Korean Journal of Microbiology (2015) Vol. 51, No. 3, pp. 187-193
DOI http://dx.doi.org/10.7845/kjm.2015.5037

pISSN 0440-2413
eISSN 2383-9902

보 문

Exploration and functional expression of homologous lipases of *Candida antarctica* lipase B

Seongsoon Park*

Department of Chemistry, Center for NanoBio Applied Technology, and Institute of Basic Sciences, Sungshin Women's University, Seoul 01133, Republic of Korea

Candida antarctica lipase B의 상동체 효소 탐색과 발현

박성순*

성신여자대학교 화학과

(Received August 20, 2015; Accepted September 2, 2015)

ABSTRACT: *Candida* (also known as *Pseudozyma*) *antarctica* lipase B (CAL-B) has been intensely studied in academic and industrial fields. However, the research related to its homologous enzymes has been rarely reported. In the current investigation, protein sequence similarity search of CAL-B has been conducted and six homologous protein sequences were identified. After the syntheses of their codon-optimized genes, the synthetic genes have been cloned into a periplasmic expression vector to express in *Escherichia coli*. Among six homologous sequences, four sequences were successfully expressed in *E. coli*. The hydrolytic activities of the expressed proteins towards 4-nitrophenyl acetate and 4-nitrophenyl butyrate were measured and compared with those of CAL-B to identify whether the expressed proteins work as a hydrolase. It has been revealed that the expressed proteins can hydrolyze the substrates and the specific activities were determined as (1.3–30) × 10^{-2} μmol/min/mg, which are lower than those of CAL-B. Among these homologous enzymes, *Pseudozyma hubeiensis* SY62 exhibits the comparable enantioselectivity to that of CAL-B towards the hydrolysis of (±)-1-phenylethyl acetate.

Key words: heterologous expression, homology, lipase

Lipases (E.C. 3.1.1.3), which catalyze the hydrolysis of triglycerides in nature, are one of the most widely used enzymes in academic and industrial fields (Bornscheuer and Kazlauskas, 2006; Wu *et al.*, 2013). Lipases structurally belong to an α/β-hydrolase fold family, and possess the catalytic triad composed of Ser-His-Asp/Glu. The amino acids of the catalytic triad are connected by hydrogen bonding to increase the nucleophilicity of the catalytic serine. The catalytic serine works as a nucleophile and attacks the carbonyl carbon atom of an ester substrate during catalysis.

Many lipases exhibit high stereoselectivity as well as chemo- and thermo-stability. Especially, *Candida antarctica* lipase B (CAL-B, also called as *Pseudozyma antarctica* lipase B; Boekhout, 1995), one of the fungal lipases, exhibits extraordinary broad enantioselectivity towards a wide range of chiral secondary alcohols and high stability in various organic solvents including alkanes, ethers, and acetonitrile. Such properties make CAL-B suitable in the organic synthetic applications. Hence, researchers have employed CAL-B in the preparation of a variety of chiral building blocks (Rotticci, 2003; Bornscheuer and Kazlauskas, 2006). For instance, it has been demonstrated that CAL-B catalyzes kinetic resolution of racemic alcohols and amines, desymmetrization of diols and diacetates, and polymerization of lactones (Schmid and Verger, 1998; Gross *et al*., 2001; van der Mee *et al*., 2006; Takwa *et al*., 2011; Andualema and Gessesse, 2012).

Although CAL-B is classified as one of the lipases (Ollis *et al*., 1992) and also structurally belongs to α/β-hydrolase fold family, CAL-B exhibits distinct characteristics compared to

***For correspondence.** E-mail: spark@sungshin.ac.kr;
Tel.: +82-2-920-7646; Fax: +82-2-920-2047

other lipases. For instance, the kinetics of typical lipases exhibit an interfacial activation behavior (Verger, 1997), while CAL-B does not follow the interfacial activation mechanism (Martinelle *et al*., 1995). The interfacial behavior of lipases is structurally understood by the presence of a helical segment, which is referred to as a lid, covering the active site (Grochulski *et al.*, 1993). Before a hydrophobic substrate approaching, the lid is covering the active site and the lipase remains as a closed structure. As a hydrophobic substrate approaches to a lipase, the lid opens up and the lipase is converted to the open structure. Both crystal structures for the closed and open forms of many lipases have been reported (Brady *et al*., 1990; Brzozowski *et al*., 1991; Grochulski *et al.*, 1994). Typical lipases exhibit low activity at a low concentration of a hydrophobic substrate because of their closed structures. According to the increase of the concentration of the hydrophobic substrate, the activity of lipase rapidly increases at a particular point. This kinetic behavior of lipase is known as an interfacial activation. Interestingly, CAL-B does not exhibit such interfacial activity and a crystal structure of the closed form of CAL-B has not been reported (Martinelle *et al.*, 1995; Uppenberg *et al*., 1995). Such distinctive characteristics of CAL-B probably suggest that CAL-B can accept a broad range of compounds including hydrophilic compounds as a substrate.

Although CAL-B has been commercialized and utilized in many researches, the homologous lipases of CAL-B have not been studied. Finding and studying homologous enzymes of interest will likely provide not only an evolutionary knowledge but also an opportunity of creating a new enzyme by gene shuffling. Gene shuffling is one of the strong tools to produce diversity of an enzyme and then to create a new enzyme through directed evolution (Crameri *et al.*, 1998; Carbone and Arnold, 2007). This paper describes the discovery of the homologous enzymes of CAL-B by comparison of protein sequences in a pool of protein sequences and the functional expression of the homologous enzymes in *E. coli*.

Materials and Methods

Chemicals, buffers, and lysozyme were purchased from Sigma-Aldrich Korea. Restriction enzymes and pfu DNA polymerase were purchased from Elpisbiotech. The Ni-NTA agarose resin and antibodies were purchased from Qiagen Korea Ltd. and Abcam, respectively. A centrifugal device (Amicon Ultra-15) was purchased from Millipore Korea. DNA oligomers were obtained from Sigma-Proligo. The synthetic genes were prepared by GenScript USA. DNA sequencing was performed by Solgent Co.

Exploration of homologous sequences of CAL-B by Protein BLAST

The protein sequence of CAL-B was put into Protein BLAST (http://blast.ncbi.nlm.nih.gov) and homologous sequences were explored with default parameters. Six sequences with more than 50% sequence identity were selected. The corresponding DNA sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nuccore). After the codons of the nucleotide sequences of the homologous proteins were optimized for *E. coli*, the codon-optimized genes were synthesized by GenScript and used as the template DNAs for PCR amplifications.

Construction of the expression vectors of the homologous lipase genes of CAL-B

A pair of gene specific primers for each gene containing restriction sites for *Nco*I and *Sal*I were used to introduce the restriction sites in the genes and the amplified genes by PCR were cloned into pBAD/gIIIa vector. The following primers were used for PCR: 5'-AGCACCATGGCTCTGCCGCA-3' and 5'-GATGGTCGACGAAGCGCGTAACGATACCG-3' for CCF54401; 5'-AGCACCATGGCTCTGCCGTCA-3' and 5'-GATGGTCGACCAGGATAACGCCAGAACAGGT-3' for CBQ 70828; 5'-AGCACCATGGCTCTGCCGATTG-3' and 5'-GATGGTCGACGAAACCCGTATTAACGCCGC-3' for GAC93661; 5'-AGCACCATGGCTCTGCCGAGT-3' and 5'-GATGGTCGACGAACGTGGTAAAACCAGAACAGG-3' for EST06015; 5'-AGCACCATGGCTGCCAGCTCT-3' and 5'-GATGGTCGACATTGATCGTACCGCTACAGGTTT-3' for XP_011387292; 5'-AGCACCATGGCTCTGCCGCG-3' and 5'-GATGGTCGACCATGATAACGCCGCTACACG-3' for GAC96817. The reaction mixture for PCR contains the synthesized gene (10 ng), dNTP (0.2 mM), a pair of primers (each 0.4 μM), and pfu polymerase (1 μl of 5 unit/μl) in a standard pfu reaction buffer (50 μl). The PCR was carried out using the following conditions:

initial denaturation at 95°C for 30 sec, followed by twenty five cycles (95°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min), and a final extension at 72°C for 7 min. After digestion of the PCR products with *Nco*I and *Sal*I, the digested products were ligated with the pBAD/gIIIa restricted by the same enzymes. The constructed expression vectors were transformed into Top 10 *E. coli* competent cells.

Expression and purification of homologous lipases of CAL-B

Overnight culture of Top10 (2.5 ml) was inoculated to LB medium (250 ml; ampicillin, 100 μg/ml) and incubated at 37°C for 3 h. A sterile L-arabinose solution (1.25 ml; 4% w/v) was added to induce protein expression and then incubated at 20°C for 20 h. The cells were harvested by centrifugation (10 min, 10,000 rpm, 4°C). The cell pellet (~0.4 g) was resuspended in a lysis buffer (2.5 ml; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 10 mM; pH 8.0) containing lysozyme (1 mg/ml). After incubation on ice for 45 min, the suspension was frozen and thawed. The viscous lysate was drawn through a sterile 20-gauge syringe needle several times and centrifuged (20 min, 10,000 rpm, 4°C). Ni-NTA agarose resin (1 ml of 50% slurry) was added to the cleared lysate (2.5 ml) and the mixture was shaken at 4°C for 1 h. The lysate-Ni-NTA mixture was loaded on a Poly-Prep column (Bio-Rad), drained, and then washed three times with a wash buffer (2 ml; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 20 mM; pH 8.0). The proteins were eluted from the column with an elution buffer (2 ml; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 250 mM; pH 8.0). Eluate (2 ml) was exchanged from the elution buffer to a BES buffer (5 mM, pH 7.2) using a centrifugal device.

Determination of the amount of functional enzymes

The amount of purified enzymes was determined by Bio-Rad Protein Assay kit (Bio-Rad) according to the manufacturer's instructions.

Measurement of the hydrolytic activity towards the hydrolysis of 4-nitrophenyl acetate and 4-nitrophenyl butyrate

The assay solution was prepared by mixing 4-nitrophenyl acetate or 4-nitrophenyl butyrate (20 μl of 200 mM in acetonitrile), acetonitrile (870 μl), and a BES buffer (5 mM, pH 7.2, 11,110 μl). After mixing the assay solution (100 μl) and the enzyme solution (5 μl), the absorbance change was measured at 404 nm for 10 min by a microplate reader. The specific activity was calculated according to the method of Janes *et al*. (1999).

Results

Exploration of homologous sequences of CAL-B and heterologous expression in *E. coli*

The amino-acid sequence of a crystal structure (pdb code: 1TCB) of CAL-B was used for the blast search. Searching the sequence revealed that six protein sequences possess above 50% identity: CCF54401, 78%; CBQ70828, 75%; GAC93661, 74%; EST06015, 72%; XP_011387292, 71%; GAC96817, 67% (Fig. 1). These six sequences were selected for the current investigation because a structure alignment from the FSSP (families of structurally similar proteins) database revealed that enzymes with above 50% sequence identity well conserve their functions (Bateman *et al.*, 2002). As CAL-B is one of the fungal proteins, all identified sequences were originated from fungi such as *Ustilago hordei*, *Sporisorium reilianum* SRZ2, *Pseudozyma hubeiensis* SY62, *Pseudozyma brasiliensis* GHG 001, and *Ustilago maydis* 521 (Table 1). The homologous sequences possess the identical amino acids corresponding to the amino acids of the catalytic triad and oxyanion hole of CAL-B (i.e. Ser-His-Asp for the catalytic triad, and Thr and Gln for the oxyanion hole). In addition, the identified sequences contain a sequence for a potential signal peptide and the sequences were removed during gene syntheses.

The sequences were cloned into an expression vector (pBAD/gIIIa) using a pair of sequence-specific primers containing the *Nco*I and *Sal*I restriction sites for each gene. The encoded proteins contain additional alanine at the C-terminal, and valine, aspartate and 6×His at the N-terminal. After addition of L-arabinose for the induction of the protein expression, the culture was incubated at 20ºC for 20 h. The expression of the homologous proteins were confirmed with an anti-6×His antibody by Western blotting analyses (Fig. 2). Among six homologous genes, only four homologous proteins were

```
1TCB_A      1   ----------------------------LPSGSDPAFSQPKSVLDAGLTCQGASPSSVSKPILLVPGTGTTGPQSFD  49
CCF54401    1   MKLSMIVSLLTLAAS-AVTASPLVKRLPQGFHPPFSTPKSLLDAGLTCQNGSPSSQSKPILLVPGTGATGKQSFD  74
CBQ70828    1   MKFLTALTVLASCSA-LASATPLVKRLPSGSDPAYTLSKAQLDSVLACQNGSPSSQKNPILLVPGTGTTGPQSFD  74
GAC93661    1 [102]MKFTSTVTALAALVC-AASATPLVKRLPIGPDPAFSVPQSQLASVLECQNGSPSSQSNPILLVPGTGVTGRQSFD  176
EST06015    1   MKFASIITALVALASsAVVASPLVKRLPSGSDPALSTPQAVLDAGLFCQNGSPSAQNKPILLVPGTGVDGRQNFD  75
XP_757569   1   MKTTSVISALVTLAS-IIRAAPL-----ASSDPAFSTPKATLDAGLECQTGSPSSQTKPILLVPGTGANGTQTFD  69
GAC96817    1   MKTIPFVSALIVVLS-VVTATPLLSLPRPSSDPPITTPKSVLDRGLECQTGSPSRQTRPILLVPGTGVTGQQSFA  74

1TCB_A     50   SNWIPLSTQLGYTPCWISPPPFMLNDTQVNTEYMVNAITALYAGSGNNKLPVLTWSQGGLVAQWGLTFFPSIRSKVDRLM  129
CCF54401   75   SNWIPLSARLGYNPCWISPPPFMLNDSQVNAEYIVNAVNILYAGSRSKKVPVLTWSQGGLAAQWALTFFPSIRSKVDRLM  154
CBQ70828   75   SNWIPLSTQLGYSPCWVSPPPFMLNDTQVNAEYIVNAVKVLSSASGA-KVPVLTWSQGGLAAQWALTFFPSIRTQVDRLM  153
GAC93661  177   SNWIPLSSQLGYSPCWISPPPFMLNDSQVNAEYIVNAVNTLYAGSGFKKVPVLTWSQGGLATQWALTFFPSIRSKVDRFM  256
EST06015   76   SNWIPLSTMLGYSPCWVSPPPFMLNDSQINAEYVVNAVRRLYAGSGSTKLPVLTWSQGGLVTQWALTFFPSTRNQIDRLV  155
XP_757569  70   SSWIPLSAKLGFSPCWISPPPFMLNDSQVNVEYIVNAVQTLYAGSGSKKVPVLTWSQGGLATQWALTFFPSIRNQVDRLM  149
GAC96817   75   SNWIPLSTLLGYSPCWISPPPFMLNDSQINSEYIVNAVHTLYSGSGG-KVPVVTWSQGGLAVQWALTFFPSIRGKVDRLM  153

1TCB_A     130  AFAPDYKGTVLAGPLDALAVSAPSVWQQTTGSALTTALRNAGGLTQIVPTTNLYSATDEIVQPQVSNSPLDSSYLFNg-K  208
CCF54401   155  AFAPDYKGTIEAGLLNAVGLSSQSVWQQTAGSAFVTALQNAGGLNQIVPTTNLYSATDEIVQPQITNSPLDSSHLFN-AK  233
CBQ70828   154  AFAPDYKGTVLAAFLTTPGLASESVWQQQAGSALTTALANAGGLTKIVPTTNLYSATDDIVQPQTFNGPLDSGYLNGgAK  233
GAC93661   257  AFAPDYKGTVEAVFLTVPGLASQSVWQQQAQSAYLTALQNAGGLTKIVPTTNLYSVFDDIVQPQVSNSPLDSSYLFN-AK  335
EST06015   156  AFVPDYKGTVNAYPLTATDTASPSIWQQRAGSAFTTALRNAGGLNKIVPTTNTYSATDEIVQPQITNSPIDSSFLFN-AK  234
XP_757569  150  AFAPDYKGTIEAGLLSTFGLASQSVWQQQAGSAFVTALKNAGGLTSFVPTTNLYSFFDEIVQPQVFNSDADSSYLGN-SK  228
GAC96817   154  AFAPDYKGTVEAGLLSTFGLAAESVWQQQAGSAFLTALKNAGGLTRIVPTTNLYSSVDEIVQPQFTNSPVDSSYLDN-SM  232

1TCB_A     209  NVQAQAVCGPLFVIDHAGSLTSQFSYVVGRSALRSTTGQARSADYGITDCNPLPANDLTPEQKVAAAALLAPAAAAIVAG  288
CCF54401   234  NIQAQSVCGPLFIIDHAGSLTSQFSYVVGKSALASPTGQAQSKDYRSSDCNPLPADPLSPQNKKEASALLLVAGANLVAG  313
CBQ70828   234  NIQAQSVCGPLFVVDHAGTLTSQFSFVVGRSALRSTTGQAQSKDYGVTDCNPLPADSLTPDQKLRAEGLLLVAGANVAAG  313
GAC93661   336  NIQAQTVCGPAFLIDHAGSLTSQFSYIVGKSALASGTGEAQSSDYSITDCNPLPADPLTPQQKAEASGLLLVAGANVVAG  415
EST06015   235  NIQSQSVCGPAFVADHAQSLVNQFAYVVGRSALRSSTGQAQSSDYSIADCNPLPADPLTPQQKAESSTLLLVAAANVAAG  314
XP_757569  229  NIQAQTVCGGFFVIDHAGSLTSQFSYVVGKSALTSSSGVANSADYSSKDCKASPADDLSAKQKADASALLFVAAGNLLAG  308
GAC96817   233  NVQMQQICGGGFIVDHAGSLTSQFSWMVGRSALGSKTGEAVSGEFGKQDCNATAAEPLSAQQKSDAGGLLLVAAGNLLGG  312

1TCB_A     289  PKQNCEPDLMPYARPFAVGKRTCSGIVTP-  317
CCF54401   314  PKQNCEPDLKPYARRFAIGKRTCSGIVTRf  343
CBQ70828   314  PKQNCEPDLMPYARQYAVGKRTCSGVIL--  341
GAC93661   416  PKQNCEPDLKPYARRFAIGKKTCSGVNTGf  445
EST06015   315  PKTNCEPDLKPYARPFAPGSQTCSGFTTF-  343
XP_757569  309  PKQNCEPDLKPYARQFAVGKKTCSGTIN--  336
GAC96817   313  PKVKCEPDLMEYARKYATGMKTCSGVIM--  340
```

Fig. 1. Alignment of CAL-B with its homologous proteins. The yellow highlighted sequences are assumed as a signal sequence and the sequences were removed in syntheses of the genes. The red colored letters are considered as the catalytic triad and the blue ones as the oxyanion holes.

Table 1. Identified homologous sequences of CAL-B

Origin (organism)	Locus number (NCBI protein)	Sequence identity	Number of amino acids[a]	Molecular weight (Da)
Ustilago hordei	CCF54401	78%	318	33,782
Sporisorium reilianum SRZ2	CBQ70828	75%	316	33,016
Pseudozyma hubeiensis SY62	GAC93661	74%	318	33,666
Pseudozyma brasiliensis GHG001	EST06015	72%	317	33,588
Ustilago maydis 521	XP_011387292	71%	314	32,910
Pseudozyma hubeiensis SY62	GAC96817	67%	316	33,379

[a] The signal peptides were removed.

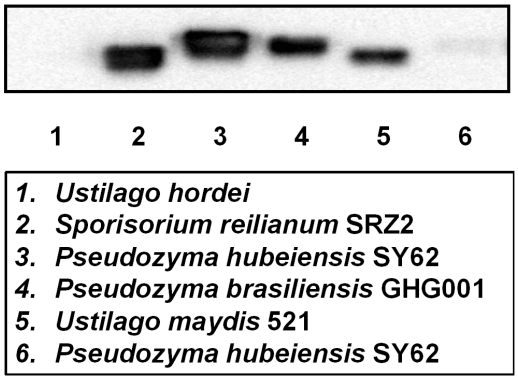


Fig. 2. Western blotting analyses of the expressed proteins. The corresponding bands (lanes 1 and 6) to the proteins from *U. hordei* and *P. hubeiensis* SY62 were not or poorly detected, respectively.

overexpressed. The proteins from *Ustilago hordei* and *Pseudozyma hubeiensis* SY62 were not or poorly expressed, respectively. Thus, the rest four proteins were used for further study. The four expressed proteins were purified using Ni-NTA agarose resins (Fig. 3) and the protein amount was determined by the Bradford method after the buffer was exchanged from the elution buffer to a 5 mM BES buffer (pH 7.2).

Measurement of hydrolytic activities of the homologous proteins and CAL-B

Hydrolytic activities of the expressed proteins were determined using 4-nitrophenyl acetate and 4-nitrophenyl

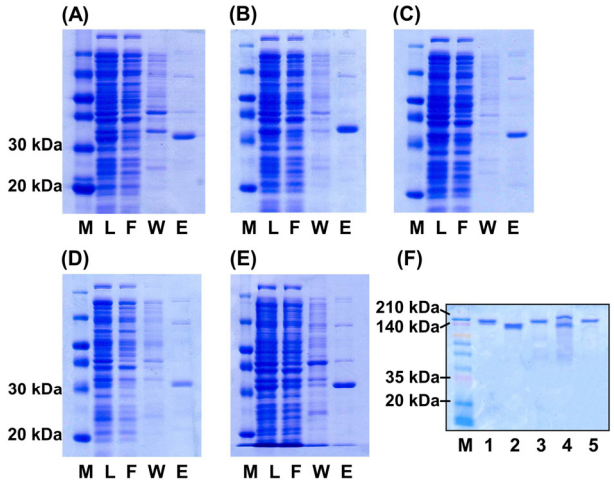
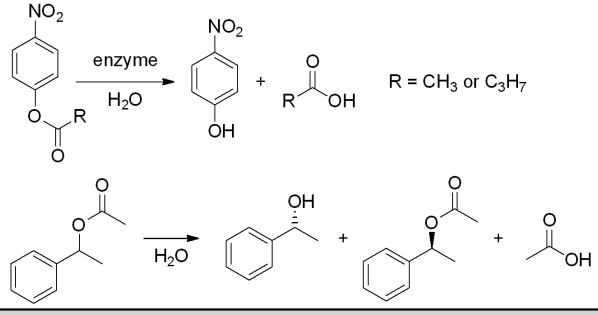


Fig. 3. SDS-PAGE and Native PAGE analyses of the recombinant homologous proteins and CAL-B expressed in *E. coli*. (A) protein from *Sporisorium reilianum* SRZ2, (B) protein from *Pseudozyma hubeiensis* SY62, (C) protein from *Pseudozyma brasiliensis* GHG00, (D) protein from *Ustilago maydis* 521, (E) lipase B from *Candida antarctica*, (F) Native PAGE analyses (Lanes: 1, protein from *Sporisorium reilianum* SRZ2; 2, protein from *Pseudozyma hubeiensis* SY62; 3, protein from *Pseudozyma brasiliensis* GHG00; 4, protein from *Ustilago maydis* 521; 5, lipase B from *Candida antarctica*). SDS-PAGE and Native PAGE were performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; L, lysis buffer fraction; F, flow-through fraction; W, wash buffer fraction; E, elution buffer fraction.

butyrate as a substrate. 4-Nitrophenyl acetate is one of the typical esterase substrates and CAL-B also readily hydrolyzes it. 4-Nitrophenyl butyrate contains a longer carbon chain than 4-nitrophenyl acetate and thus is better substrate for lipases (Jung and Park, 2008). The hydrolysis product of 4-nitrophenyl acetate and 4-nitrophenyl butyrate is 4-nitrophenol, which can be detected by a UV/Vis spectrophotometer. Table 2 shows the specific activities of the expressed proteins and CAL-B. All expressed proteins can hydrolyze both substrates and thus they can be considered as a hydrolase. Each enzyme exhibited different relative specific activities. Enzyme from *S. reilianum* SRZ2 possesses similar specific activities towards both substrates. Enzyme from *P. hubeiensis* SY62 exhibited three times higher activity towards 4-nitrophenyl butyrate, while enzyme from *P. brasiliensis* GHG001 and enzyme from *U. maydis* 521 displayed higher activity towards 4-nitrophenyl acetate by a factor of about 5. However, all homologous enzymes exhibited lower specific activities than those of CAL-B towards both substrates. The enantiomeric preferences of the homologous enzymes towards (±)-1-phenylethyl acetate were measured. CAL-B possesses high enantioselectivity towards (±)-1-phenylethyl acetate (E > 200). Among four homologous enzymes, enzyme from *P. hubeiensis* SY62 exhibits the comparable enantioselectivity (E > 200) towards (±)-1-phenylethyl acetate and the others showed lower enantioselectivity (E = 21-80).

Table 2. Hydrolytic activity and selectivity of CAL-B and its homologous enzymes[a]



Enzyme	Specific activity for 4-nitrophenyl acetate (μmol/min/mg)	Specific activity for 4-nitro phenyl butyrate (μmol/min/mg)	Enantiomeric ratio (E) towards (±)-1-phenylethyl acetate
CAL-B	(1.6 ± 0. 057[b])	(2.6 ± 0.12)	>200
enzyme from *S. reilianum* SRZ2	(5.9 ± 0. 12) × 10^{-2}	(8.0 ± 0. 13) × 10^{-2}	80
enzyme from *P. hubeiensis* SY62	(9.8 ± 0. 65) × 10^{-2}	(3.0 ± 0. 083) × 10^{-1}	>200
enzyme from *P. brasiliensis* GHG001	(7.1 ± 0. 056) × 10^{-2}	(1.3 ± 0. 068) × 10^{-2}	21
enzyme from *U. maydis* 521	(1.8 ± 0. 12) × 10^{-1}	(3.5 ± 0. 21) × 10^{-2}	53

[a] Reaction condition: see the 'Materials and Methods' section.
[b] Errors are standard deviations for at least three measurements.

Discussion

Besides finding new enzymes from nature, it has become an important approach to create functionally improved mutant enzymes for industrial use. For the creation of mutant enzymes of interest, two approaches are mainly concerned: rational design and directed evolution (Bornscheuer and Pohl, 2001). Rational design of enzymes is based on the structural and mechanistic knowledges. On the other hand, directed evolution, which does not require the detailed mechanistic knowledges, uses generating random mutants and screening the mutants to find a functionally improved one. Researchers have used error-prone PCR, mutator strain, or gene shuffling to generate random mutants (Neylon, 2004). Among these approaches, gene shuffling is considered as one of the strongest tools to increase the genetic diversity. However, a homologous family is required to use gene shuffling to keep a conserved fold of an enzyme (Carbone and Arnold, 2007). Since the expression systems of CAL-B have been developed in low eukaryotes and in *E. coli* (Hoegh *et al*., 1995; Rotticci-Mulder *et al*., 2001; Zhang *et al*., 2003; Chodorge *et al*., 2005; Blank *et al*., 2006; Liu *et al*., 2006; Jung and Park, 2008), a directed evolution approach based on error prone PCR has been successfully employed to improve the functional properties of CAL-B (Zhang *et al*., 2003; Wu *et al*., 2013). However, the employment of gene shuffling on generating random mutants of CAL-B is still worth to be exploited. In the present study, six homologous hydrolases of CAL-B were identified from a protein sequence pool and four of them were successfully expressed in *E. coli*. Although the enzymes are originated from fungi, functional expression in *E. coli* benefits researchers from decrease of expression time and culture cost (Jung and Park, 2008). Identification of functionally active homologous enzymes of CAL-B could provide a useful source in direct evolution based on gene shuffling. In addition, the detailed sequence comparison of CAL-B with the homologous enzymes may provide a useful information for rational design to improve the property of CAL-B.

적 요

Candida (*Pseudozyma*로도 알려짐) *antarctica* lipase B (CAL-B)는 학문적으로 그리고 산업적으로 많이 활용되고 있다. CAL-B 자체에 대한 연구는 많이 진행되어온 반면, CAL-B 상동체에 관한 연구는 그리 알려진 바가 없다. 본 연구에서는 단백질 유사성 검색을 통해서 CAL-B의 상동체 탐색을 수행하였고, 6종의 단백질 서열을 찾았다. 해당하는 유전자들을 대장균에 대한 코돈 최적화를 수행하였고, 이를 바탕으로 유전자 합성을 진행하였다. 이들 유전자를 대장균 발현용 벡터에 클로닝한 후, 대장균 내에서 단백질 발현을 시도하여 이들 중 4종의 단백질이 성공적으로 발현되었다. 이들 단백질들이 가수분해 효소로서의 활성이 있는지 확인하기 위해서, 4-nitrophenyl acetate와 4-nitrophenyl butyrate를 반응기질로 하여 가수분해 반응성을 확인하였다. 이들 단백질들의 비활성(specific activity) 값은 (1.3–30) × 10^{-2} μmol/min/mg로 측정되었고, 이는 CAL-B의 비활성 수치보다는 다소 낮은 값에 해당하였다. (±)-1-phenylethyl acetate의 가수분해 반응에 대한 입체선택성은 이들 상동체 효소들 중에서 *Pseudozyma hubeiensis* SY62에서 유래된 효소만이 CAL-B의 입체선택성과 유사함이 확인되었다.

Acknowledgements

This work was supported by the Sungshin University Research Grant of 2015.

References

Andualema, B. and Gessesse, A. 2012. Microbial lipases and their industrial applications: review. *Biotechnol.* **11**, 100–118.

Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S.R., Griffiths-Jones, S., Howe, K.L., Marshall, M., and Sonnhammer, E.L.L. 2002. The Pfam protein families database. *Nucleic Acids Res*. **30**, 276–280.

Blank, K., Morfill, J., Gumpp, H., and Gaub, H.E. 2006. Functional expression of *Candida antarctica* lipase B in *Escherichia coli*. *J. Biotechnol.* **125**, 474–483.

Boekhout, T. 1995. *Pseudozyma* Bandoni emend. Boekhout, a genus for yeast-like anamorphs of ustilaginales. *J. Gen. Appl. Microbiol*. **41**, 359–366.

Bornscheuer, U.T. and Kazlauskas, R.J. 2006. Hydrolases in Organic Synthesis. 2nd. Wiley-VCH, Weinheim, Germany.

Bornscheuer, U.T. and Pohl, M. 2001. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* **5**, 137–143.

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., *et al.* 1990. A serine protease triad forms the

catalytic centre of a triacylglycerol lipase. *Nature* **343**, 767−770.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., and Thim, L. 1991. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **351**, 491−494.

Carbone, M.N. and Arnold, F.H. 2007. Engineering by homologous recombination: exploring sequence and function within a conserved fold. *Curr. Opin. Struct. Biol.* **17**, 454−459.

Chodorge, M., Fourage, L., Ullmann, C., Duvivier, V., Masson, J.M., and Lefèvre, F. 2005. Rational strategies for directed evolution of biocatalysts-application to *Candida antarctica* lipase B (CALB). *Adv. Synth. Catal.* **347**, 1022−1026.

Crameri, A., Raillard, S.A., Bermudez, E., and Stemmer, W.P.C. 1998. DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* **391**, 288−291.

Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B., and Cygler, M. 1993. Insights into interfacial activation from an open structure of *Candida rugosa* lipase. *J. Biol. Chem.* **268**, 12843−12847.

Grochulski, P., Li, Y., Schrag, J.D., and Cygler, M. 1994. Two conformational states of *Candida rugosa* lipase. *Protein Sci.* **3**, 82−91.

Gross, R.A., Kumar, A., and Kalra, B. 2001. Polymer synthesis by *in vitro* enzyme catalysis. *Chem. Rev*. **101**, 2097−2124.

Hoegh, I., Patkar, S., Halkier, T., and Hansen, M.T. 1995. Two lipases from *Candida antarctica*: Cloning and expression in *Aspergillus oryzae. Can. J. Bot.* **73**, 869−875.

Janes, L.E., Cimpoia, A., and Kazlauskas, R.J. 1999. Protease-mediated separation of cis and trans diastereomers of 2(*R,S*)-benzyloxymethyl-4(*S*)-carboxylic acid 1,3-dioxolane methyl ester: intermediates for the synthesis of dioxolane nucleosides. *J. Org. Chem.* **64**, 9019−9029.

Jung, S. and Park, S. 2008. Improving the expression yield of *Candida antarctica* lipase B in *Escherichia coli* by mutagenesis. *Biotechnol. Lett*. **30**, 717−722.

Liu, D., Schmid, R.D., and Rusnak, M. 2006. Functional expression of *Candida antarctica* lipase B in the *Escherichia coli* cytoplasm − a screening system for a frequently used biocatalyst. *Appl. Microbiol. Biotechnol.* **72**, 1024-103.

Martinelle, M., Holmquist, M., and Hult, K. 1995. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta* **1258**, 272−276.

Neylon, C. 2004. Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. *Nucleic Acids Res*. **32**, 1448−1459.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., and Silman, I. 1992. The alpha/beta hydrolase fold. *Protein Eng*. **5**, 197−211.

Rotticci, D. 2003. Understanding and engineering the enantioselectivity of *Candida antarctica* lipase B towards sec-alcohols, Ph.D. thesis, KTH, Stockholm, Sweden.

Rotticci-Mulder, J.C., Gustavsson, M., Holmquist, M., Hult, K., and Martinelle, M. 2001. Expression in *Pichia pastoris* of *Candida antarctica* lipase B and lipase B fused to a cellulose-binding domain. *Protein Expr. Purif.* **21**, 386−392.

Schmid, R.D. and Verger, R. 1998. Lipases: interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed.* **37**, 1608−1633.

Takwa, M., Wittrup Larsen, M., Hult, K., and Martinelle, M. 2011. Rational redesign of *Candida antarctica* lipase B for the ring opening polymerization of D,D-lactide. *Chem. Commun*. **47**, 7392−7394.

Uppenberg, J., Oehrner, N., Norin, M., Hult, K., Kleywegt, G.J., Patkar, S., Waagen, V., Anthonsen, T., and Jones, T.A. 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochem*. **34**, 16838−16851.

van der Mee, L., Helmich, F., de Bruijn, R., Vekemans, J.A.J.M., Palmans, A.R.A., and Meijer, E.W. 2006. Investigation of lipase-catalyzed ring-opening polymerizations of lactones with various ring sizes: kinetic evaluation. *Macromolecules* **39**, 5021−5027.

Verger, R. 1997. Interfacial activation of lipases: facts and artifacts. *Trends Biotechnol*. **15**, 32−38.

Wu, Q., Soni, P., and Reetz, M.T. 2013. Laboratory evolution of enantiocomplementary *Candida antarctica* lipase B mutants with broad substrate scope. *J. Am. Chem. Soc.* **135**, 1872−1881.

Zhang, N., Suen, W.C., Windsor, W., Xiao, L., Madison, V., and Zaks, A. 2003. Improving tolerance of *Candida antartica* lipase B towards irreversible thermal inactivation through directed evolution. *Protein Eng.* **16**, 599−605.