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# *In Vitro* Evolution of Lipase B from *Candida antarctica* Using Surface Display in *Hansenula polymorpha*

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**Abstract** Lipase B from *Candida antarctica* (CalB) displayed on the cell surface of *H. polymorpha* has been functionally improved for catalytic activity by molecular evolution. CalB was displayed on the cell surface by fusing to a cell-wall anchor motif (CwpF). A library of CalB mutants was constructed by *in vivo* recombination in *H. polymorpha*. Several mutants with increased whole-cell CalB activity were acquired from screening seven thousand transformants. The two independent mutants CalB10 and CalB14 showed an approximately 5 times greater whole-cell activity than the wild-type. When these mutants were made as a soluble form, CalB10 showed 6 times greater activity and CalB14 showed an 11 times greater activity compared with the wild-type. Sequence analyses of mutant *CALB* genes revealed amino acid substitutions of $Leu^{278}Pro$ in CalB10 and $Leu^{278}Pro/Leu^{219}Gln$ in CalB14. The substituted $Pro^{278}$ in both mutants was located near the proline site of the α10 helix. This mutation was assumed to induce a conformational change in the α10 helix and increased the $k_{cat}$ value of mutant CalB approximately 6 times. Site-directed mutagenized CalB, LQ ($Leu^{219}Gln$) was secreted into the culture supernatant at an amount of approximately 3 times more without an increase in the CalB transcript level, compared with the wild-type.

**Keywords:** CalB, directed evolution, *Hansenula polymorpha*, lipase, surface display

Lipases (triacylglycerol lipase EC 3.1.1.3) catalyze the hydrolysis of water-insoluble esters and triacylglycerides at the water-oil interface. They also catalyze the enantio- and regio-selective hydrolysis and synthesis of esters. Microbial lipases are stable under the artificial conditions of high temperature and presence of non-aqueous organic solvents because of their extracellular nature. As such, they are used for industrial purposes in the detergent and food industries and for fine chemical preparation [14, 19, 28]. *Candida antarctica* produces two types of lipases that are widely different in their characteristics. Whereas lipase A is more thermostable, and nonstereo-specific, lipase B (CalB) is more thermolabile with a high stereospecificity [6, 23]. The crystal structure of CalB has been resolved [24]. CalB has a catalytic triad consisting of Ser105, Asp187, and His224 [15]. The active site is a narrow funnel type and, therefore, displays higher activity toward carboxylic acid esters than toward triglycerides [17]. CalB is one of the most widely used biocatalyst, with many applications including stereoselective transformation and polyester synthesis [2]. For these reasons, CalB has been functionally expressed in *Aspergillus oryzae* for industrial-scale production [6] and commercialization.

Because of the importance of CalB in organic synthesis, CalB mutants with improved activity and stability have been generated using random or site-directed mutagenesis [13], circular permutation [16], or directed evolution [22, 29]. Recently, directed evolution has emerged as a key technology to generate enzymes with new or improved properties [3, 27, 30]. For the directed evolution of enzymes, an efficient expression system and a reliable screening system are necessary to create a high molecular diversity and obtain a good candidate enzyme. Display of heterologous proteins on the cell surface of microorganisms has been a valuable tool for screening useful ligands from polypeptide libraries and development of whole-cell biocatalyst, adsorbent, and live oral vaccines [27]. We reasoned that surface display of a library of enzyme variants can also be useful in circumventing the common obstacle in successful selection of the desired variant from the variant pool, *i.e.*, occurrence of frequent false positives resulting from the increased

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transcription, translation, and/or secretion without the increase in the specific enzyme activity. In contrast to the secreted enzyme that consistently secretes to the medium, the amount of enzyme anchored on the cell surface would saturate to a fixed level and become relatively insensitive to the increase in the transcription, translation, and/or membrane translocation for reasonably translocation-competent proteins.

A cell surface display system in *H. polymorpha* has been developed that can be used as a screening system and as a biocatalyst to perform bioprocesses [12]. The methylotrophic yeast *H. polymorpha* has been studied as an efficient host for production of foreign proteins [4, 7, 25]. This yeast has several distinctive features as an expression host including availability of strong promoters from the genes involved in methanol metabolism, stable maintenance of multiple copies of foreign genes in the chromosomes, and ease of growth to a high cell density of 100–130 g/l [5]. An efficient method for construction of gene libraries was also developed through *in vivo* recombination of two linear fragments, both of which contained a part of telomere-originated ARS as a recombination target [11]. This method could simplify the construction of a mutant library by elimination of the gene manipulation in *E. coli*.

In this study, we have displayed CalB on the cell surface of *H. polymorpha* using the cell-wall GPI (glycosylphosphatidyl-inositol)-anchored protein motif CwpF and screened for functionally improved CalB mutants by directed evolution.

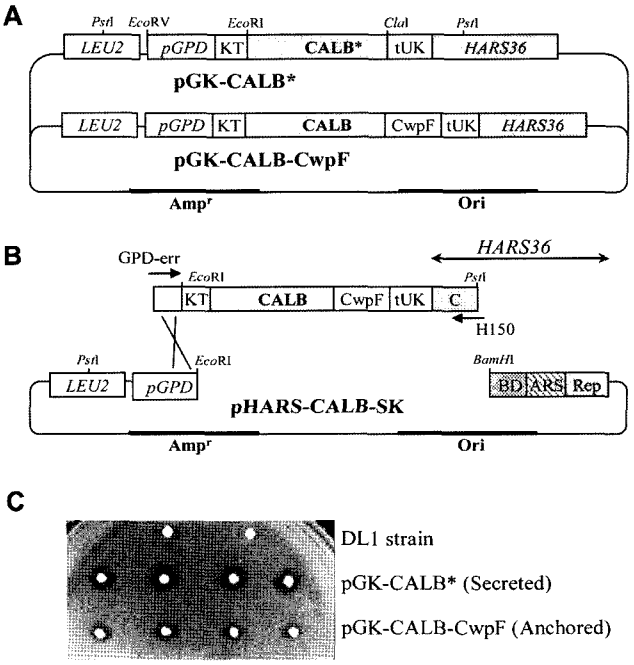


**Fig. 1.** Schematic diagram of plasmids for secreted and anchored forms of lipase, and lipase activity on halo plate.
**A.** Expression plasmids, pGK-CALB* and pGK-CALB-CwpF. **B.** Acceptor vector and mutagenized PCR fragment used for the *CALB* mutant library construction. Abbreviations, *pGPD*, *GAPDH* promoter; KT, killer toxin signal sequence; tUK, unknown terminator [10]; C, chromosome end of *HARS36*; BD, bent DNA domain; ARS, autonomously replicating sequence of *HARS36*; Rep, telomeric repeat of *HARS36* [21]. **C.** Lipase activity of wild-type CalB in secreted and anchored forms in *H. polymorpha*.

## Materials and Methods

### Strains and Media

The *Hansenula polymorpha* strain used was DL1-L, a *LEU2* defective strain of DL1 (ATCC26012). Cells were routinely grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) with shaking at 37°C. Transformants were selected on SD minimal medium (0.67% Bacto yeast nitrogen base w/o amino acid and 2% glucose).

### Plasmid Construction and Yeast Transformation

General DNA manipulations were performed as described by Sambrook *et al.* [18], and total yeast DNA was isolated according to the method of Holm *et al.* [8]. DNA sequencing was performed using an ABI Model 373A automatic DNA sequencer (model 373A; Applied Biosystems). To construct a *CALB* expression vector, the *CALB* gene from a chromosome of *C. antarctica* (ATCC28323) was obtained by PCR amplification using primers CalBN (5'-GGC TCT TCA GCC ACT CCT TTG GTG AAG-3') and CalBF (5'-GCG GAT CCG GGG GTG ACG ATG CCG GAG-3'). The PCR-amplified *CALB* fragment was treated with SapI-Klenow/BamHI and subcloned into the EcoRI-Klenow/BamHI site of both pGA-GOD-CwpF and pGA-GOD* [12] containing the *Kluyveromyces lactis* killer toxin signal sequence to construct the display vector pGK-CALB-CwpF and the secretion vector pGK-CALB* (Fig. 1A). The plasmid used for single copy integration of the *CALB* gene into the *LEU2* locus of *H. polymorpha* was constructed from AMIpLD1 [1], a plasmid with a defective *LEU2*. The EcoRV/ClaI fragment of each *CALB* expression plasmid was introduced into the EcoRV/ClaI site of AMIpLD1. The two resulting plasmids were named pLGK-CALB-CwpF and pLGK-CALB*.

### Random Mutagenesis and Library Construction

PCR random mutagenesis was optimized to produce two to five base substitutions per *CALB* gene using a PCR random mutagenesis kit according to the instructions provided by the supplier (Clontech, U.S.A.). pGK-CALB-CwpF containing both the *CALB* and *CwpF* genes was used as a template for the first round of PCR random mutagenesis. The forward primer was GPD-err (5'-GCA GAG CTA ACC AAT AAG G-3') and the reverse primer was H150 (5'-TGC AGT TGA ACA CAA CCA C-3').

PCR was performed at 94°C for 30 sec, 25 cycles of 94°C for 30 sec and 68°C for 1 min, and then 68°C for 10 min. The amplified DNA fragment was purified by agarose gel elution and used as a template for the second PCR, which was performed using Premix Taq polymerase (Bioneer, Taejon, Korea) for DNA amplification. Amplification was performed using a program of 94°C for 3 min, 20 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 30 sec, followed by 72°C for 7 min.

The acceptor vector for *in vivo* recombination was constructed using the pGK-CALB* plasmid. The 6.5 kb HindIII/PstI fragment of pGK-CALB* was subcloned into the pBluescript SK(+) plasmid and designated as pHARS-CALB-SK (Fig. 1B). For *in vivo* recombination, 100 ng of a 5-kb gel-eluted EcoRI/BamHI fragment of pHARS-CALB-SK was transformed with 100 ng of the PCR-amplified insert fragment. The amplification reaction of insert DNA used for transformation was performed using Premix Taq polymerase (Bioneer) with 94°C for 3 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, and then 72°C for 7 min for extension.

### Activity Assay of Lipase

Transformants were inoculated on YPD plate containing 1% tributyrin and incubated for 24 h to test for lipase activity. The lipase activity of the culture broth was determined by measuring the release of *p*-nitrophenol by the action of an enzyme on *p*-nitrophenyl palmitate (*p*NPP). Yeast cells were cultured at 37°C for 18 h in YPD medium, and then the culture medium and the cell pellets were isolated by centrifugation, followed by washing and suspension of the cell pellets with 0.1 M-Tris/HCl buffer at pH 7.5. The reaction mixture was prepared by addition of a 100 μl enzyme solution to the substrate solution, which was composed of 10 μl of 10 mM *p*NPP, 40 μl of ethanol, and 950 μl of 0.05 M Tris-HCl buffer at pH 7.5. After incubation at 25°C for 1 h, the amount of released *p*-nitrophenol was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme releasing one μmole of *p*-nitrophenol per min. The apparent concentration of lipase was determined by a Bradford assay using BSA as a standard and a lipase molecular weight of 33,000 to permit calculation of $k_{cat}$ from the relationship $k_{cat}=V_{max}/[enzyme]$. The thermal stability of lipase was measured by incubation of a 0.5 ml aliquot of 50 μg/ml purified CalB at 50°C. A 50 μl sample was taken at various times and immediately cooled on ice. The residual lipase activity was measured using *p*NPP as a substrate.

### Purification of CalB Expressed in *H. polymorpha*

Yeast cells harboring the lipase secretion plasmids were grown for 18 h in YPD medium and centrifuged at 3,000 rpm for 10 min. The culture supernatant was concentrated by ultrafiltration using a YM10 membrane (Millipore) and then dialyzed with 50 mM sodium phosphate buffer at pH 6.5. Ammonium sulfate ($(NH_4)_2SO_4$) was added to the concentrated supernatant to a concentration of 1 M, and then the supernatant was loaded onto a Butyl-Sepharose CL-4B column previously equilibrated with 1 M ammonium sulfate in 50 mM sodium phosphate buffer at pH 6.5. Protein was eluted in a 1 M to 0 M ammonium sulfate gradient with a flow rate of 1 ml/min. Fractions with the highest lipase activity were combined and concentrated by ultrafiltration. The resulting protein sample was then loaded onto a 16×600 mm Superdex G-200 column (Pharmacia) previously equilibrated with 50 mM Tris-HCl buffer at pH 7.5 and 150 mM NaCl, and then eluted with the same buffer. Fractions with the highest lipase activity were pooled and used for further study. The total protein concentration was determined by a Bradford assay with BSA as the standard.

## RESULTS

### Surface Expression of CalB

The CalB expression plasmid pGK-CALB-CwpF (Fig. 1A) contains an expression cassette for CalB under control of the *GAPDH* promoter, the killer toxin signal sequence, and the cell-wall anchoring motif (CwpF) for surface display in *H. polymorpha* [12]. The plasmids pGK-CALB* for CalB secretion and pGK-CALB-CwpF for CalB surface display were introduced into *H. polymorpha* DL1-L cells. A plate assay was first performed to determine whether the transformants showed lipase activity on YPD agar plate containing tributyrin. Cells producing lipase developed a halo around the colony caused by degradation of tributyrin. Fig. 1C shows that cells harboring the plasmid pGK-CALB-CwpF produced a compact halo around the colony, whereas cells harboring pGK-CALB* produced a large halo, indicating that secretion of CalB is repressed by fusion of the CwpF anchor. Surface-displayed CalB showed considerably lower lipase activity in the whole-cell fraction.

### Random Mutagenesis of CALB

The *in vivo* recombination method was applied for construction of a CalB mutant library on the cell surface. To avoid the background variation caused by differential copy number integration of the transforming DNA in the chromosome, a modified *in vivo* recombination system using one-end overlapped fragments was developed [11]. The library constructed by this method showed the same lipase expression level due to the same integration locus and copy number. Error-prone PCR was performed to synthesize the insert fragment containing the mutant *CALB* gene library. Error-prone PCR was performed under the condition that two to five base substitutions were introduced on the *CALB* gene using the primer pair GPD-err and H150 (Fig. 1B). Approximately $3\times10^4$ transformants

**Table 1.** DNA mutations found in the sense strand of sequenced mutants of the *CALB* gene after PCR random mutagenesis (from 4.7-kb sequencing).

| | Mutations | | | | Type of mutations | | |
|---|---|---|---|---|---|---|---|
| Wild-type | T | C | A | G | Transitions | Transversions | Total |
| T | - | 11 | 3 | 1 | 11 | 4 | 15 |
| C | 6 | - | 2 | 2 | 6 | 4 | 10 |
| A | 2 | - | - | 2 | 2 | 2 | 4 |
| G | 2 | - | 3 | - | 3 | 2 | 5 |
| Total | 34 | | | | 22 | 12 | 34 |

were acquired by co-transformation of the PCR product with the EcoRI-BamHI-digested acceptor vector fragment from pHARS-CALB-SK. To analyze the diversity of the library, ten colonies from the mutant pool were randomly selected and subjected to DNA sequencing. The mutant *CALB* gene integrated into the chromosome was recovered by PCR using the primer pair GPD-err and H150. A total of 34 base substitutions was found from the sequencing of 4.7 kb of the sense strand. Of these 34 mutations, 17 resulted from conversion of T and C, and transitions outnumbered transversions 22 to 12 with 17 T↔C transitions and 5 A↔G transitions (Table 1). Among the ten transformants originally selected from the mutant pool, 3 clones carried 5 substitutions, 2 clones had 3 substitutions, and each remaining clone contained 0, 1, 4, 6, and 7 substitutions per 500 bp of the *CALB* gene, respectively.

### Screening of a CalB Mutant from the Library

Transformants were screened by halo assay on tributyrin plate to obtain a clone with improved lipase activity. A total of 23 colonies were selected for further analysis from 7,000 transformants tested. After culture of each transformant, the lipase activity of the whole-cell fraction was determined. Finally, CalB10 and CalB14 colonies exhibiting improved activities were selected. DNA sequencing revealed that

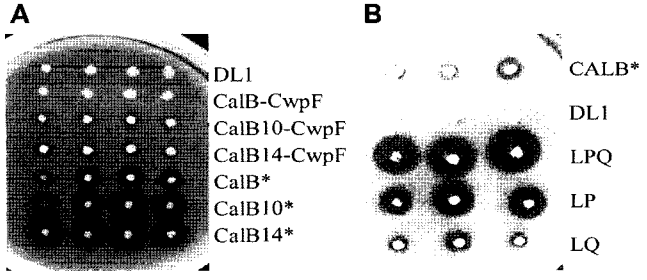


**Fig. 2.** Lipase activity of mutant strains selected from the library. **A.** Lipase activity of selected mutants CalB. **B.** Lipase activity of point mutated CalB. DL1, vector only; CalB-CwpF, strain harboring pLGK-CALB-CwpF; CalB10-CwpF and CalB14-CwpF, strain harboring pLGK-CALB10-CwpF and pLGK-CALB14-CwpF, respectively; CalB*, strain harboring pLGK-CALB*; CalB10* and CalB14*, strain harboring pLGK-CALB10* and pLGK-CALB14*, respectively. LP, LQ, and LPQ, strains harboring pLGK-LP, pLGK-LQ, and pLGK-LPQ, respectively.

**Table 2.** Lipase activity of the wild-type and mutant CalB.

| Construct | Activity of supernatant (U/l) | Construct | Activity of supernatant (U/l) |
|---|---|---|---|
| CalB* | 17,458 | LP | 98,890 |
| CalB10* | 118,401 | LQ | 48,850 |
| CalB14* | 190,854 | LPQ | 200,300 |
| Construct | Activity of the whole-cell fraction (U/l) | | |
| CalB-CwpF | 174 | | |
| CalB10-CwpF | 831 | | |
| CalB14-CwpF | 765 | | |

CalB*, strain harboring wild-type pLGK-CALB*; CalB10* and CalB14*, strains harboring pLGK-CALB10* and pLGK-CALB14*. CalB-CwpF, strain harboring pLGK-CALB-CwpF; CalB10-CwpF and CalB14-CwpF, strains harboring pLGK-CALB10-CwpF and pLGK-CALB14-CwpF. LP, LQ, and LPQ, strains harboring pLGK-LP, pLGK-LQ, and pLGK-LPQ, respectively.

CalB10 and CalB14 had the same amino acid replacement $Leu^{278}Pro$, and CalB14 had an additional $Leu^{219}Gln$ mutation.

Each selected *CALB* gene was PCR-amplified from the chromosome and introduced into the single-copy integration plasmid AMIpLD1 [1], to prevent influence from the copy number and the integration locus. Each *CALB* gene was also cloned as a secreted form and cloned into the same AMIpLD1plasmid to study whether these mutants exhibit an improved lipase activity as a soluble form. The plasmids were transformed in *H. polymorpha* and analyzed for lipase activity. Transformants displaying CalB10 and CalB14 showed larger activity halos on tributyrin plate in both cases, displaying or secreting forms than those obtained by wild-type CalB (Fig. 2A).

When the lipase activity of the whole-cell fraction was checked, each mutated CalB showed approximately 5 times higher activity than the wild-type (Table 2). The secreted forms of CalB10 and CalB14 exhibited 6 and 11 times greater lipase activities than the wild-type, respectively. The secreted form of CalB14 exhibited a much higher activity than CalB10, although they did not exhibit a significant difference in the anchored forms, indicating that the $Leu^{219}Gln$ mutation in CalB14 is effective only in the soluble form of the enzyme.

### Site-directed Mutagenesis of CALB

To determine if the amino acid change was the only reason for the increase of lipase activity and to determine the effect of the $Leu^{219}Gln$ and $Leu^{278}Pro$ substitutions, two individual mutations were separately introduced into the wild-type *CALB* gene. The *CALB* gene was synthesized by PCR to mutate the base encoding $Leu^{219}Gln$ and $Leu^{278}Pro$. The lipase mutant containing the $Leu^{278}Pro$ substitution was named LP and the $Leu^{219}Gln$ mutant was named LQ. The LP mutant was then mutated again to obtain an additional substitution of $Leu^{219}Gln$. This mutant was named

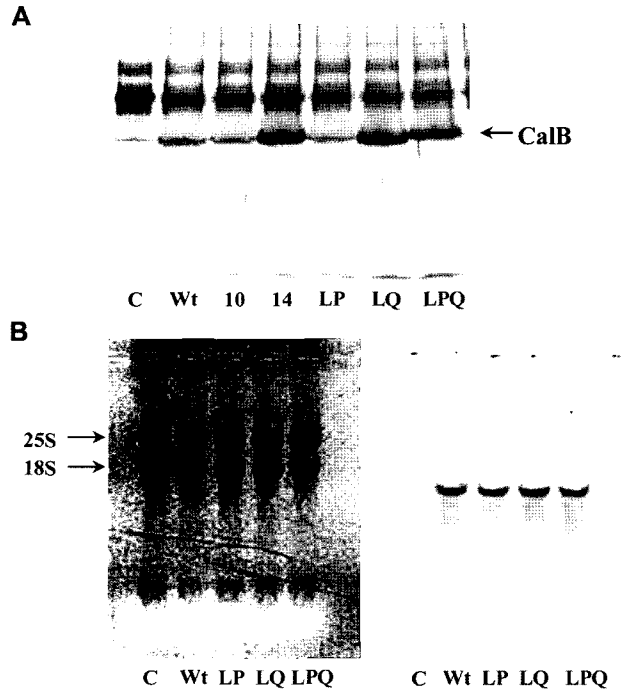


**Fig. 3.** SDS-PAGE analysis of the culture supernatant (**A**) and Northern blot analysis (**B**) of lipase mutants.
C, DL1 strain; Wt, strain harboring pLGK-LP*; 10 and 14, strain harboring pLGK-CALB10* and pLGK-CALB14*. LP, LQ, and LPQ, strains harboring pLGK-LP, pLGK-LQ, and pLGK-LPQ, respectively.

LPQ. Each mutant gene was cloned as a secreted form in the AMIpLD1 vector for single-copy integration into the *H. polymorpha LEU2* locus. The lipase activity of LP was similar to CalB10 and the activity of LPQ was similar to CalB14, indicating that the increase of catalytic activity resulted from the amino acids substitutions (Fig. 2B, Table 2). The transformant containing the LQ lipase showed a three times greater lipase activity compared with the wild type lipase, indicating that each amino acid substitution induced an independent increase in lipase activity.

The culture supernatant of each transformant was analyzed by SDS-PAGE. The transformant containing CalB14, LQ, and LPQ exhibited an exceptionally thick CalB band, whereas the wt, CalB10, and LP did not (Fig. 3A). We therefore deduced that protein production was increased because of the $Leu^{219}Gln$ amino acid substitution. The increase of protein production can be explained by increased transcription including enhancement of mRNA production or mRNA stability, or increased translation or protein stability. When the mRNA level of each transformant was analyzed by Northern blot analysis, each mutant showed a similar band intensity (Fig. 3B). The amount of mRNA synthesized during the culture time was similar for the wt and all the three mutants, LP, LQ, and LPQ. Therefore, the increase of protein production by the $Leu^{219}Gln$ mutation was not caused by an increase in mRNA.

**Table 3.** Kinetic parameters of purified wild-type and mutants for hydrolysis of pNPP.

| | $K_m$ (mM) | $V_{max}$ ($s^{-1}$) | $k_{cat}$ ($s^{-1}pmol^{-1}$) | $k_{cat}/K_m$ ($10^6 s^{-1}M^{-1}$) | Value relative to wild-type |
|---|---|---|---|---|---|
| CalB wt | 20.4 | 1.99 | 130.9 | 6.41 | 1.0 |
| CalB10 | 21.4 | 12.9 | 850.9 | 39.8 | 6.2 |
| CalB14 | 23.8 | 13.7 | 898.0 | 37.7 | 5.9 |

### Kinetic Constants and Stabilities of Purified CalB

Initial velocity studies were performed to determine the kinetic parameters of the selected mutants using *p*NPP as a substrate (Table 3). Each lipase was purified through Butyl-Sepharose CL-4B resin and gel filtration chromatography. The amino acid substitutions in CalB10 and CalB14 had a negligible effect on $K_m$ values. Both mutants had similar $k_{cat}$ values, which showed that the substitution $Leu^{219}Gln$ found in CalB14 was not related to the increase of catalytic activity. This substitution apparently influences only expression or secretion of the protein. The $k_{cat}$ value of these two mutants was approximately 6-fold higher than the wild type, indicating that the increased catalytic activity of the mutant results from the increase in $V_{max}$ and this increase is due to the $Leu^{278}Pro$ substitution.

Thermostability of both the wild-type and mutant lipases was also investigated. When the rate of inactivation was measured at 50°C in Tris-HCl buffer (pH 7.5), the half-lives of inactivation were 11 min for CalB10, 16 min for CalB14, and 25 min for the wild-type (Fig. 4).

## DISCUSSION

Directed evolution is one of the most effective methods currently available for protein engineering to obtain desirable properties. We applied two techniques for efficient enzyme

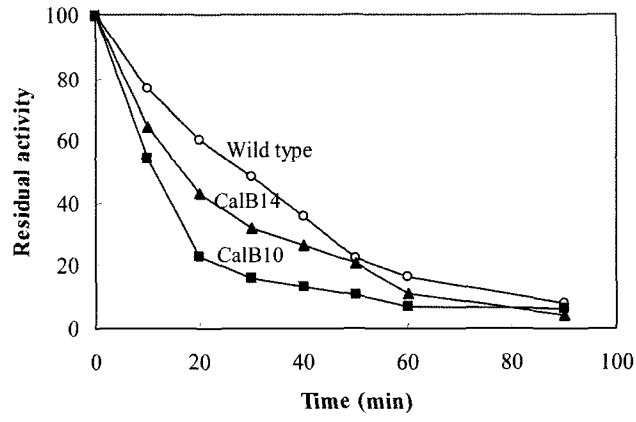


**Fig. 4.** Thermal stability of the wild-type and mutants CalB.
The residual enzyme activity after exposure to 50°C for various time intervals was assayed at 25°C.

evolution; namely, *in vivo* recombination and surface display. *In vivo* recombination in *H. polymorpha* helps overcome the problems usually encountered in library construction in this yeast, such as variation in the copy number and the integration locus [11]. *In vivo* recombination also improves the diversity of the mutant library and simplifies the construction of the mutant library by elimination of the gene manipulation procedure in *E. coli*.

Surface display of lipase has been attempted using various sources of lipases. Lipase from *Humicola lanuginosa* failed to be displayed on the cell surface as an active form because of hindrance of the C-terminal lipid-binding domain [20]. To solve this type of problem, lipase from *Rhizopus oryzae* was displayed on the cell surface by use of a spacer sequence between the lipase and the C-terminal anchor [26]. We expressed CalB as a fusion form with the anchor in the methylotrophic yeast *H. polymorpha*. Lipase activity in the whole-cell fraction was considerably lower than in the excreted lipase, similar to other reported lipase results. This lessened the whole-cell activity, but could be advantageous in screening of the improved clones. Activity halos of the secretion forms diffused rapidly during the incubation, which created difficulties in discriminating improved clones from the background. Directed evolution of CalB displayed on the cell surface was successfully performed, and two lipase mutants with increased whole-cell activities were isolated from the mutant CalB library. It was found that these two mutants, CalB10 and CalB14, showed a five times greater whole-cell activity and 6 and 11 times greater activities as a soluble form, respectively. The fact that surface-displayed CalB10 and CalB14 did not show any difference in whole-cell activities indicates that the increase of lipase production rarely affects the amount of lipase displayed on the cell surface. This fact may be a useful characteristic of the surface display of enzymes for directed evolution since only variants with increased activity, not the ones with increased protein amount, could be selected.

CalB10 contains the $Leu^{278}Pro$ substitution, whereas CalB14 contains the $Leu^{278}Pro/Leu^{219}Gln$ substitutions. The catalytic triad of CalB is made up of $Ser^{105}$, $Asp^{187}$, and $His^{224}$, and the active-site channel is formed by three structural parts including helices α5 and α10, and a loop region where $Ile^{189}$ projects into the channel (Fig. 5A). The C-terminal helix α10 (amino acids 268 to 287) is near the active site of the enzyme. It is dominated by alanine residues and is kinked in the middle at a proline residue ($Pro^{280}$). The mutated $Pro^{278}$ of both mutants located near the proline residue of the α10 helix may introduce an additional kink into the helix. Since the residue is also close to the substrate binding site, the mutation with Pro would change the size and shape of the active-site channel [23], resulting in an increase in the lipase activity. In the crystal structure of lipase B from *C. antarctica*, $Leu^{278}$ exhibits a large

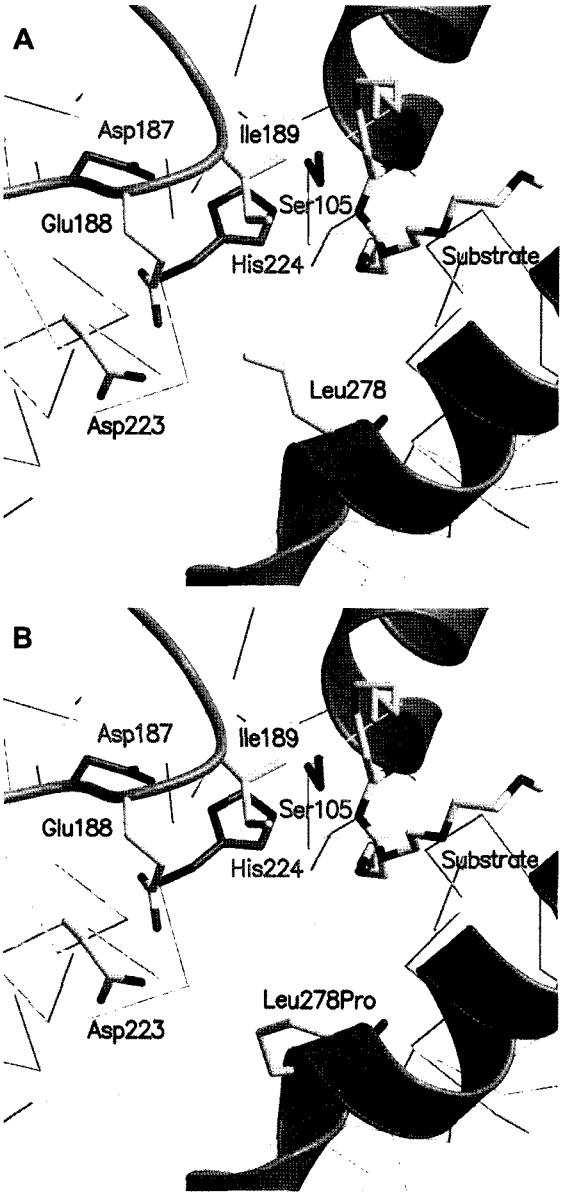


**Fig. 5.** The active site of (**A**) wild-type and (**B**) mutant CalB ($Leu^{278}Pro$).
The catalytic triad, Ser, Asp, and His, are shown in the diagram, and $Glu^{188}$ and $Asp^{223}$, which could be affected by $Leu^{278}Pro$, are included.

chi angle deviation from ideality, indicating a distorted configuration. In addition, a decrease in van der Waals volume of the mutated residue in this study may provide a subtle change in the active site pocket, which would have a larger space for the substrate (Fig. 5B). This local change in the active site, which is surrounded by the charged residues $Asp^{223}$ and $Glu^{188}$, may contribute to the increase in the lipase activity, corresponding to the 6-fold increase of $k_{cat}$ without any effect on $K_m$.

$Leu^{219}Gln$ mutation was found to cause additional enhancement of enzyme production in the soluble form.

The reason for the increase of protein production is currently unknown, but the increase did not arise from an increased amount of mRNA or a codon change. The codon used for $Leu^{219}$ was not a rare codon in *H. polymorpha* and the increase of LQ lipase expression was also found in *S. cerevisae* (data not shown). A phospholipase from *Vipera ammodytes* venom showed increased protein production in *E. coli* due to a change in the secondary structure of mRNA [9]. However, the secondary structure of mRNA analyzed by the RNAdraw program did not show any difference between mutant and wild-type lipases. Further work is required to explain how only one amino acid change can alter the protein production approximately three-fold.

We found that enzyme evolution can be achieved for enzymes displayed on cell surfaces. The system is expected to facilitate high throughput selection of mutants by reducing the occurrence of frequent false-positive clones. *In vitro* evolution of a surface-displayed enzyme also enables screening of a library for enzymes showing a high substrate affinity or different substrate specificities using a flow cytometer. Therefore, the novel screening system we have developed in this study can provide a general tool for multipurpose enzyme evolution.

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