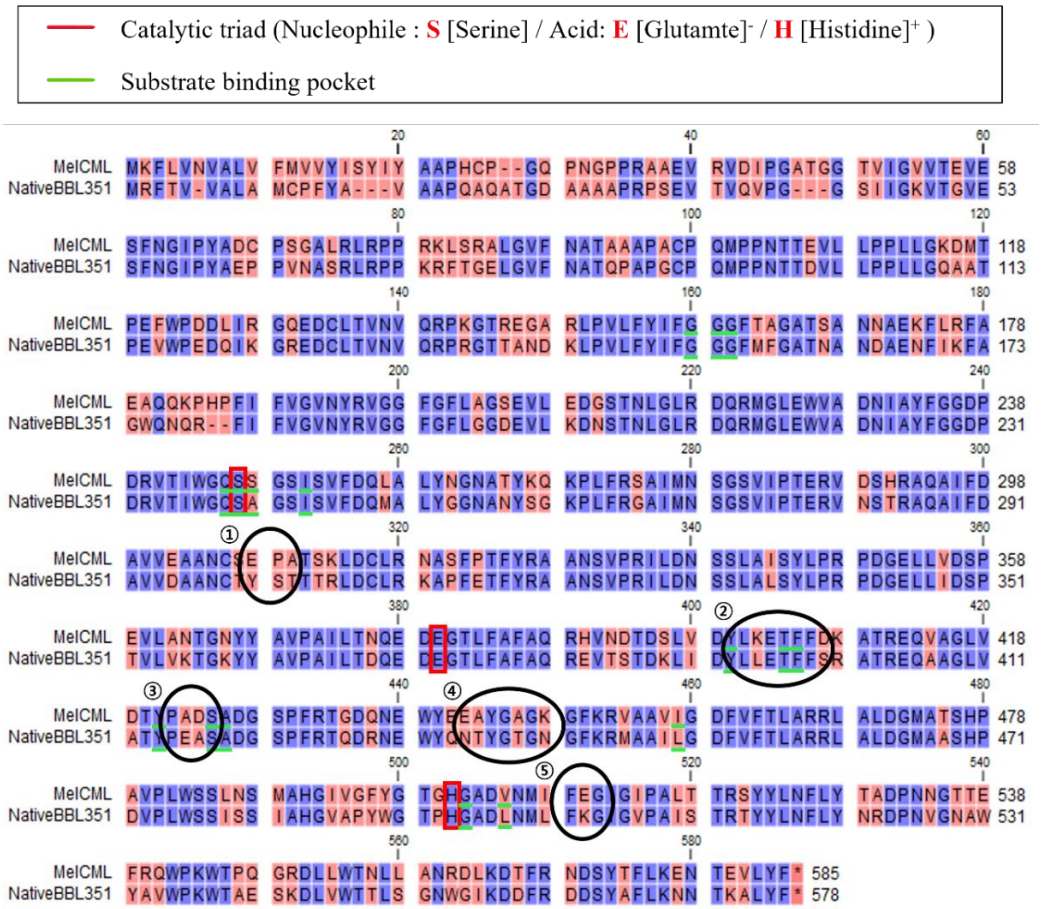


Fig. 24. The amino acid sequence-based comparison of CML and BBL351.

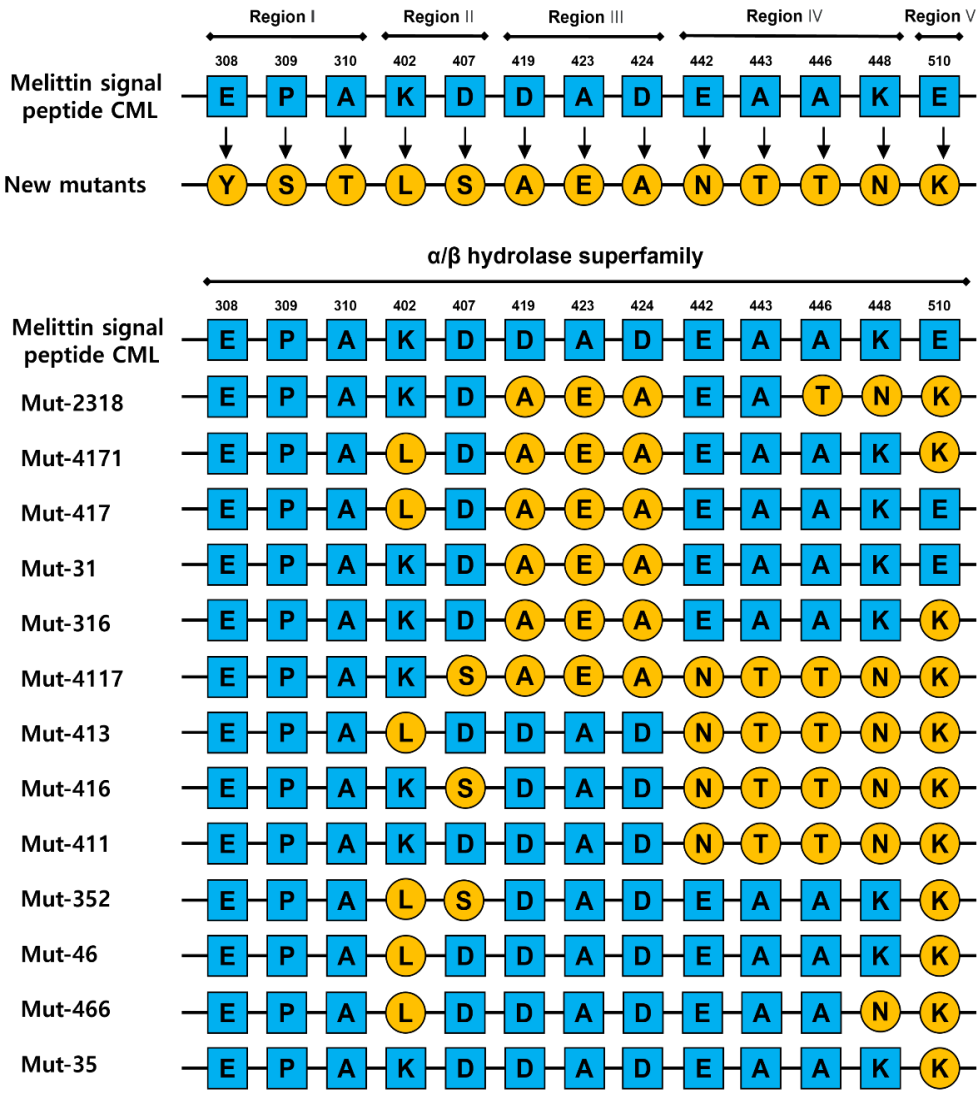


Fig. 25. Mutated residues of 13 sequence-based CML mutants. Ellipse represents mutated amino acid sequences and rectangle represents unchanged amino acid sequences.

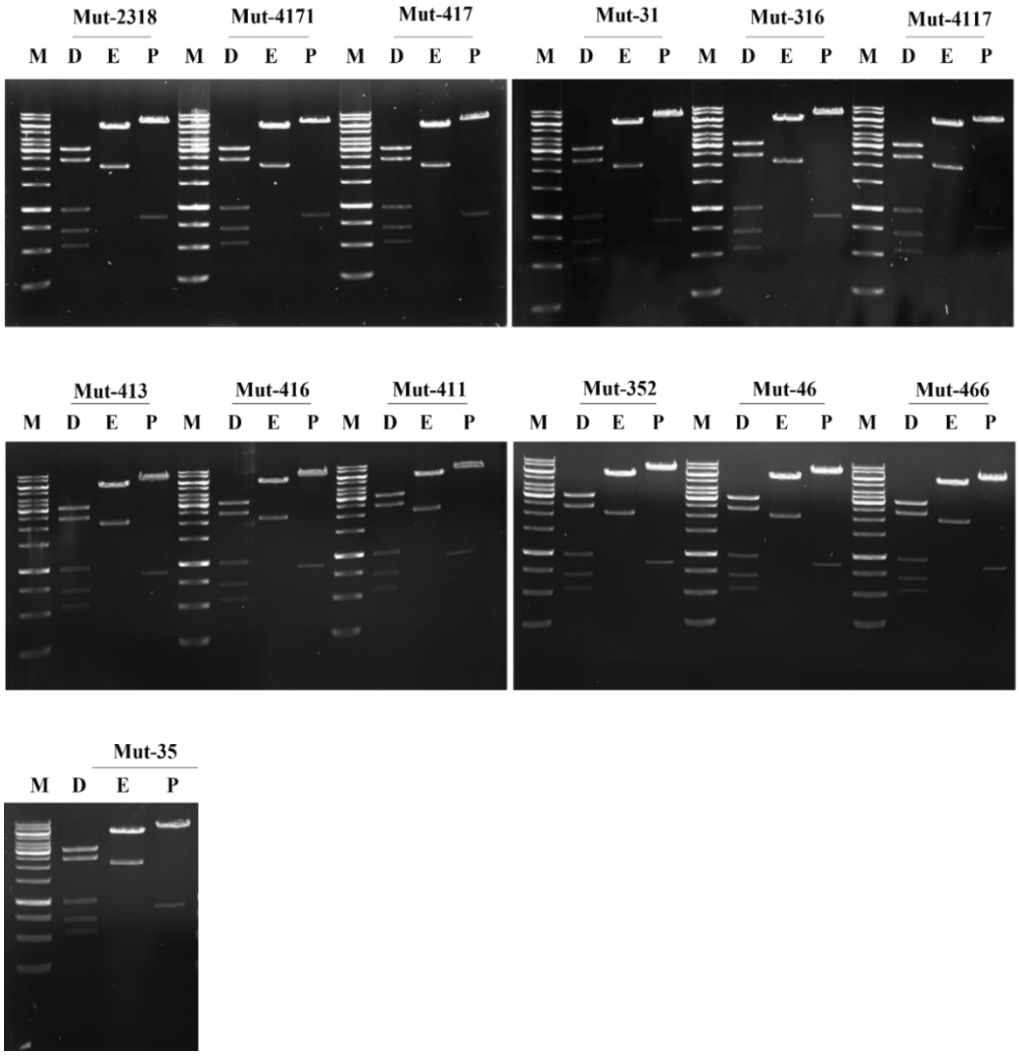


Fig. 26. The restriction endonuclease digestion pattern of sequence-based mutant lipase genes into baculovirus donor vector. Lane, M, 1Kb DNA Ladder; D, *Dra*I; E, *Eco*RV; P, *Pst*I

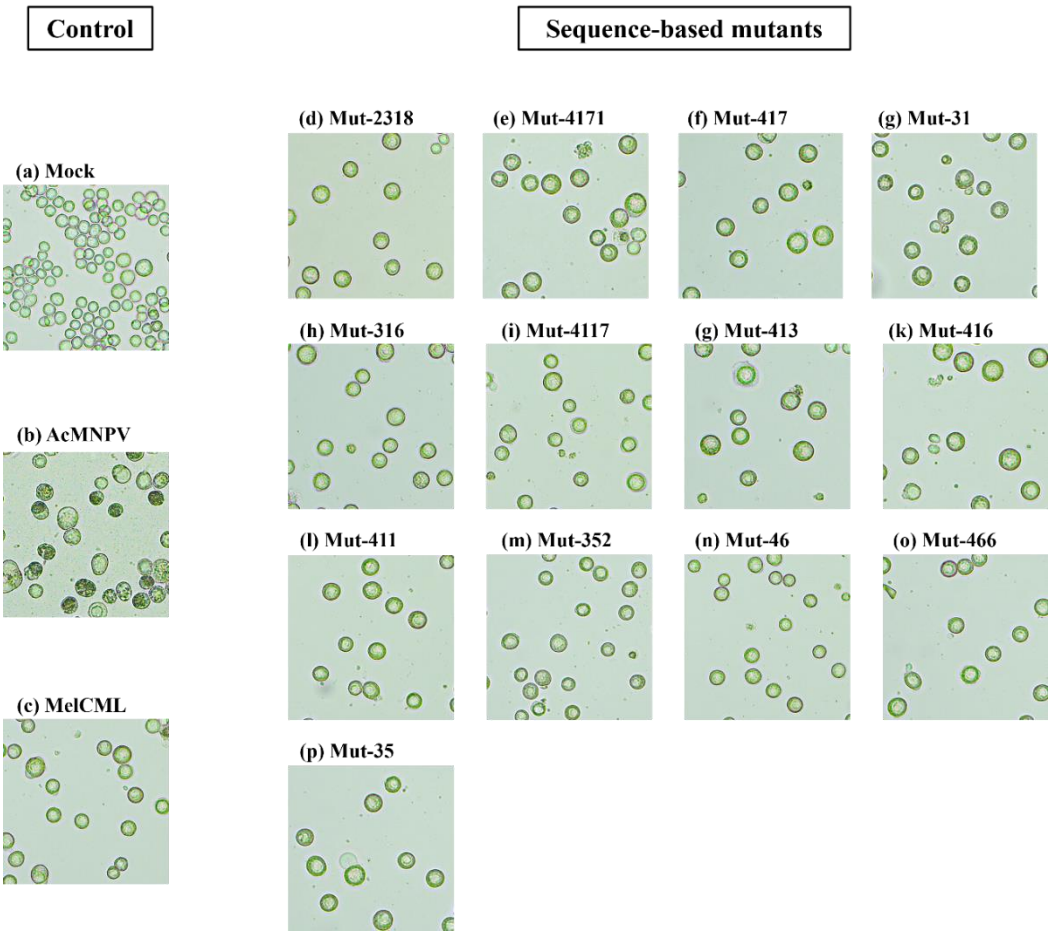


Fig. 27. The Sf9 cells were infected with sequence-based recombinant baculoviruses. (a) Mock, not-infected Sf9 cells; (b) AcMNPV, Sf9 cells infected AcMNPV; (c) ~ (p), Sf9 cells infected with recombinant baculoviruses, AcEasy_MelCML, AcEasy_Mut-X (X, 2318, 4171, 417, 31, 316, 4117, 413, 416, 411, 352, 46, 466, and 35).

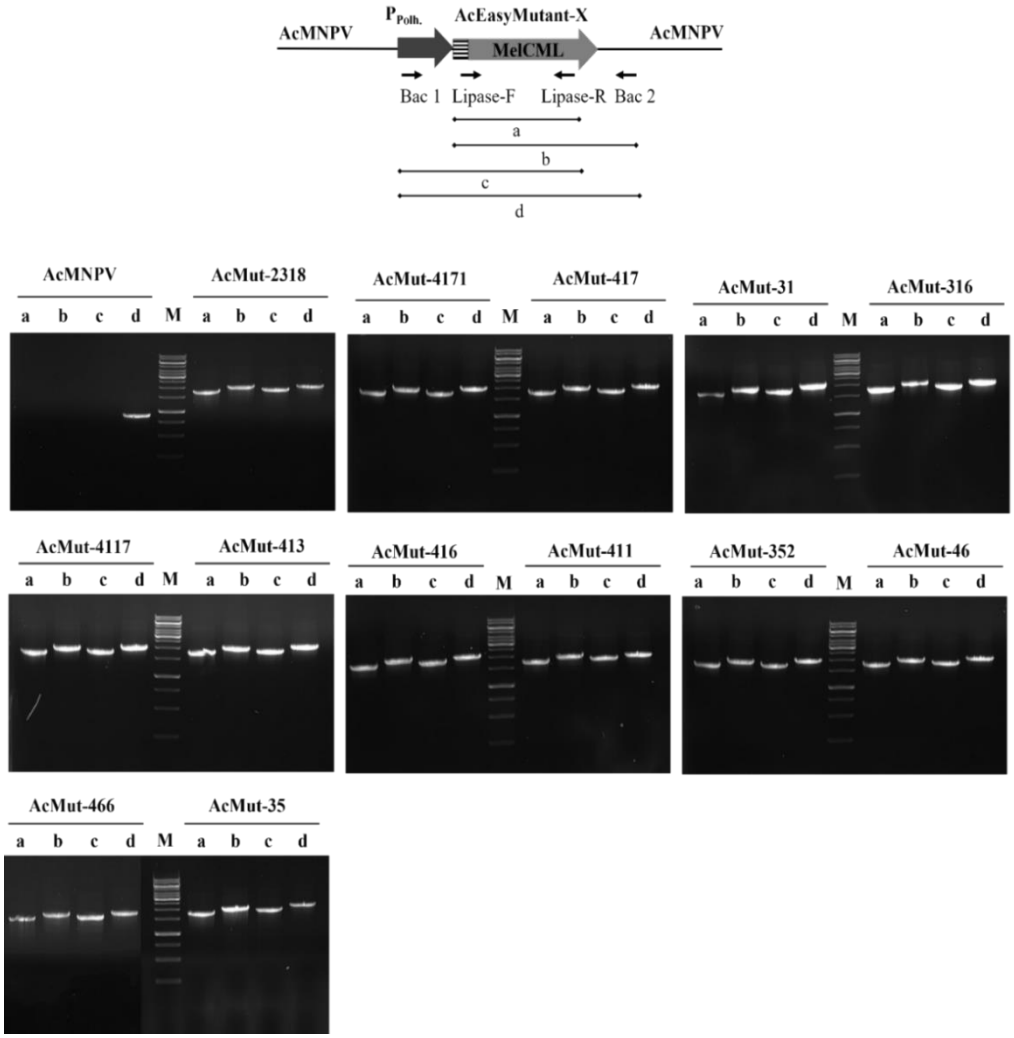


Fig. 28. Confirmation of internal genome structure of sequence-based recombinant baculoviruses by viral DNA PCR. PCR amplifications were performed at four positions using specific primer sets. Lane: M, 1Kb DNA Ladder.

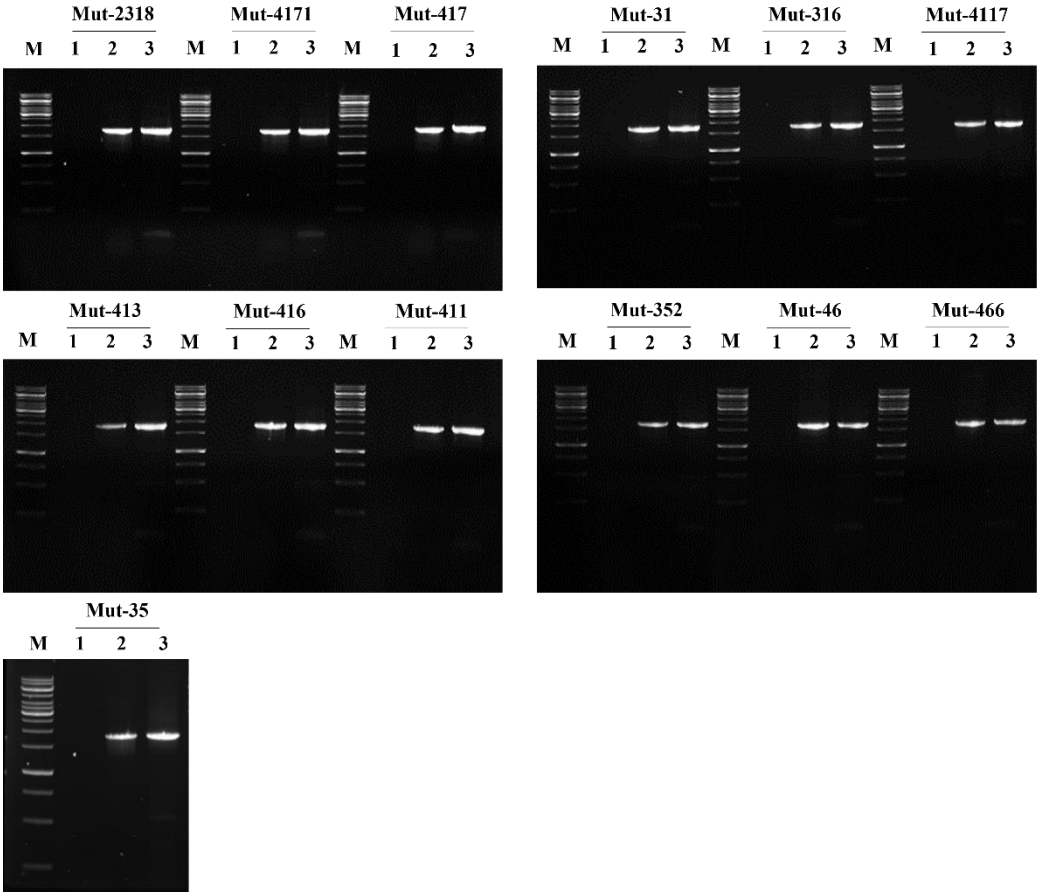


Fig. 29. Confirmation of transcription for sequence-based recombinant baculoviruses by RT-PCR. Lane: M, 1Kb DNA Ladder; 1, AcMNPV 2, pDualBac_Mut-X (X, 2318, 4171, 417, 31, 316, 4117, 413, 416, 411, 352, 46, 466, and 35); 3, AcEasy_Mut-X (X, 2318, 4171, 417, 31, 316, 4117, 413, 416, 411, 352, 46, 466, and 35)

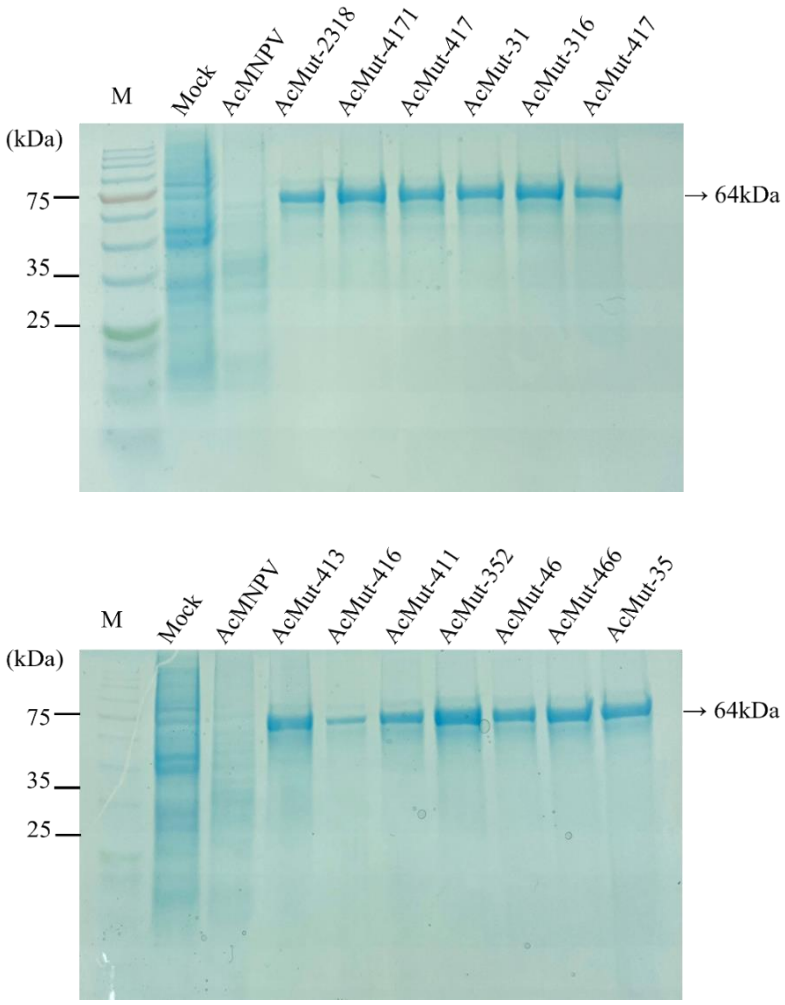


Fig. 30. Analysis of sequence-based mutated lipase proteins expression in High-Five cells by SDS PAGE. Lane: M, 3-color prestained protein size marker; Mock, not-infected High-Five cell.

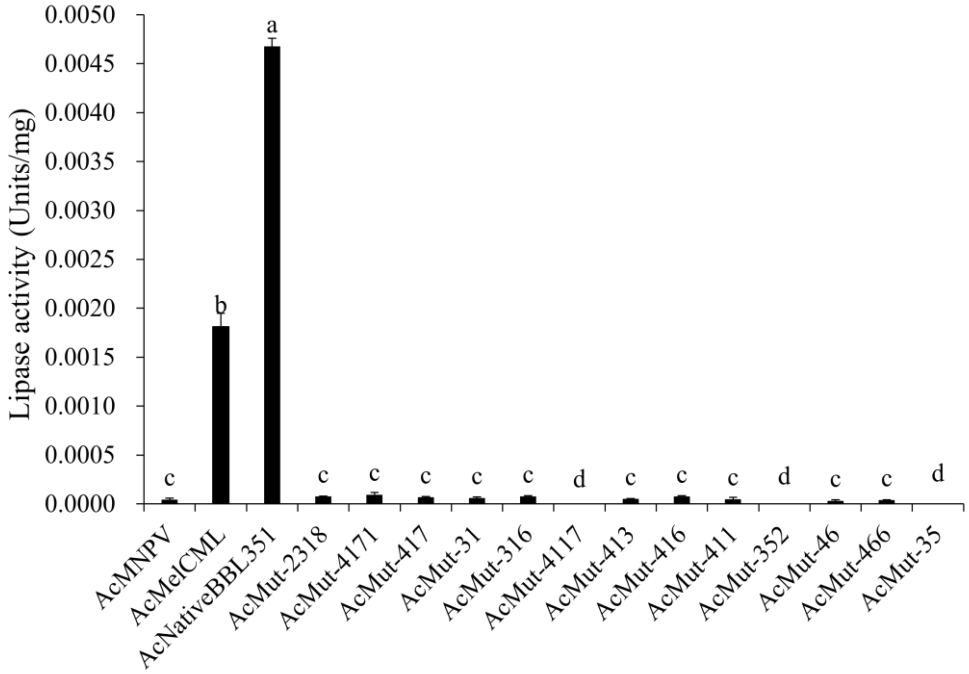
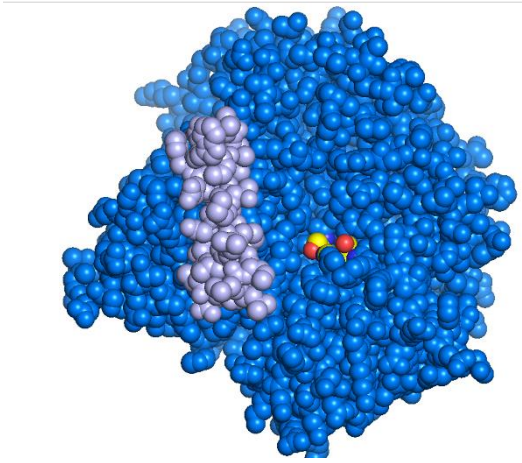


Fig. 31. Lipase activity of sequence-based mutants. Expressed as secretory protein in High-Five cells infected with recombinant baculoviruses harvested after post-infection 3 days.

(a) Open state



(b) Closed state

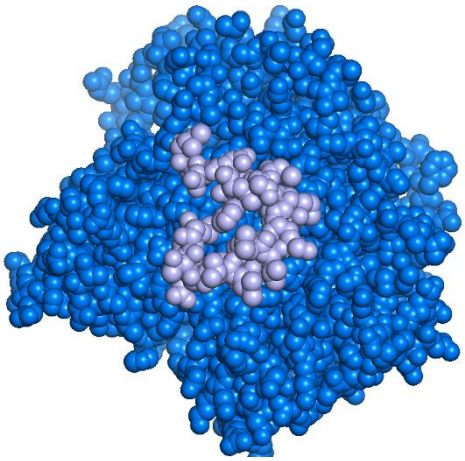


Fig. 32. Predicted three-dimensional structure of CML. (a) open state; (b) closed state

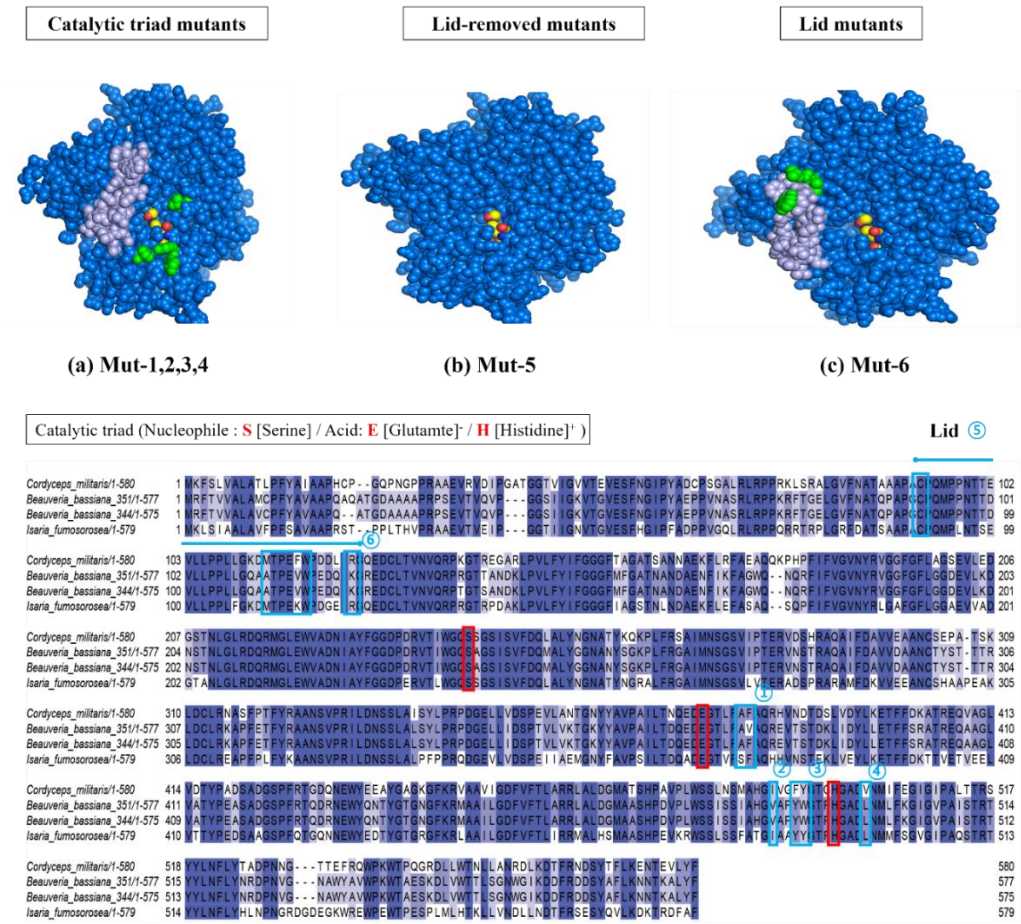


Fig. 33. The amino acid comparison of CML and BBL351 based on the three-dimensional structure of CML. Mutant sites were indicated in three-dimensional structure of CML, (a) Mut-1,2,3, and 4, catalytic triad-related mutants; (b) Mut-5, lid-deleted mutant; (c) Mut-6, lid-related mutant.

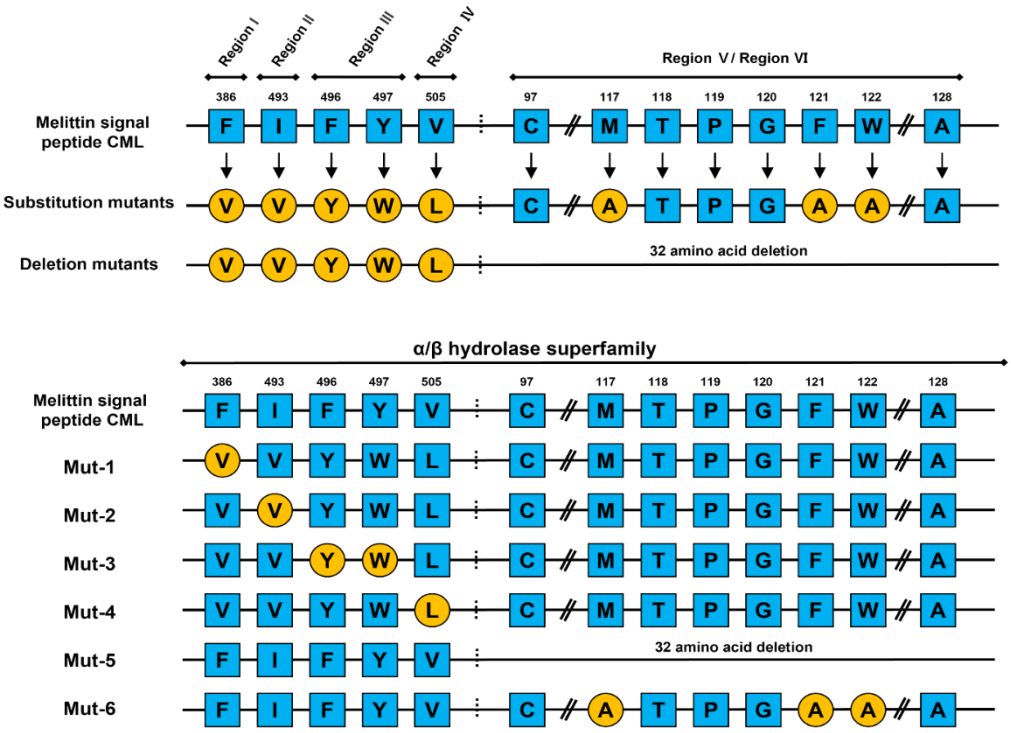


Fig. 34. Mutated residues of 6 structure-based CML mutants. Ellipse represents mutated amino acid sequences and rectangle represents unchanged amino acid sequences.

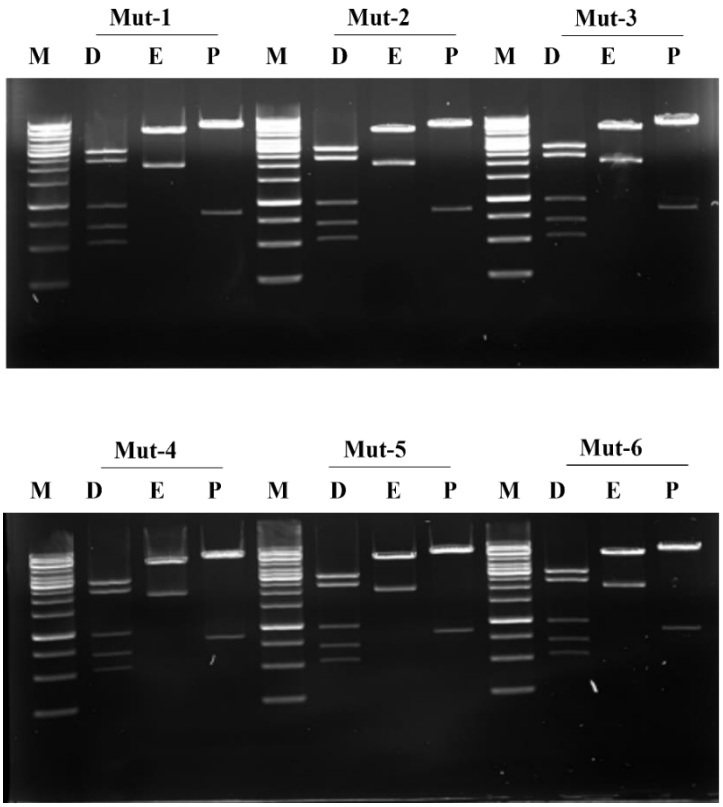


Fig. 35. The restriction endonuclease digestion pattern of structure-based mutant lipase genes into baculovirus donor vector. Lane, M, 1Kb DNA Ladder; D, *Dra*I; E, *Eco*RV; P, *Pst*I

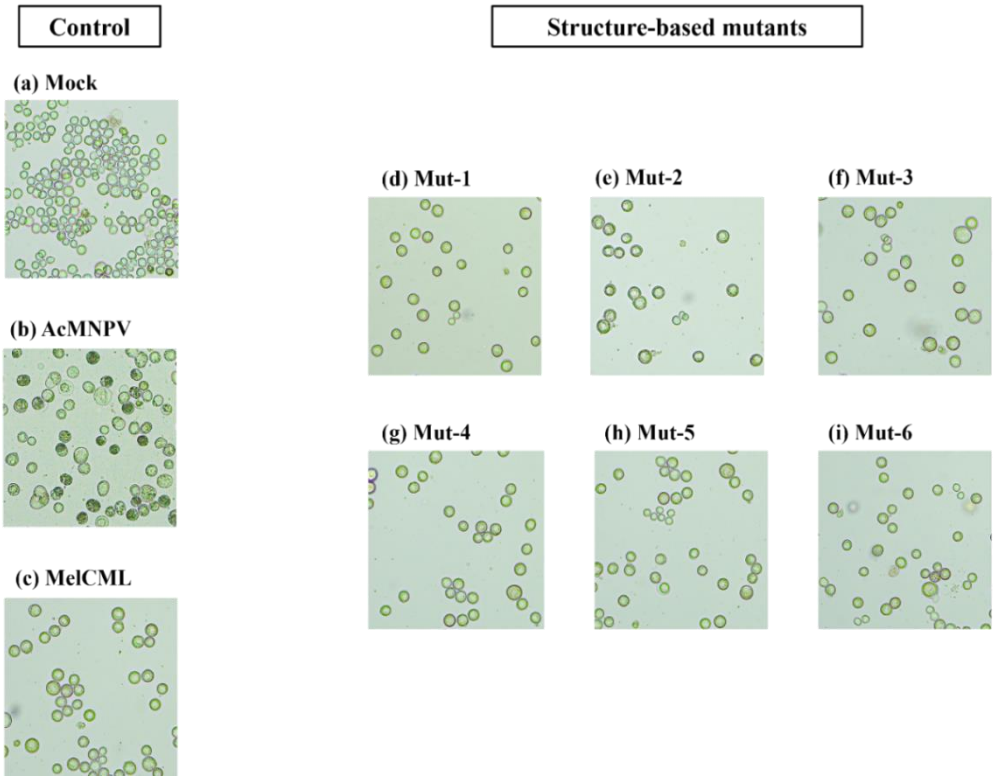


Fig. 36. The Sf9 cells were infected with structure-based recombinant baculoviruses. (a) Mock, not-infected Sf9 cells; (b) AcMNPV, Sf9 cells infected AcMNPV; (c) ~ (i), Sf9 cells infected with recombinant baculoviruses, AcEasy_MelCML, AcEasy_Mut-X (X, 1,2,3,4,5, and 6).

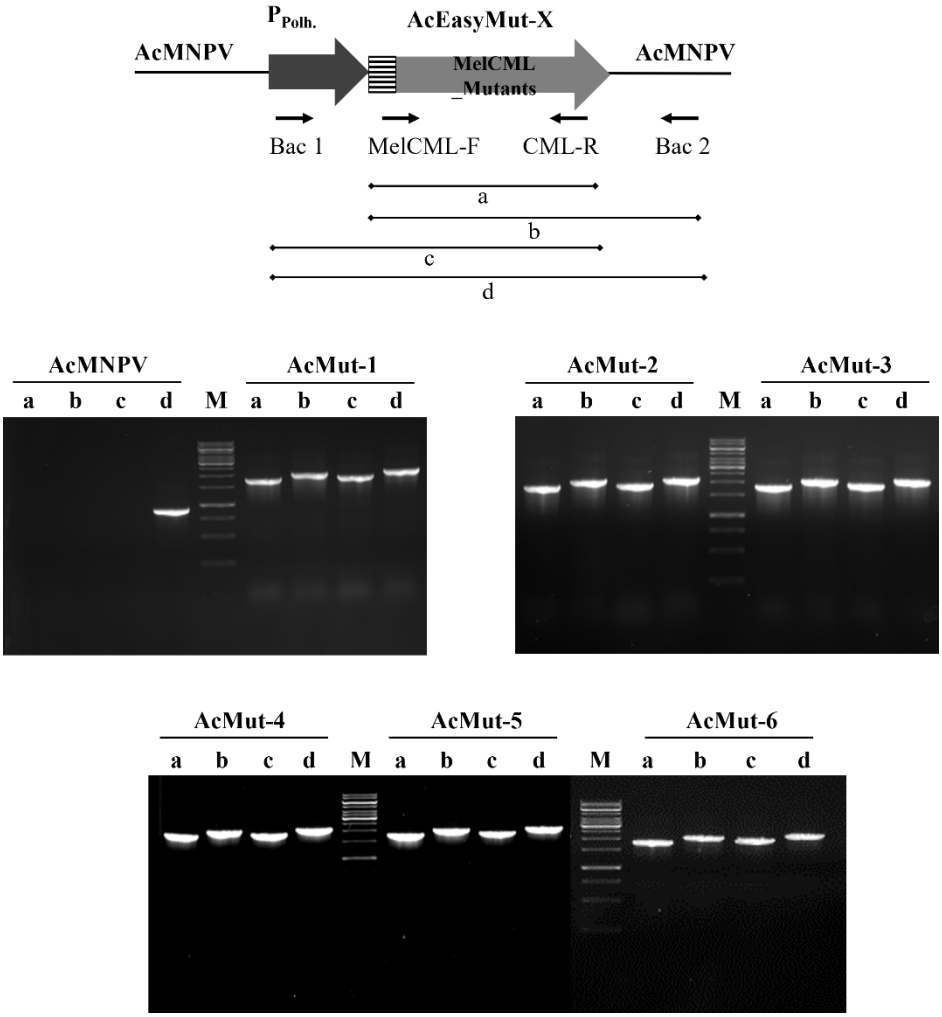


Fig. 37. Confirmation of internal genome structure of structure-based recombinant baculoviruses by viral DNA PCR. PCR amplifications were performed at four positions using specific primer sets. Lane: M, 1Kb DNA Ladder.

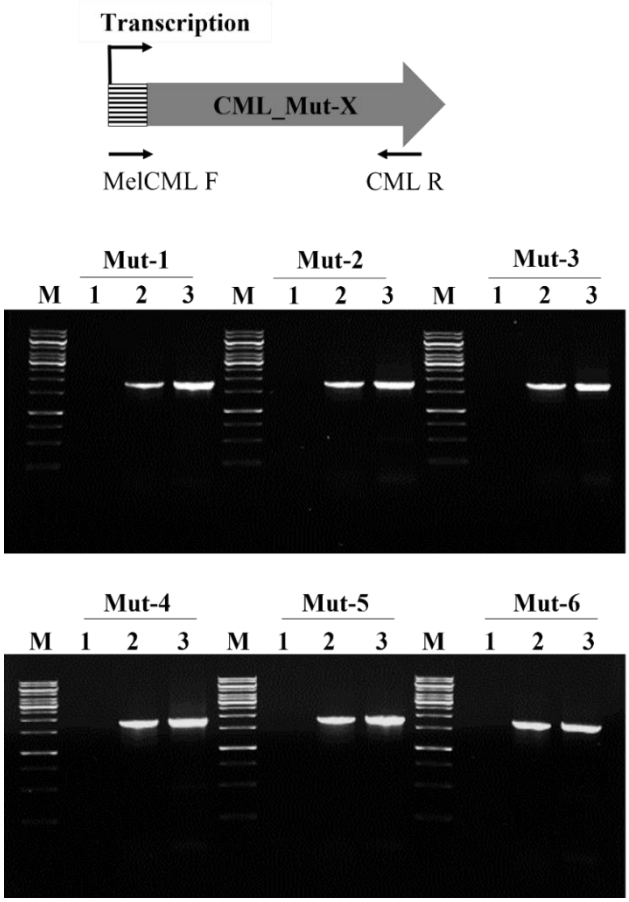


Fig. 38. Confirmation of transcription for structure-based recombinant baculoviruses by RT-PCR. Lane: M, 1Kb DNA Ladder; 1, AcMNPV; 2, pDualBac_Mut-X (X, 1,2,3,4,5, and 6); 3, AcEasy_Mut-X (X, 1,2,3,4,5, and 6).

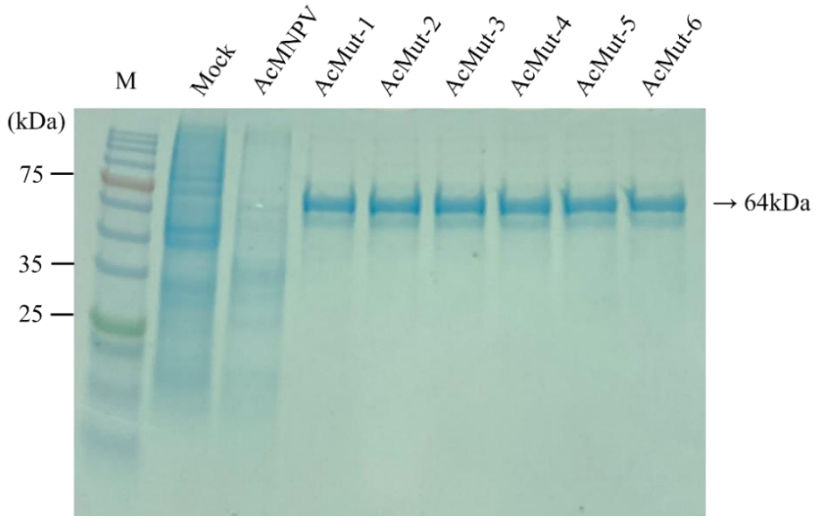


Fig. 39. Analysis of structure-based mutated lipase proteins expression in High-Five cells by SDS PAGE. Lane: M, 3-color prestained protein size marker; Mock, not-infected High-Five cell.

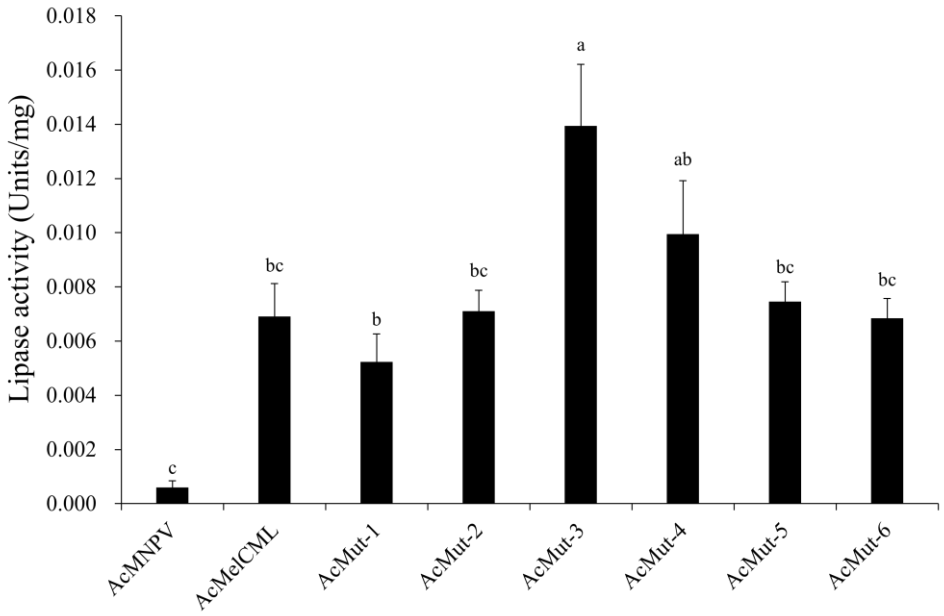


Fig. 40. Lipase activity of structure-based mutants. Expressed as secretory protein in High-Five cells infected with recombinant baculoviruses harvested after post-infection 3 days.

DISCUSSION

The enzyme industry is called the highlight of the fourth industry. Approximately 4,000 enzymes are known so far; about 200 enzymes are applied industrially (Mondal, Baksi, Koris, & Vatai, 2016). It is expected to secure competitiveness for producing valuable enzymes by improving the activity and developing mass production of entomopathogenic fungi-derived lipases.

Originally, entomopathogenic fungal enzymes were used to invade the insects' body, especially lipases, mainly used to decompose insect epicuticles which are composed of wax layers. However, since regioselectivity had been revealed in lipase, it gained a high value industrially. Therefore, in this study, CML with regioselectivity, and BBL351, which are closely related to CML, were introduced into an insect-derived baculovirus expression system for a comparative investigation of two types of entomopathogenic lipases.

Both CML and BBL351 showed high activity at *p*-nitrophenyl butyrate. It demonstrated that the activity of each lipase was not dependent on the short length of the substrate but on its suitability with the substrate. Based on these biological properties, signal peptide optimization and mass expression conditions setting experiments were conducted.

To establish mass expression conditions, the lipase genes derived from *C. militaris* and *B. bassiana* were expressed using the baculovirus expression system and insect cell lines that showed higher yields other than bacteria, yeast, and fungi. By comparing two insect cell types, selecting a high-efficiency expression system was possible to compare the activity of lipase expressed in each cell line and measure the total amount of protein secreted. The regioselectivity lipase, CML, improved its activity through amino acid

sequence comparison with BBL351, which showed high activity and tertiary structure analyses. These analyses allowed CML to construct mutants with higher enzyme activity through mutagenesis. Sequence-based mutants showed that lipase activity was lowered in all mutants. It demonstrated that all five selected mutant sites were important and played a crucial role in lipase activity. Therefore, rather than comparing the sequences one-dimensionally, as the results exhibited, it was important to compare the amino acid based on the three-dimensional structure of each lipase and possible to find mutants with increased activity.

The relationship between protein structure and lipase activity will be identified through accurate structural analysis. Lipases and mutant proteins identified through these studies might be widely used to control the properties of lipase based on protein structure and primary data for analyzing the structure-activity relationship of the protein. Lipases with regioselectivity, such as CML, affect the production of alternative energy, such as bio-diesel, a fat-derived drug such as omega-3 and DHA, as well as new food additives such as salatrim by selectively reconfiguring fat acid of triacylglycerol through transesterification reactions.

Therefore, it is important to efficiently control the lipase properties for commercial application. It can be used as a novel valuable natural source by explaining the three-dimensional structure of lipase proteins that react specifically to lipids, constructing structural-activity relationships (SARs), and establishing an eco-friendly mass-expression system that optimizes reaction efficiency and stability.

ABSTRACT IN KOREAN

곤충병원성 곰팡이 유래 지방질 효소의 생물학적 특성 및 대량 발현 조건 설정 연구

서울대학교
농생명공학부 곤충학전공

김 상 희

초 록

라이페이스는 일반적으로 지질이라 알려진 트라이아실글리세롤을 글라이세롤과 지방산으로 가수분해 또는 에스터화하는 중요한 효소 중 하나로 현재 다양한 산업에서 효과적으로 사용되고 있다. 최근에, 곤충병원성 곰팡이인 번데기 동충하초(*Cordyceps militaris*)로 부터 유래된 라이페이스에서 트리아실글리세롤의 *sn*-1,3 위치에 특이적으로 작용하는 위치특이성이 있는 것이 보고되었다. 위치특이성이 있다고 알려진 *C. militaris* 유래 지방질 효소 유전자인 CML과 높은 유사성을 가지는 곤충병원성 곰팡이인 *Beauveria bassiana* JEF-

351 균주 유래의 지방질 효소 유전자 BBL-351을 베큘로바이러스 발현 벡터 시스템을 이용하여 발현하고, *p*NPP assay를 통하여 CML과 BBL351의 지방질 분해 활성을 비교분석 하였다. 또한 각 효소의 생물학적인 특성을 규명하기 위해, 기질에 따른 가기질 분해활성도를 비교한 결과, CML과 BBL351 모두 기질의 탄소의 개수가 4개인 *p*-Nitropheyl butyrate에서 높은 활성을 나타내었다. BBL351의 지방질 분해활성이 CML보다 약 2배 높았으며, 두 효소 모두 High-Five 세포에서 Sf9 세포보다 높은 발현 효율을 보였다. 또한, 곤충 세포를 이용한 이들 지방질 효소의 효율적인 대량발현을 위한 signal peptide 치환을 이용해 분비 signal 최적화 및 대량발현 조건을 확립하였다.

한편, CML과 BBL351의 단백질 3차구조 및 아미노산 비교를 기반으로 mutagenesis를 통하여 보다 높은 효소 활성을 가지는 CML을 선별하였다. 총 13종의 서열기반의 mutant와 6종의 구조기반의 mutant를 제작하여 각각의 분해활성도를 측정하였다. 그 결과, 총 19종의 mutant 중에서 구조기반의 mutant인 Mut-3, Mut-4에서 본래의 CML보다 높은 지방질 분해 활성을 나타내는 것을 확인할 수 있었다.

본 연구를 통해 확인된 곤충병원성 곰팡이 유래 지방질 효소 및 mutant 단백질은 해당 단백질의 구조-활성 관계 분석을 위한 기초자료로서 뿐만 아니라, 단백질 구조에 기반한 지방질 효소의 특성 제어에 폭넓게 이용될 수 있는 결과로, 다양한 산업에서 유용하게 사용할 수 있는 새로운 자연 공급원이 될 수 있음을 시사하였다.

Key words: 라이페이스, 곤충병원성 곰팡이, 동충하초, 보베리아 바시아나, 베큘로 바이러스 발현 시스템

학번: 2021-29945

LITERATURES CITED

Akanbi, T. O., Adcock, J. L., & Barrow, C. J. (2013). Selective concentration of EPA and DHA using *Thermomyces lanuginosus* lipase is due to fatty acid selectivity and not regioselectivity. *Food Chemistry, 138*(1), 615-620.

Aranda, F., Gomez-Alonso, S., Del Álamo, R. R., Salvador, M., & Fregapane, G. (2004). Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: Comparison with other Spanish cultivars. *Food Chemistry, 86*(4), 485-492.

Arnold, R., Shahani, K., & Dwivedi, B. (1975). Application of lipolytic enzymes to flavor development in dairy products. *Journal of Dairy Science, 58*(8), 1127-1143.

Behie, S. W., & Bidochka, M. J. (2014). Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and environmental microbiology, 80*(5), 1553-1560.

Bilgo, E., Lovett, B., St Leger, R. J., Sanon, A., Dabiré, R. K., & Diabaté, A. (2018). Native entomopathogenic *Metarhizium spp.* from Burkina Faso and their virulence against the malaria vector *Anopheles coluzzii* and non-target insects. *Parasites & vectors, 11*(1), 1-6.

Castro, T., Mayerhofer, J., Enkerli, J., Eilenberg, J., Meyling, N. V., de Andrade Moral, R., . . . Delalibera Jr, I. (2016). Persistence of Brazilian isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *M. robertsii* in strawberry crop soil after soil drench application. *Agriculture, Ecosystems & Environment, 233*, 361-369.

Chambers, A. C., Aksular, M., Graves, L. P., Irons, S. L., Possee, R. D., & King, L. A.

(2018). Overview of the baculovirus expression system. *Current Protocols in Protein Science, 91*(1), 5.4. 1-5.4. 6.

Chandra, P., Singh, R., & Arora, P. K. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microbial Cell Factories, 19*(1), 1-42.

Choi, Y., Park, J.-Y., & Chang, P.-S. (2021). Integral stereoselectivity of lipase based on the chromatographic resolution of enantiomeric/regioisomeric diacylglycerols. *Journal of Agricultural and Food Chemistry, 69*(1), 325-331.

Choudhury, P., & Bhunia, B. (2015). Industrial application of lipase: a review. *Biopharm J, 1*(2), 41-47.

Facchini, F. D. A., Vici, A. C., Pereira, M. G., Jorge, J. A., & de Moraes, M. d. L. T. (2016). Enhanced lipase production of *Fusarium verticillioides* by using response surface methodology and wastewater pretreatment application. *Journal of Biochemical Technology, 6*(3), 996-1002.

Gamayurova, V., Zinov'eva, M., Shnaider, K., & Davletshina, G. (2021). Lipases in esterification reactions: a review. *Catalysis in Industry, 13*(1), 58-72.

Gandhi, N. N. (1997). Applications of lipase. *Journal of the American Oil Chemists' Society, 74*(6), 621-634.

Gandhi, N. N., Patil, N. S., Sawant, S. B., Joshi, J. B., Wangikar, P. P., & Mukesh, D. (2000). Lipase-catalyzed esterification. *Catalysis Reviews, 42*(4), 439-480.

Grabherr, R., & Ernst, W. (2001). The baculovirus expression system as a tool for generating diversity by viral surface display. *Combinatorial chemistry & high throughput screening, 4*(2), 185-192.

He, L., Shao, W., Li, J., Deng, F., Wang, H., Hu, Z., & Wang, M. (2021). Systematic analysis

of nuclear localization of *Autographa californica* multiple nucleopolyhedrovirus proteins. *Journal of General Virology, 102*(3), 001517.

Herniou, E. A., Olszewski, J. A., Cory, J. S., & O'Reilly, D. R. (2003). The genome sequence and evolution of baculoviruses. *Annual review of entomology, 48*, 211.

Hitchman, R. B., Possee, R. D., & King, L. A. (2009). Baculovirus expression systems for recombinant protein production in insect cells. *Recent patents on biotechnology, 3*(1), 46-54.

Hou, C. T. (2002). Industrial uses of lipase. In *Lipid biotechnology* (pp. 449-460): CRC Press.

Huang, J., Yang, Z., Guan, F., Zhang, S., Cui, D., Guan, G., & Li, Y. (2013). A novel mono- and diacylglycerol lipase highly expressed in *Pichia pastoris* and its application for food emulsifier preparation. *Process Biochemistry, 48*(12), 1899-1904.

Ismail, O. Z., & Bhayana, V. (2017). Lipase or amylase for the diagnosis of acute pancreatitis *Clinical biochemistry, 50*(18), 1275-1280.

Jaber, L. R., & Enkerli, J. (2017). Fungal entomopathogens as endophytes: can they promote plant growth *Biocontrol Science and Technology, 27*(1), 28-41.

Jaihan, P., Sangdee, K., & Sangdee, A. (2016). Selection of entomopathogenic fungus for biological control of chili anthracnose disease caused by *Colletotrichum spp. European journal of plant pathology, 146*(3), 551-564.

José, C., Toledo, M. V., & Briand, L. E. (2016). Enzymatic kinetic resolution of racemic ibuprofen: past, present and future. *Critical reviews in biotechnology, 36*(5), 891-903.

Keyhani, N. O. (2018). Lipid biology in fungal stress and virulence: Entomopathogenic

fungi. *Fungal biology, 122*(6), 420-429.

Khan, S., Guo, L., Maimaiti, Y., Mijit, M., & Qiu, D. (2012). Entomopathogenic fungi as microbial biocontrol agent. *Molecular Plant Breeding, 3*(7).

Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current opinion in biotechnology, 13*(4), 345-351.

Klieber, J., & Reineke, A. (2016). The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *Journal of Applied Entomology, 140*(8), 580-589.

Kosikowski, F. V. (1976). Flavor development by enzyme preparation in natural and processed cheddar cheese. In: Google Patents.

Kost, T. A., Condreay, J. P., & Jarvis, D. L. (2005). Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature biotechnology, 23*(5), 567-575.

Kost, T. A., & Kemp, C. W. (2016). Fundamentals of baculovirus expression and applications. *Advanced technologies for protein complex production and characterization*, 187-197.

Lacey, L. (1998). The effect of selected allelochemicals on germination of conidia and blastospores and mycelial growth of the entomopathogenic fungus, *Paecilomyces fumosoroseus* (*Deuteromycotina: Hyphomycetes*). *Mycopathologia, 142*(1), 17-25.

Lanser, A. C., Manthey, L. K., & Hou, C. T. (2002). Regioselectivity of new bacterial lipases determined by hydrolysis of triolein. *Current microbiology, 44*(5), 336-340.

Lee, S. Y., Debnath, T., Kim, S.-K., & Lim, B. O. (2013). Anti-cancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell

HT-29. *Food and chemical toxicology, 60*, 439-447.

Linko, Y.-Y., Lämsä, M., Wu, X., Uosukainen, E., Seppälä, J., & Linko, P. (1998). Biodegradable products by lipase biocatalysis. *Journal of biotechnology, 66*(1), 41-50.

Litwin, A., Nowak, M., & Różalska, S. (2020). Entomopathogenic fungi: unconventional applications. *Reviews in Environmental Science and Bio/Technology, 19*(1), 23-42.

Lu, H.-Y., Chen, Y.-H., & Liu, H.-J. (2012). Baculovirus as a vaccine vector. *Bioengineered, 3*(5), 271-274.

Mazza, M., Pomponi, M., Janiri, L., Bria, P., & Mazza, S. (2007). Omega-3 fatty acids and antioxidants in neurological and psychiatric diseases: an overview. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 31*(1), 12-26.

Mehta, A., Bodh, U., & Gupta, R. (2017). Fungal lipases: a review. *Journal of Biotech Research, 8*, 58.

Mondal, S., Baksi, S., Koris, A., & Vatai, G. (2016). Journey of enzymes in entomopathogenic fungi. *Pacific Science Review A: Natural Science and Engineering, 18*(2), 85-99.

Nanssou Kouteu, P. A., Baréa, B., Barouh, N., Blin, J. l., & Villeneuve, P. (2016). Lipase activity of tropical oilseed plants for ethyl biodiesel synthesis and their typo-and regioselectivity. *Journal of Agricultural and Food Chemistry, 64*(46), 8838-8847.

Nelson, J. H. (1972). Enzymatically produced flavors for fatty systems. *Journal of the American Oil Chemists Society, 49*(10), 559-562.

O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1994). *Baculovirus expression vectors: a laboratory manual*: Oxford University Press on Demand.

Park, J. H., Park, K.-M., Chang, Y., Park, J.-Y., Han, J., & Chang, P.-S. (2018). Cloning and protein expression of the *sn*-1 (3) regioselective lipase from *Cordyceps militaris*. *Enzyme and Microbial technology, 119*, 30-36.

Park, J. Y., Park, K. M., Yoo, Y., Yu, H., Lee, C. J., Jung, H. S., . . . Chang, P. S. (2019). Catalytic characteristics of a *sn*-1 (3) regioselective lipase from *Cordyceps militaris*. *Biotechnology Progress, 35*(2), e2744.

Posorske, L. (1984). Industrial-scale application of enzymes to the fats and oil industry. *Journal of the American Oil Chemists' Society, 61*(11), 1758-1760.

Possee, R. D., Griffiths, C. M., Hitchman, R. B., Chambers, A., Murguia-Meca, F., Danquah, J., . . . King, L. A. (2010). Baculoviruses: biology, replication and exploitation.

Ramakuwela, T., Hatting, J., Bock, C., Vega, F. E., Wells, L., Mbata, G. N., & Shapiro-Ilan, D. (2020). Establishment of *Beauveria bassiana* as a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests. *Biological Control, 140*, 104102.

Ramanujam, B., Rangeshwaran, R., Sivakmar, G., Mohan, M., & Yandigeri, M. (2014). Management of insect pests by microorganisms. *Proceedings of the Indian National Science Academy, 80*(2), 455-471.

Ray, A. (2012). Application of lipase in industry. *Asian Journal of Pharmacy and technology, 2*(2), 33-37.

Reetz, M. T. (2013). Biocatalysis in organic chemistry and biotechnology: past, present, and future. *Journal of the American Chemical Society, 135*(34), 12480-12496.

Ríos-Moreno, A., Garrido-Jurado, I., Resquín-Romero, G., Arroyo-Manzanares, N., Arce,

L., & Quesada-Moraga, E. (2016). Destruxin A production by *Metarhizium brunneum* strains during transient endophytic colonisation of *Solanum tuberosum*. *Biocontrol Science and Technology, 26*(11), 1574-1585.

Rodríguez-García, J., Sahi, S. S., & Hernando, I. (2014). Functionality of lipase and emulsifiers in low-fat cakes with inulin. *LWT-Food Science and Technology, 58*(1), 173-182.

Rogalska, E., Cudrey, C., Ferrato, F., & Verger, R. (1993). Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality, 5*(1), 24-30.

Rogalska, E., Ransac, S., & Verger, R. (1990). Stereoselectivity of lipases. II. Stereoselective hydrolysis of triglycerides by gastric and pancreatic lipases. *Journal of Biological Chemistry, 265*(33), 20271-20276.

Rohrmann, G. F. (1992). Baculovirus structural proteins. *Journal of General Virology, 73*(4), 749-761.

Rohrmann, G. F., Leisy, D. J., Chow, K.-C., Pearson, G. D., & Beaudreau, G. S. (1982). Identification, cloning, and R-loop mapping of the polyhedrin gene from the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*. *Virology, 121*(1), 51-60.

Rompianesi, G., Hann, A., Komolafe, O., Pereira, S. P., Davidson, B. R., & Gurusamy, K. S. (2017). Serum amylase and lipase and urinary trypsinogen and amylase for diagnosis of acute pancreatitis. *Cochrane Database of Systematic Reviews*(4).

Sarney, D. B., Barnard, M. J., MacManus, D. A., & Vulfson, E. N. (1996). Application of lipases to the regioselective synthesis of sucrose fatty acid monoesters. *Journal of the American Oil Chemists' Society, 73*(11), 1481-1487.

Semb, H., Peterson, J., Tavernier, J., & Olivecrona, T. (1987). Multiple effects of tumor necrosis factor on lipoprotein lipase in vivo. *Journal of Biological Chemistry, 262*(17), 8390-8394.

Šinkūnienė, D., & Adlercreutz, P. (2014). Effects of regioselectivity and lipid class specificity of lipases on transesterification, exemplified by biodiesel production. *Journal of the American Oil Chemists' Society, 91*(7), 1283-1290.

Skinner, M., Parker, B. L., & Kim, J. S. (2014). Role of entomopathogenic fungi in integrated pest management. *Integrated pest management*, 169-191.

Smith, G. E., Fraser, M., & Summers, M. D. (1983). Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. *Journal of Virology, 46*(2), 584-593.

Smith, G. E., Vlak, J. M., & Summers, M. D. (1983). Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. *Journal of Virology, 45*(1), 215-225.

Speranza, P., & Macedo, G. A. (2012). Lipase-mediated production of specific lipids with improved biological and physicochemical properties. *Process Biochemistry, 47*(12), 1699-1706.

Sugihara, A., Shimada, Y., & Tominaga, Y. (1991). A novel *Geotrichum candidum* lipase with some preference for the 2-position on a triglyceride molecule. *Applied microbiology and biotechnology, 35*(6), 738-740.

Takasu, S., Mutoh, M., Takahashi, M., & Nakagama, H. (2012). Lipoprotein lipase as a candidate target for cancer prevention/therapy. *Biochemistry research international, 2012*.

Tan, T., Lu, J., Nie, K., Deng, L., & Wang, F. (2010). Biodiesel production with immobilized lipase: a review. *Biotechnology advances, 28*(5), 628-634.

Treacy, J., Williams, A., Bais, R., Willson, K., Worthley, C., Reece, J., . . . Thomas, D. (2001). Evaluation of amylase and lipase in the diagnosis of acute pancreatitis. *ANZ journal of surgery, 71*(10), 577-582.

Tuli, H. S., Kashyap, D., & Sharma, A. K. (2015). Cordycepin: a cordyceps metabolite with promising therapeutic potential. *Fungal Metabolites, Reference Series in Phytochemistry; Springer Nature: Basingstoke, UK*, 1-22.

van Beek, N., & Davis, D. C. (2016). Baculovirus insecticide production in insect larvae. In *Baculovirus and Insect Cell Expression Protocols* (pp. 393-405): Springer.

Van Iddekinge, B. H., Smith, G., & Summers, M. (1983). Nucleotide sequence of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. *Virology, 131*(2), 561-565.

Ye, L., Zhang, B., Seviour, E. G., Tao, K.-x., Liu, X.-h., Ling, Y., . . . Wang, G.-b. (2011). Monoacylglycerol lipase (MAGL) knockdown inhibits tumor cells growth in colorectal cancer. *Cancer letters, 307*(1), 6-17.

Yoo, 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.

Zhang, S., Hao, A.-J., Zhao, Y.-X., Zhang, X.-Y., & Zhang, Y.-J. (2017). Comparative mitochondrial genomics toward exploring molecular markers in the medicinal fungus *Cordyceps militaris*. *Scientific reports, 7*(1), 1-9.

Britannica, T. Editors of Encyclopaedia (2022, August 19). *enzyme*. *Encyclopedia Britannica*. https://www.britannica.com/science/enzyme.