Improved activity and thermostability of Candida antarctica lipase B by DNA family shuffling

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DNA family shuffling was used to create chimeric lipase B proteins with improved activity toward the hydrolysis of diethyl 3-(3',4'-dichlorophenyl)glutarate (DDG). Three homologous lipases from Candida antarctica ATCC 32657, Hyphozyma sp. CBS 648.91 and Crytococcus tsukubaensis ATCC 24555 were cloned and shuffled to generate a diverse gene library. A high-throughput screening assay was developed and used successfully to identify chimeric lipase B proteins having a 20-fold higher activity toward DDG than lipase B from Candida antarctica ATCC 24557 and a 13-fold higher activity than the most active parent derived from Crytococcus tsukubaensis ATCC 24555. In addition, the stability characteristics of several highly active chimeric lipases were also improved as a result of family shuffling. For example, the half-life at 45°C and melting point (Tm) of one chimera exceeded those of lipase B from Candida antarctica ATCC 24557 by 11-fold and 6.4°C, respectively, which closely approached the stability characteristics of the most thermostable parent derived from Hyphozyma sp. CBS 648.91.

Keywords: Candida antarctica lipase B/enantioselectivity/family shuffling/lipase/thermostability

Introduction

Candida antarctica lipase B (CALB) is one of the most widely used biocatalysts in organic synthesis on both the laboratory and the commercial scale. It has a broad range of applications including polymerizations, resolutions of alcohols and amines, modifications of sugars and sugar-related compounds, desymmetrization of complex drug intermediates and ring opening of β-lactams (Anderson et al., 1998; Homann et al., 2001; Rotticci et al., 2001a; Jaeger and Eggert, 2002; Park et al., 2003). Recently, we used the wild-type CALB on a pilot scale in the desymmetrization of ~200 kg of the prochiral diethyl 3-(3',4'-dichlorophenyl)glutarate (Figure 1), an intermediate in the synthesis of an NK1/NK2 antagonist (Homann et al., 2001). Although the enzymatic process was used successfully for the production of initial drug supplies, the relatively low specific activity and moderate stability of the catalyst at elevated temperature would have compromised its use on a commercial scale.

Numerous approaches to optimizing CALB activity, selectivity and stability have been used with various degrees of success. For example, immobilization of the lipase on solid supports and the use of non-aqueous solvents have often resulted in noticeable changes in the catalyst’s activity and stability, although in a rather unpredictable manner (Morgan et al., 1997; Anthonsen and Hoff, 1998; Lundhaug et al., 1998; Parker et al., 1998; Koops et al., 1999; Reetz et al., 2003). Several rational protein engineering approaches applied to CALB have produced mutant proteins with improved thermostability, activity and altered enantioselectivity (Patkar et al., 1997, 1998; Rotticci et al., 2001b), but these improvements were relatively modest when compared with results obtained by directed evolution technologies. Indeed, the application of random mutagenesis [error-prone polymerase chain reaction (PCR)] or DNA family shuffling, coupled with high-throughput screening, has often resulted in remarkable improvements in numerous characteristics of enzymes (Moore and Arnold, 1996; Cramer et al., 1998; Cherry et al., 1999; Ness et al., 1999; Schmidt-Dannert and Arnold, 1999; Abecassis et al., 2000; Petrounia and Arnold, 2000; Reetz and Jaeger, 2000; Joern et al., 2002; Ness et al., 2002; Cherry and Fidantsef, 2003; Hult and Berglund, 2003; Zha et al., 2003). Error-prone PCR applied to CALB has generated mutants with >20-fold improvement in half-life at 70°C (Zhang et al., 2003).

We now report the application of DNA family shuffling coupled with a high-throughput screening assay to create chimeric thermostable lipase B with enhanced activity toward hydrolysis of DDG.

Materials and methods

Construction of recombinant yeast producing homologous lipase B

An EcoRI/SalI fragment of pWS2 (Zhang et al., 2003) containing the lipase B gene from Candida antarctica ATCC 32657 was cloned into the EcoRI/Xhol site of the YEpFLAG-1 vector resulting in a recombinant plasmid designated pWS2a. The homologous lipase B gene from Hyphozyma sp. CBS 648.91 (Hoegh et al., 1995; Hashida et al., 1999) was amplified by PCR with forward (5’-AGTACGAATTCACACCCTTC-CCCACGGG-3’) and reverse (5’-AGTACCTCGAGTCATCC-AGTGATGACGCCC-3’) primers. EcoRI and XhoI restriction enzyme recognition sites in these primers are shown in italics.

![Fig. 1. Hydrolysis of prochiral diethyl 3-(3',4'-dichlorophenyl)glutarate by CALB.](Image)
A recombinant plasmid designated pWS60 was constructed by cloning this PCR fragment, containing the *Hyphozyma* lipase B gene, into the EcoRI/XhoI site of YEpFLAG-1. The homologous lipase B gene from *Crytodorococcus tsukubaensis* ATCC 24555 was cloned by PCR amplification of an ~700 bp PCR fragment using forward (5’-CCGCGTTCATGCACAACTACG-3’) and reverse (5’-GGTAGCCATGAGGTCAAGG-3’) primers. The full-length DNA sequence was completed using the Universal Genome Walker kit (Clontech, Palo Alto, CA). The sequence’s forward and reverse PCR primers were derived from internal conserved DNA sequence regions of the lipase B genes from *C. antarctica* strains ATCC 32657, ATCC 28323, ATCC 32189 and *Hyphozyma* sp. strain CBS6489.1. The full-length gene of ATCC 24555 was subsequently amplified by PCR using forward (5’-ATCAGAGTTTCTTACCCCTCCCACGGG-3’) and reverse (5’-ATCACCTTGCAGTACCTCCAGTGATGACGCCC-3’) primers. The resultant PCR fragment was then cloned into the *EcoRI/XhoI* site of YEpFLAG-1 resulting in the recombinant plasmid designated pWS57. Total genomic DNA isolation, PCR amplification and fragment purification, plasmid DNA isolation and nucleotide sequencing were conducted as described previously (Zhang et al., 2003). All three plasmids (pWS52a, pWS57 and pWS60) were transformed into Saccharomyces cerevisiae BJ3505 using the YEASTMAKER Yeast Transformation System according to the manufacturer’s instructions (Clontech).

**DNA family shuffling library construction**

The chimeric library was constructed using a modified DNA family shuffling method (Abecassim et al., 2000; Joern et al., 2002). Three ~1.2 kb DNA fragments containing the lipase B genes from pWS52a, pWS57 and pWS60 were amplified by using YcC-21 (5’-AGCACAAATAACGGGTTATTG-3’) and YcC-21 (5’-CCGCCGTTCATGCTCAAC-3’) primers. The reaction mixture (100 µl) contained 1 µl of *Pfu* polymerase (2.5 units/µl) (Stratagene, La Jolla, CA), 250 ng of each primer, 50 ng of each plasmid DNA, 0.2 mM dNTP mix and 10 µl of 10× *Pfu* buffer. The resulting parent DNA fragments were then digested with bovine pancreas DNase I (Roche, Indianapolis, IN) as follows. A 25 µl volume of solution containing 1 µg of each parent DNA was mixed with 1.25 µl of 1 M Tris–HCl (pH 7.5) and 1.25 µl of 0.2 M MnCl₂. Digestion was initiated with the addition of 1 µl of DNase I (0.1 unit/µl) into the mixture at 15°C. Following incubation for 20–30 s, the digestion was stopped by the addition of 12.5 µl of a solution containing 50 mM EDTA and 30% glycerol. The digested fragments were separated by gel electrophoresis. The desired 200–450 bp DNA fragments were isolated and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

Reassembly of the DNase I digested fragments was conducted in a 50 µl reaction mixture containing 42 µl of fragment DNA, 5 µl of 10× *Pfu* buffer, 2 µl of dNTP mix (10 mM each) and 1 µl (2.5 units) of *Pfu* polymerase. A progressive hybridization PCR cycling program (Joern et al., 2003) was used for reassembly. The reassembled reaction mixture (1 µl) along with primers YcC-21 and YcC-21 was used to amplify the full-length genes using a PCR cycling program described previously (Zhang et al., 2003).

Purified YEpFLAG-1 (50 ng) cut with *EcoRI/NotI* was combined with purified PCR fragments (300 ng) containing the full-length reassembled genes. The resulting DNA mixture was transformed into freshly prepared *S. cerevisiae* BJ3505 (200 µl) using the YEASTMAKER Yeast Transformation System according to the manufacturer’s instructions. The transformed cells were plated on to SD-trp (QBIOgene, Carlsbad, CA) agar plates for isolation of the recombinant yeast clones.

**Enzyme expression, library screening and characterization of positive clones**

Saccharomyces cerevisiae transformants were inoculated into 96-deep well plates (Corning, Acton, MA) containing 0.6 ml of expression medium (1% yeast extract, 8% peptone, 1% glucose, 3% glycerol and 20 mM CaCl₂). Cells were grown with agitation (200 r.p.m.) for 2 days at 30°C, followed by 7 days at 20°C. Supernatants containing the secreted enzymes were then clarified by centrifugation at 2500 g for 15 min.

Enzyme libraries were screened using the following protocol. Enzyme supernatant (10 µl) was transferred from each well of the 96-well growth plate into the corresponding well of the assay plate containing 180 µl of 4.45 mM MOPS (3-morpholinopropanesulfonic acid) buffer (pH 7.6), 2 µl of 0.5% bromothymol blue and 10 µl of a 120 mM solution of DDG dissolved in N,N-dimethylformamide (DMF). Addition of the enzyme solutions resulted in lowering of the pH from 7.6 to ~7.0. The plates were then sealed with a ChemiSeal sealing film (USA Scientific, Ocala, FL) and incubated at 30°C with agitation at 200 r.p.m. for 3 h followed by centrifugation at 2500 g for 15 min. Supernatant (100 µl) was transferred to the corresponding well in the measurement plate for determination of *A₆₂₀* using a plate reader (Perkin-Elmer HTS7000). Enantioselectivity and DDG conversion were determined using a 96-deep well plate format as follows: culture supernatants (10 µl) were mixed with 180 µl of 4.45 mM MOPS buffer (pH 7.6) and 10 µl of 400 mM DDG in DMF. This reaction mixture was then agitated at 200 r.p.m. for 3 h at 30°C. The reaction was stopped by adjusting the pH to ~3 with 10 µl of 0.2 M sulfuric acid. Samples were then extracted with ethyl acetate (420 µl) and analyzed by reversed-phase or chiral HPLC.

SDS–PAGE was conducted using a 12% pre-cast gel (Ready Gel Cell system; Bio-Rad, Hercules, CA). The gel was stained with GelCode Blue Stain reagent (Pierce, Rockford, IL). The relative amount of recombinant lipase B protein in the culture supernatants was determined by comparing the intensity of protein bands among different samples using an Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY).

**Analytical methods**

Chiral HPLC was conducted using a Phenomenon Chirex NGLY&DNB column (150×4.6 mm i.d.) equilibrated with hexane–2-propanol–trifluoroacetic acid (97:3:0.1). Reversed-phase HPLC was performed using a Phenomenex Luna 5µ C18 column (150×4.6 mm) equilibrated with acetonitrile–water–trifluoroacetic acid (60:40:0.1).

The protein melting point (*Tₘ*) was determined using circular dichroism by fitting the temperature dependence of ellipticity into a two-state folding–unfolding model as described previously (Zhang et al., 2003).

**Enzyme activity assays**

The enzyme specific activity for the hydrolysis of DDG was determined by measuring the reaction rates at protein concentrations of 0.4–2 µg/ml and substrate concentrations of 3–50 mM in 50 mM MOPS buffer, pH 7.0, at 38°C. The reaction rates were independent of substrate concentrations under this condition.
condition. The product formation was quantified by HPLC. Data were analyzed using Grafit 5 software, version 5 (Erithacus Software, Horley, UK).

The enzyme specific activity for the hydrolysis of tributyrin was determined by measuring the initial rate of hydrolysis using volumetric titration at 25°C. The reaction mixture, containing 29 ml of 5 mM potassium phosphate buffer (pH 7) and 0.9 ml of tributyrin, was emulsified in a pH-stat cuvette using high-speed stirring. The reaction was initiated by the addition of 100 µl of purified enzyme (2–5 µg). The pH was maintained at 7.0 by automatic addition of 25 mM NaOH using a pH-stat system (718 STAT Titrino; Metrohm, Herisau, Switzerland).

The enzyme specific activity for the hydrolysis of p-nitrophenyl butyrate (p-NB) was determined as described previously (Zhang et al., 2003).

The enzyme activity for the hydrolysis of Tween 80 was determined using Tween 80 agar plates as follows. Cells or culture supernatants were spotted onto Tween 80 agar plates (1% yeast extract, 4% peptone, 1% glucose, 3% glycerol, 2% Tween 80, 20 mM CaCl2 and 1.8% agar) and incubated at 30°C overnight. Active clones were identified by the formation of white fatty acid calcium salt precipitates in agar when Tween 80 was enzymatically hydrolyzed.

**Protein purification and analysis**

Recombinant lipase B from three parents and seven selected clones was purified by using an Anti-FLAG M1 monoclonal antibody affinity gel (Sigma, St Louis, MO) column and a BioCAD SPRINT perfusion chromatography system as described previously (Zhang et al., 2003). Protein purity was determined by SDS–PAGE. Enzyme concentration was determined using Bio-Rad protein assay reagent with bovine serum albumin (BSA) as standard.

Native gel electrophoresis was conducted using the SDS–PAGE procedure, except that SDS was excluded from the running buffer and native proteins were used for running samples. Following electrophoresis, the gels were stained with Gelcode Blue Stain reagent for 1 h and rinsed with water. The lipase activity was determined by incubating each protein band on Tween 80 agar plates overnight at 30°C.

Western blots were conducted as follows: purified enzymes (1µg) were separated on 12% SDS–PAGE gels and electrophotically transferred on to PVDF membranes (Bio-Rad). The membranes were soaked for 1 h in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.4) containing 1% (w/v) casein and then allowed to react with Anti-FLAG M1 primary antibodies (7 µg/ml) (Sigma) in TBS buffer containing 0.05% Tween 20 and 1 mM CaCl2 for 2 h. The membranes were washed with TBS buffer and allowed to react for 1 h with 1:2500 diluted secondary antibodies, conjugated with horseradish peroxidase (HRP) (Promega, Madison, WI). The membranes were then washed with TBS buffer and HRP activity was detected by the addition of the substrate, 3,3'-5,5'-tetramethylbenzidine (Promega).

Protein deglycosylation with N-glycanase was conducted by following the protocol provided by the enzyme supplier (Prozyme, San Leandro, CA). Purified enzyme (5 µg) was denatured by boiling in a solution of 0.1% SDS, 50 mM β-mercaptoethanol for 5 min. NP-40 (final concentration 0.75%) and N-glycanase (5 nU) were then added to the reaction mixture followed by incubation at 37°C for 16 h.

**Results and discussion**

**Cloning, sequencing and expression of homologous lipase B**

To take full advantage of natural diversity, we selected several homologous lipase B genes as candidates for DNA family shuffling. The genes from C.antarctica ATCC 32657 (Zhang et al., 2003) and Hyphozyma sp. CBS 648.91 (Hoegh et al., 1995; Hashida et al., 1999) were cloned by PCR as outlined in Materials and methods and determined to have a DNA and a protein sequence identity of 67.1 and 73.9%, respectively. Two additional homologous lipase B genes were cloned from C.antarctica (ATCC 28323 and ATCC 32189) by PCR using a primer pair derived from the beginning and the end of the CALB from strain ATCC 32657. Sequence analysis of these two cloned genes revealed that they have sequence identities with those of CALB from strain ATCC 32657 >91% at the DNA level and >97% at the protein level. To expand the pool of homologous lipase B genes, we decided to clone and sequence a relatively unknown lipase from C.tsubukensis ATCC 24555. This strain was shown to produce a thermally stable enzyme with an immunological response similar to that of CALB from C.antarctica ATCC 32657 (Ishii, 1993). Since attempts to clone this lipase using primers derived from the beginning and the end of CALB from strain ATCC 32657 were unsuccessful, we decided to utilize the primers derived from the internal conserved regions of four homologous lipase B genes described above. This strategy yielded an ~700 bp fragment that had >70% amino acid sequence identity with the CALB from strain ATCC 32657. This fragment was then expanded to a full-length 951 bp gene by using the Universal Genome Walker kit. Comparison of this novel gene with that of CALB from ATCC 32657 revealed a sequence identity of 68.9 and 77.7% at the DNA and protein levels, respectively.

Of the five homologous lipase B genes cloned, three with the highest divergence, C.antarctica ATCC 32657 (P52), C.tsubukensis ATCC 24555 (P57) and Hyphozyma sp. CBS 648.91 (P60), were selected for DNA family shuffling. Sequence analysis of these three genes revealed identities varying from 67.1 to 69.6% at the DNA level and from 73 to 81.1% at the protein level (Table 1). All three genes were actively expressed in an S.cerevisiae Yeast FLAG expression system as secreted fusion proteins with an eight amino acid FLAG peptide at their N-terminus as described previously (Zhang et al., 2003).

**Family shuffling and characterization of the shuffled library**

Both in vitro and in vivo recombination of homologous genes in yeast were used to create a shuffled library (Abecassis et al., 2000; Joern et al., 2002). Sequence analysis of 10 randomly picked clones revealed that nine out of 10 clones were hybrids. The number of crossovers per gene varied from one to 10 with

<table>
<thead>
<tr>
<th>Parent pair</th>
<th>DNA</th>
<th>Protein</th>
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<tbody>
<tr>
<td>1–2 p</td>
<td>68.9</td>
<td>77.7</td>
</tr>
<tr>
<td>1–3 p</td>
<td>67.1</td>
<td>73.9</td>
</tr>
<tr>
<td>2–3</td>
<td>69.6</td>
<td>81.1</td>
</tr>
</tbody>
</table>

* C.antarctica ATCC 32657.
* Crytococcus tsukubaensis ATCC 24555.
* Hyphozyma sp. CBS 648.91.
an average of 3.4. Only two point mutations were detected in the 10 clones sequenced. This accounts for a mutational frequency of about 0.2 base substitutions per gene. The recombination efficiency and the point mutation rate were similar to those reported for family shuffling of three homologous dioxygenases (Joern et al., 2002). Moreover, when 420 randomly picked clones were analyzed for Tween 80 and p-NB hydrolytic activity, distinct activity profiles characterized by a wide range of activity variation for each substrate were observed among different clones, indicating high functional diversity of the library (data not shown).

Assay development and the library screening

The hydrolysis of DDG results in the formation of an acid, prompting the use of a pH indicator-based assay for high-throughput screening. Although a number of high-throughput pH-based hydrolytic assays have been reported (Janes et al., 1998; Moris-Varas et al., 1999), they had to be modified substantially to fit the specific assay needs. In the case of lipase-catalyzed hydrolysis of DDG, the MOPS buffer and the bromothymol blue indicator (both have \( pK_a = 7.1 \)) were chosen to ensure proportionality between proton concentration and the absorbance. The concentrations of buffer, substrate, enzyme and indicator, in addition to the reaction time, were all optimized to achieve maximum assay sensitivity, while maintaining the ability to distinguish between clones with similar activity. The reaction progress was monitored by following the color transition from blue (alkaline) to yellow (acidic) visually and by measuring the change in absorbance at 620 nm.

Of the ~2500 clones screened using the above assay, 69 were identified with a >2-fold improvement in the rate of DDG hydrolysis compared with the most active parent, P57. Even though the enantioselectivity of the 69 clones varied widely, 16 clones and all three parents catalyzed the hydrolysis of DDG to the desired (S)-monoglutamate product with >99% ee. Interestingly, two clones had reverse enantiomeric preference and produced (R)-monoglutamate with moderate enantiomeric purity (55–60% ee).

In order to determine whether the enhanced activity of the 16 clones with desired enantioselectivity was due to their improved catalytic efficiency and/or their elevated protein expression level, we analyzed their crude enzyme preparations by SDS–PAGE. The results showed a significant variation in lipase expression level among different clones (data not shown). We therefore selected the seven most active clones for further characterization. Four clones, 2A10, 2B3, 8C7 and 12E10, had an elevated lipase protein expression level compared with that of the most active parent, P57. The expression level of the remaining three clones, 3A4, 4D6 and 5D10, was similar to that of P57.

Characterization of lipase B parents and selected clones

Enzyme purification. Three parent proteins, P52, P57 and P60, and the seven selected clones were purified using an Anti-FLAG M1 column as described in Materials and methods. Although the purification by affinity chromatography was expected to yield nearly homogeneous protein, SDS–PAGE analysis revealed the presence of at least two protein populations in every sample except for the two parent proteins, P52 and P60. We hypothesized that the mixed protein population was probably due to a heterogeneous glycosylation pattern commonly observed during heterologous gene expression in yeast (Romanos et al., 1992; Morawski et al., 2000). This hypothesis was supported by western blot analysis of the recombinant protein preparations with Anti-FLAG M1 monoclonal antibodies, which confirmed the presence of an N-terminal FLAG peptide in all protein populations detected by SDS–PAGE. Hence the purified protein fractions contained completely processed mature recombinant lipase and were free of any other proteins (data not shown). To verify further that the heterogeneity of the purified protein fractions detected by SDS–PAGE was caused by their distinct glycosylation pattern, we treated all protein fractions with N-glycanase, an enzyme that specifically removes the sugars from N-linked glycosylation sites. As expected, SDS–PAGE of the deglycosylated protein fractions revealed a single band of decreased molecular weight (Figure 2), further supporting the variable glycosylation hypothesis. SDS–PAGE of all seven selected clones exhibited similar patterns to those of P57 (data not shown). Consistent with earlier observations (Romanos et al., 1992; Morawski
et al., 2000), this variable degree of glycosylation did not appear to have any noticeable effect on lipase activity. The discrete protein fractions of all native recombinant enzymes separated by native gel electrophoresis were found to be active in the Tween-80 assay (see Materials and methods). Consequently, no further attempts were made to separate the fractions with different glycosylation patterns.

**Enzyme characterization.** The specific activities of the purified recombinant lipase B derived from three parents and seven selected clones for the hydrolysis of DDG, p-NB and tributyrin are presented in Table II. The selected clones had 3–13-fold higher specific activity for the hydrolysis of DDG than that of the most active P57 parent and up to 20-fold higher than that of P52. Not surprisingly, the activity for the hydrolysis of p-NB and tributyrin varied significantly from one clone to another, but generally stayed within the range of activities for the three parents.

*Candida antarctica* lipase B is a moderately thermostable enzyme retaining catalytic activity for many hours when incubated between 30 and 40°C. Above 40°C, the enzyme becomes progressively unstable, especially in aqueous media and in the presence of a high concentration of a xenobiotic substrate (Homann et al., 2001). Therefore, it was of interest to determine whether the clones selected for increased activity toward the hydrolysis of DDG also have acquired improved thermostability. We evaluated the thermal unfolding of each clone by measuring the temperature dependence of the circular dichroism response at 226 nm between 20 and 90°C. The $T_{m}$ of the three parents and seven selected clones are shown in Table III. Interestingly, it was noted that the heterogeneous glycosylation pattern of the seven selected clones did not alter the unfolding process that fit well to a two-state folding–unfolding model. The P57 parent protein, however, exhibited two separate transition phases. Since it was beyond the scope of this work to isolate sufficient quantities of various glycosylation populations of P57 for $T_{m}$ determination, no conclusion can be drawn regarding whether the two transition phases of P57 were caused by the heterogeneous glycosylation populations and/or the unique primary structure of this protein. The $T_{m}$ values of the seven clones were within the range of values observed for the three parent proteins (Table III). This result supports previous observations that DNA family shuffling often creates progeny proteins with combinations of multiple

**Fig. 3.** Temperature inactivation profile of clone 3A4 and three parents at 45°C. Each enzyme solution (150 μg/ml) was incubated at 45°C. Aliquots (80 μl) were removed at 0, 2, 4, 8, 12, 24 h after incubation and their activities were determined by using the p-NB assay. Each value was derived from an average of triplicate assays with standard deviation shown as error bars.

**Table III.** $T_{m}$ and $t_{1/2}$ of recombinant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$T_{m}$ (°C)</th>
<th>$t_{1/2}$ (h)$^{b}$</th>
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<tr>
<td>P52</td>
<td>56.2</td>
<td>2</td>
</tr>
<tr>
<td>P57</td>
<td>44 (62)</td>
<td>11</td>
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<tr>
<td>P60</td>
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<tr>
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<td>62.6</td>
<td>ND</td>
</tr>
<tr>
<td>8C7</td>
<td>58.9</td>
<td>ND</td>
</tr>
<tr>
<td>12E10</td>
<td>61.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^{a}$Determined from two separate transition phases in the unfolding profile.
$^{b}$Half-life at 45°C.
$^{c}$Not determined.
parental properties (Ness et al., 1999, 2002). To our satisfaction, all seven clones had an improved $T_m$ (up to 6.4°C) compared with that of P52 (CALB).

We then compared the rates of irreversible inactivation at 45°C of the three parent lipases with that of 3A4, one of the two most thermostable clones. The inactivation profile of 3A4 presented in Figure 3 was virtually identical with that of the most thermostable parent, P60, with $t_{1/2}$ for both enzymes being ~24 h. In comparison, $t_{1/2}$ of the two remaining parent proteins, P57 and P52, were significantly shorter, 11 and 2 h, respectively, as would be expected based on the lower $T_m$ of these proteins (Table III).

Sequence analysis. Sequence analysis of the seven selected clones revealed that they were all chimeras containing DNA elements from two to three parents, resulting from two to five crossover events (Figure 4A). No point mutations were found in any of the seven chimeric genes. Six out of seven chimeras had >95% protein sequence identity with P57, the most active among the three parents toward DDG hydrolysis. The remaining highly active chimera, 3A4, had only moderate protein sequence identity with P57 (85.5%) but the highest identity (95.6%) with the least active parent, P60. The chimeras bore the least resemblance to the P52 parent with protein sequence identity ranging from 73.9 to 81.2%. This sequence relationship is illustrated in a phylogenetic tree clearly showing that six chimeras were closely related to parent P57, while the remaining chimera, 3A4, was most similar to parent P60 (Figure 4B). The amino acid sequence alignment of the three parents and the two most active chimeras, 4D6 and 3A4, revealed that the lipase catalytic triad, six cysteine residues and the single N-linked glycosylation site of CALB (Uppenberg et al., 1994) are all conserved. However, when compared with P52, blocks of residues derived from P57 and P60 were observed throughout the entire amino acid sequences of 4D6 and 3A4 (Figure 5).

Structural mapping. To elucidate the role of amino acid substitutions on activity of the two most active chimeric proteins, the sequence variations between the chimeras and their closest parents, i.e. 3A4/P60 (Figure 6A) and 4D6/P57 (Figure 6B), were mapped on to the published crystal structure of CALB (Uppenberg et al., 1994). The protein sequence identity between 3A4/P60 and 4D6/P57 was ~95% for both chimera/parent pairs. The sequence comparison between 3A4 and P60 revealed that 3A4 had an insertion of Gly254 to Ala286 of P57 that aligns with Ser256 to Val288 of P60 (Figure 5). This stretch of amino acid residues comprises an omega loop and helix 10. Since part of helix 10 in P60, especially Ile280 and Ile287, bound the substrate binding pocket (Figure 6A), it seems likely that the Ile280Leu and Ile287Leu substitutions in 3A4 optimize the binding of DDG leading to a significant improvement in activity. The effect of
more distal variations on activity is more difficult to rationalize.

Structural and sequence comparisons of the 4D6 chimera with the P57 parent revealed that two substitutions, Asp188Glu and Ile189Val of 4D6, are located in the vicinity of the catalytic triad (Ser105, Asp187 and His224). The remaining substitutions occurred on the protein surface at a significant distance from the active site. Interestingly, all seven chimeras selected for their high activity toward DDG contained the same Asp188Glu substitution (sequences of 2A10, 2B3, 5D10, 8C7 and 12E10 not shown), tempting one to conclude that Asp188Glu substitution was responsible for converting a moderately active P57 parent protein into the highly active chimeras. However, the activity of the P52 and P60 parent proteins with Glu at the corresponding Asp188 of P57 is lower than that of P57. Apparently, the greater activity of chimeras depends not on the single site substitution, but on the directed evolution of this change in concert with the appropriate context provided by the rest of the protein.

In summary, DNA family shuffling was used to improve the activity of lipase B from \textit{C. antarctica} towards the hydrolysis of DDG. An efficient high-throughput method was used to screen a library of \textasciitilde 2500 clones leading to the identification of seven highly selective chimeras with 5–20-fold enhanced specific activity towards the substrate of interest. The thermal stability of the selected chimeras, characterized by an increase in $T_m$ and a decrease in the rate of irreversible thermoinactivation at 45°C, was also significantly improved compared with that of CALB. To our knowledge, this is the first report describing the use of DNA family shuffling of homologous lipase B genes to generate chimeric enzymes with improved properties. Further study of these hybrid enzymes should expand our understanding of the structure–function relationship for these valuable biocatalysts.

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**References**


