Multiple mutagenesis of non-universal serine codons of the *Candida rugosa LIP2* gene and biochemical characterization of purified recombinant LIP2 lipase overexpressed in *Pichia pastoris*

Guan-Chiun LEE*, Li-Chiun LEE†, Vasyl SAVA*[1] and Jei-Fu SHAW*[2]

*Institute of Botany, Academia, Sinica, Nankang, Taipei, Taiwan 11529, and †Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan 20224

The 17 non-universal serine codons (CTG) in the *Candida rugosa LIP2* gene have been converted into universal serine codons (TCT) by overlap extension PCR-based multiple site-directed mutagenesis. An active recombinant LIP2 lipase was overexpressed in *Pichia pastoris* and secreted into the culture medium. The recombinant LIP2 showed distinguishing catalytic activities when compared with recombinant LIP4 and commercial *C. rugosa* lipase. The purified enzyme showed optimum activity at pH 7 and a broad temperature optimum in the range 30–50 °C. The enzyme retained 80 % of residual activity after being heated at 70 °C for 10 min. Recombinant LIP2 demonstrated high esterase activity towards long-chain (C_{12}–C_{16}) *p*-nitrophenyl esters. Tributyrin was the preferred substrate among all triacylglycerols tested for lipolysis. Among cholesteryl esters, LIP2 showed highest lipolytic activity towards cholesteryl laurate. The esterification of myristic acid with alcohols of various chain lengths showed that the long-chain n-octadecanol (C_{18}) was the preferred substrate. In contrast, the esterification of n-propanol with fatty acids of various chain lengths showed that the short-chain butyric acid was the best substrate. From comparative modelling analysis, it appears that several amino acid substitutions resulting in greater hydrophobicity in the substrate-binding site might play an important role in the substrate specificity of LIP2.

Key words: isoforms, recombinant lipase, site-directed mutagenesis, substrate specificity.

INTRODUCTION

Candida rugosa (formerly *Candida cylindracea*) lipase (CRL) is a very important industrial enzyme that is widely used in biotechnological applications such as the production of fatty acids, the synthesis of various esters, and the resolution of racemic mixtures [1–7]. However, crude enzyme preparations obtained from the various commercial suppliers exhibit remarkable variation in their catalytic efficiency and stereospecificity [8]. Seven lipase genes, namely *LIP1* to *LIP7*, have been described in *C. rugosa*. However, only three lipases (LIP1, LIP2 and LIP3) have been identified [9–13] in commercial crude enzyme preparations. The purified isoenzymes displayed different substrate specificities and thermal stabilities [10,12,14–16].

We have observed variations in the protein composition of three commercial preparations of CRL that caused the essential differences in catalytic efficiency, substrate specificity and thermostability [17]. These data were derived using different inducers of the growth of *C. rugosa*, suggesting that certain genes may be modulated by the culture conditions. The multiplicity of CRL has been attributed to variations in gene expression, in addition to further glycosylation, partial proteolysis or other post-translational modifications [17]. Using competitive reverse transcription–PCR (RT-PCR), we have demonstrated that levels of gene expression usually follow the order (from highest to lowest): *LIP1*, *LIP3*, *LIP2*, *LIP5* and *LIP4*. It was observed that expression of *LIP1* and *LIP3* was always high and constitutive, while the expression of the other genes was affected by the culture conditions [18].

Several CRL genes have been cloned [19–22]. Proteins deduced from the DNA sequences showed 84 % similarity or 66 % identity in amino acid sequence across the entire protein family [23]. Lipases are mainly conserved at a catalytic triad, Ser-209, His-449 and Glu-341, and at the sites (Cys-60/Cys-97 and Cys-268/Cys-277) involved in disulphide bond formation. However, the enzymes differ in their N-glycosylation sites, isoelectric points and in some local features of their hydrophobicity profiles.

Separation of CRL isoenzymes is highly desirable to allow their use under well defined conditions. However, high identity in their protein sequences causes similarities in the physical properties of the lipases that create technical difficulties in the isolation of individual isoenzymes from cultures of *C. rugosa*. In addition, the purification procedure may affect the properties of different isoforms [24], and the differential expression level of the five lipase genes makes it difficult to purify each isoenzyme directly from cultures of *C. rugosa* on a preparative scale for industrial applications. Therefore cloning and expression can be suggested as the most suitable approach for the production, characterization and optimization of biocatalytic properties of pure isoforms.

Unfortunately, despite the general availability of the cloned genes, the non-spore-forming yeast *C. rugosa* utilizes a non-universal codon; namely, the triplet CTG, a universal codon for leucine, is read as serine [19]. CTG triplets encode most of the serine residues, including the catalytic Ser-209, in the lipases. Therefore the heterologous expression of such genes may result in the production of inactive lipases. Thus the conversion of

Abbreviations used: CL, commercial lipase; CRL, *Candida rugosa* lipase; 3-D, three-dimensional; RT-PCR, reverse transcription–PCR.

[1] Permanent address: Bogatsky Physico-Chemical Institute, Academy of Science of Ukraine, Odessa, Ukraine.
[2] To whom correspondence should be addressed (e-mail boplshaw@gate.sinica.edu.tw).

several or even all of the CTG codons into universal serine triplets is required for the expression of a functional lipase protein in heterologous hosts.

Heterologous expression of the cloned genes can also be used as a basis for the study of structure–function relationships in CRL. By using purified commercial native CRL, the three-dimensional (3-D) structures of the LIP1 and LIP3 enzymes [9,25,26], especially the structures of enzymes co-crystallized with substrate or inhibitor [26–28], have been provided in recent years. The production of pure lipase isoenzymes and their site-specific mutagenesis are now expected to generate more detailed information with regard to lipase catalysis and specificity. However, since the 3-D structures of the LIP2, LIP4, and LIP5 isoenzymes are not yet available, computer modelling based on the structures of LIP1 and LIP3 may render valuable information for understanding differences in the biocatalytic properties of various isoforms.

Recently, an amended *lip4* gene of CRL was successfully expressed in heterologous hosts [29,30]. We have changed the 19 CTG codons in *LIP4* to universal serine codons using site-directed mutagenesis. The functional expression of the *lip4* gene was successfully accomplished in *Escherichia coli* and in the yeast *Pichia pastoris*. This possibility has not been studied for the other CRL isoforms. LIP2 has been purified by a rather tedious procedure with a low yield (4.3 %) [31]. Although it has been shown to have a higher lipase specific activity than LIP1 (the major component of commercial Sigma type VII CRL) using triolein as a substrate [12], it is difficult to obtain sufficient amounts of purified LIP2 for detailed biochemical characterization, structural analysis and industrial applications.

In the present work, the 17 CTG codons in *LIP2* were replaced with universal serine codons (TCT) by overlap extension PCR-based multiple site-directed mutagenesis, and the active recombinant lipase was overexpressed in *P. pastoris*. The purified enzyme showed distinct catalytic activities compared with LIP4 and commercial CRL. In addition, for the first time the chosen heterologous host produced this important enzyme in high yield and high purity, making it available for further studies of structure–function relationships and for industrial applications.

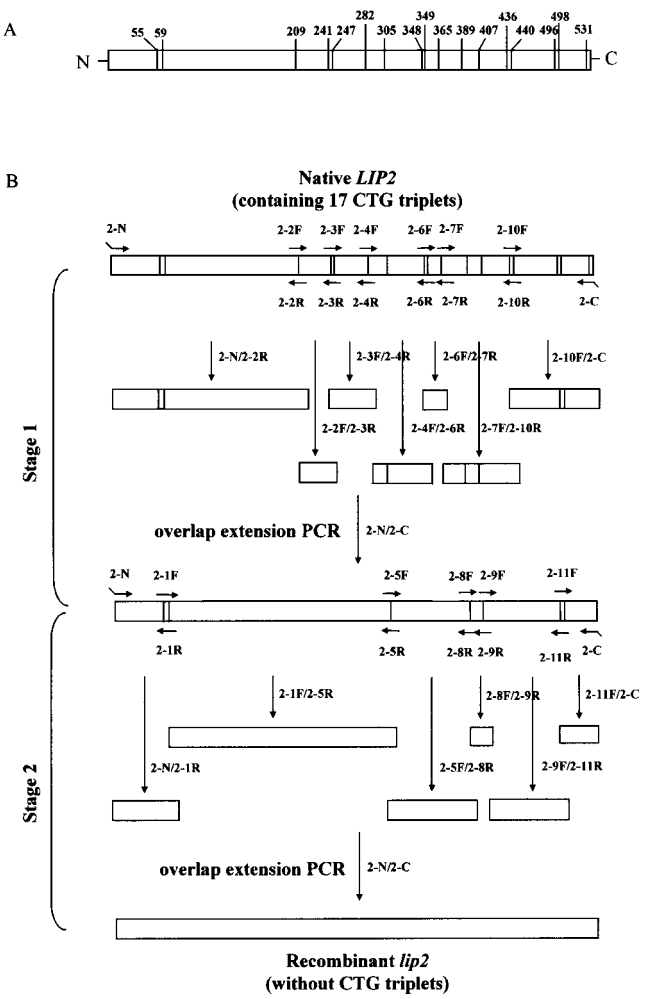

Figure 1 Simultaneous multiple mutagenesis introduced by overlap extension PCR for the replacement of the non-universal serine codons in the *C. rugosa lip2* gene

(**A**) The 17 CTG serine residues are indicated with their residue numbers along the polypeptide chain of LIP2. (**B**) The arrows indicate the mutagenic primers that were used to alter the 17 CTG triplets in the *lip2* gene. The oligonucleotide sequences of the mutagenic primers are shown in Table 1.

EXPERIMENTAL

Materials and strains

A commercial CRL (type VII) and substrates (*p*-nitrophenyl esters, cholesteryl esters, triacylglycerols, alcohols and fatty acids) were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, U.S.A.). *E. coli* TOP10F′ (Invitrogen, Carlsbad, CA, U.S.A.) was used as host for cloning, whereas *P. pastoris* strain SMD168H (Invitrogen) harboured the recombinant plasmid for gene expression. Primer oligonucleotides were produced by GENSET Singapore Biotech Pte Ltd. Enzymes for the recombinant DNA experiments were purchased from Promega Co. (Madison, WI, U.S.A.). DNA purification kits were from Qiagen Co. (Madison, WI, U.S.A.). Protein molecular mass markers were obtained from Bio-Rad (Richmond, CA, U.S.A.). A DNA sequencing kit was purchased from Perkin-Elmer Cetus (Wellesley, MA, U.S.A.). Other chemicals used were of the purest grades available.

Cloning and mutagenesis

The mature protein-coding sequence of *LIP2* (GenBank accession no. X64704), which encodes 534 amino acids, was obtained by RT-PCR. The final product was inserted between the *Kpn*I and *Xba*I sites of the *P. pastoris* expression vector pGAPZα C (Invitrogen) to generate pGAPZα C-LIP2 plasmid. This plasmid construct was used as the template for mutagenesis to convert the 17 non-universal CTG serine codons in *LIP2* into universal TCT serine codons. The simultaneous multiple mutagenesis was carried out by overlap extension PCR [32]. The mutagenesis procedure is depicted in Figure 1, and consisted of two stages. In the first, two end-primers and six pairs of primers annealing to the overlap regions were used to introduce mutations at positions 209, 241, 247, 282, 348, 349, 365, 436, 440 and 531. In the second stage, the *lip2* gene was mutated at positions 55, 59, 305, 389, 407, 496 and 498 using two end-primers and five pairs of mutagenic primers (Table 1). The amplified product was purified with an agarose gel-extraction kit (Qiagen). The sequence of mutated *lip2* containing the replacements was confirmed by full-length DNA sequencing.

Transformation and expression of *lip2* in *P. pastoris*

The plasmid (10 μg) harbouring the engineered *lip2* was linearized with *Eco*RV and transformed into *P. pastoris* SMD168H by electroporation. High-voltage pulses (1.5 kV) were delivered to

Table 1 Mutagenic primers used for the multiple site-directed mutagenesis of the *lip2* gene

The primers were used as forward primers, except for primer 2-C, which is a reverse primer. The sequences of reverse primers were fully complementary to those of the corresponding forward primers (not shown). The mutated serine codons (TCT) and the mutated nucleotides, which will not result in amino acid substitutions, are in bold. The numbers indicate the locations of the nucleotide in the lipase-encoding sequence. The created restriction sites (*Kpn*I in 2-N and *Xba*I in 2-C) are underlined.

Primer	Positions of serine residues	Oligonucleotide sequence (5′ to 3′)
2-N		ATGGTAC^{46}CCAC**C**GCCAC**G**CTCGCCAAC
2-1F	55, 59	G^{195}CAGTTTACC**TCT**TACGGCCCG**TCT**TGCATGCAG
2-2F	209	G^{651}ACCATATACGGCGAG**TCT**GCGGGCAGCATG
2-3F	241, 247	C^{753}ATCATGCAG**TCT**GGCTGCATGGTGCCG**TCT**GACCCGGTG
2-4F	282	G^{872}CTTGCGCGGCCTT**TCT**CAGGACACGTTG
2-5F	305	T^{943}CGTTGCGGTTG**TCT**TA**T**CTCCC**G**CGGC
2-6F	348, 349	T^{1075}TTGG**G**CTC**TCTTCT**TTGAACGTGACC
2-7F	365	T^{1123}ACTTCAAGCAG**TCT**TTCATCCACGCC
2-8F	389	T^{1196}CACCCAGGG**TTCT**CCGTTCGACAC**G**GG
2-9F	407	G^{1248}TTCAAACGGATC**TCT**GCGTTGCTTGGC
2-10F	436, 440	C^{1338}TCGTT**C**CTC**TCT**AAGCAGCTT**TCT**GG**G**TTGCCC
2-11F	496, 498	G^{1515}TACACCAGCAG**CTCT**CAG**TCT**GGCAACAACTTG
2-C	531	GGTCTAGACTACACAAAGAA**AGA**CGGCGGGTTGGA^{1621}

100 μl samples in 0.2 cm electrode gap cuvettes using a Gene Pulser® apparatus supplied with the Pulse Controller (Bio-Rad). Transformants were plated on YPDS (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol and 2 % agar, pH 7.2) plates containing 100 μg/ml Zeocin™ (Invitrogen) to isolate Zeocin™-resistant clones. Individual colonies containing lipase-secreting transformants were pitched and patched on 1 % tributyrin-emulsion YPD plates. The clear zone on the opaque tributyrin emulsion identified the lipase-secreting transformants. *P. pastoris* transformed with pGAPZα C was used as a negative control.

Purification of LIP2 recombinant enzyme

Selected *P. pastoris* transformants were grown in 500-ml flasks containing 200 ml of YPD medium with 100 μg/ml Zeocin™ at 30 °C for 3 days. The culture medium was concentrated by ultrafiltration on the Labscale™ TFF System with Pellicon® XL Devices coupling Biomax-30 membranes (Millipore, Bedford, MA, U.S.A.) and then applied on to a HiPrep™ 16/10 Octyl FF column (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.) equilibrated with TE buffer (20 mM Tris/HCl, 2 mM EDTA, pH 7.0). The column was washed with 5 column volumes of TE buffer plus 4 mM CHAPS. Bound proteins were then eluted with 5 column volumes of TE buffer containing 30 mM CHAPS. The eluted protein was dialysed against TE buffer.

The dialysed proteins were then applied to a HiPrep™ 16/10 Q XL column (Amersham Pharmacia Biotech) equilibrated with TE buffer, and the proteins were eluted using a linear gradient concentration of $(NH_4)_2SO_4$ (0–300 mM) in 5 column-bed volumes. Fractions were collected and protein concentration determined using the Bio-Rad assay kit. The enzyme activity was measured using *p*-nitrophenyl butyrate as a substrate (as described in the 'Enzyme characterization' section). The molecular masses of the purified recombinant lipases were determined in denaturing conditions by SDS/PAGE. Purified protein was stored in a storage buffer (60 mM KCl, 10 mM Tris/HCl, 1.25 mM EDTA, 1 % Triton X-100 and 17 % glycerol, pH 7.5) at −20 °C. Preparation of the recombinant LIP4 lipase from *P. pastoris* has been described in a previous paper [30].

Enzyme characterization

The initial rates of *p*-nitrophenol esterase and cholesterol esterase activities were measured spectrophotometrically (Hitachi U-2010) [33,34]. The hydrolysis of *p*-nitrophenyl esters was carried out at 37 °C in 500 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 % Triton X-100 and a 5 mM solution of the corresponding *p*-nitrophenyl ester. The increase in absorbance was recorded for 10 min at 348 nm (isosbestic point of the *p*-nitrophenol/*p*-nitrophenoxide couple). One unit of activity was defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per min under the above conditions. The cholesterol esterase activity of lipase was analysed using a peroxidase/cholesterol oxidase coupled system to measure the formation of cholesterol during the hydrolysis of a cholesteryl ester. The hydrolytic reaction was carried out at 37 °C in a mixture of 900 μl of reagent solution [100 mM Tris/HCl, pH 7.0, 50 mM $MgCl_2$, 6 mM phenol, 1 mM 4-aminoantipyrine, 4 mM 3,4-dichlorophenol, 10 mM sodium cholate, 3 g/l Thesit® (Boehringer Mannheim, Mannheim, Germany), 500 units/l cholesterol oxidase (Roche Diagnostics, Mannheim, Germany), 400 units/l peroxidase (Roche Diagnostics)] and 100 μl of 100 g/l Thesit® solution containing 10 mM substrate. The increase in absorbance was recorded for 10 min at 500 nm. One unit of cholesterol esterase activity was defined as the amount of lipase necessary to hydrolyse 1.0 μmol of ester per min. The lipolytic activity was evaluated titrimetrically using triacylglycerols with fatty acids of various chain lengths as substrates. The release of non-esterified fatty acids was monitored continuously by titration using 1 mM NaOH with a pH-Stat (Radiometer Copenhagen, Bagsvaerd, Denmark), adjusted to a constant end-point value. The substrate emulsion was prepared by a modification of the Tiss method [35]. The emulsification reagent contains, in 1 litre, NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 ml), gum arabic (6 g) and distilled water. Each kinetic assay was carried out in a 37 °C thermostatted reaction vessel containing 2.5 ml of 40 mM triacylglycerol substrate emulsion, 6.5 ml of distilled water and 1 ml of the enzyme solution. One unit of lipase activity was defined as the amount of lipase necessary to produce 1 μmol of fatty acid per min under the assay conditions.

Temperature and pH effects were assessed using *p*-nitrophenyl butyrate as a substrate. The optimal temperature for the esterase reaction was investigated in the range 10–60 °C at pH 7.0. The optimal pH was investigated in the pH range 3.0–9.0 using Good's buffer (50 mM each of Bicine, CAPS, sodium acetate and BisTris propane). To analyse thermal stability, the lipase was incubated for 10 min at various temperatures in the range 37–100 °C. Residual activity was determined spectrophotometrically at 37 °C using *p*-nitrophenyl caprylrate as substrate.

Lipase-catalysed esterification

For selectivity determination in ester synthesis, two competitive assays were carried out. In order to determine the alcohol selectivity of lipase in the synthesis of alkyl myristates, the reaction mixture consisted of 1 ml of iso-octane solution containing 50 mM each of mixed alcohols (n-hexanol, n-octanol, n-dodecanol, n-hexadecanol and n-octadecanol) and 600 mM myristic acid. To determine the fatty acid selectivity of lipase in the synthesis of n-propanol esters, the reaction mixture consisted of 1 ml of iso-octane solution containing 50 mM each of mixed fatty acids (butanoic, octanoic, dodecanoic, hexadecanoic and octadecanoic acids) and 600 mM n-propanol. The reaction mixture along with 0.1 mg of freeze-dried lipase (0.8-mm mesh) in a Teflon-lined, screw-capped reaction vial was shaken in an

orbital shaker at 250 rev./min and 30 °C. Sample aliquots of 1 μl were withdrawn at specified intervals during the 24-h reaction period and analysed by gas chromatography.

Gas chromatography

Quantitative analysis of the esterification reaction mixture was carried out on a Hitachi gas chromatograph, model G-3000, equipped with a flame ionization detector. Esters synthesized by enzymic reaction were separated on a Rtx®-1 cross-linked 100 % dimethyl polysiloxane capillary column (30 m × 0.25 mm × 0.25 μm; RESTEK Corp., Bellefonte, PA, U.S.A.) using nitrogen as the carrier gas at a flow rate of 1.0 ml/min. The split ratio was 1:10, and the injector and the flame ionization detector temperatures were 220 and 330 °C respectively. To separate alkyl myristates, the column temperature programme was as follows: initially held at 100 °C for 5 min; ramped from 100 to 230 °C at 30 °C/min; held at 230 °C for 5 min; ramped from 230 to 330 °C at 20 °C/min; finally held at 330 °C for 5 min. The column temperature programme used for separating n-propanol esters was as follows: ramped from 40 to 100 °C at 17 °C/min; from 100 to 220 °C at 30 °C/min; held at 220 °C for 3 min; ramped from 220 to 270 °C at 30 °C/min; finally held at 270 °C for 5 min. Peak areas were calculated using a Hitachi integrator model D-2000. The extent of conversion of substrate was defined as: [peak area of ester product/(peak areas of ester product and residue substrate)] × 100 %. The average values were calculated from three independent experiments.

Molecular modelling

3-D structures of LIP2 and LIP4 isoenzymes were obtained using the web-based comparative protein modelling server SWISS-MODEL (Version 3.5; Glaxo Smith Kline S.A., Geneva, Switzerland; www.expasy.org/swissmod/SWISS-MODEL.html). Protein sequences were obtained from the SWISS-PROT sequence database [36]. The structures of molecular complexes of LIP1 with hexadecanesulphonyl chloride [28] and of LIP3 with cholesteryl linoleate [26] were obtained from the RCSB protein data bank [37] under the entry numbers 1LPP and 1CLE respectively. These structures were used as the corresponding templates for comparative protein modelling. Display and analysis of modelled structures was performed using a Swiss-Pdb viewer (Glaxo Smith Kline S.A.).

RESULTS

Expression of recombinant CRL

The mature protein-coding region of the *lip2* gene that we have cloned and mutated contains four amino acid substitutions (A1V, T35S, R78L and H79D) when compared with the published sequence [20]. The expression plasmid pGAPZα C-LIP2, which contains the mutated *lip2* gene, uses the promoter of the *GAP* gene [38], encoding the glyceraldehyde-3-phosphate dehydrogenase enzyme, to constitutively express recombinant LIP2 protein to high levels in *P. pastoris*. In addition, this construct produces LIP2 fused to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. Due to the cloning positions that we chose on the vector, the secretion signal cleavage will leave 13 residues on the N-terminus. The majority of expressed LIP2 was secreted into the culture medium, and the active recombinant enzyme was stably accumulated at a concentration of 2.3 mg/litre after a 3 day culture. Purification of the recombinant enzyme by ultrafiltration combined with ion-exchange chromatography and hydrophobic interaction chromatography allowed us to obtain the homogeneous protein (Table 2), which had a molecular mass of approx. 60 kDa, as determined by SDS/PAGE (results not shown).

Table 2 Purification of recombinant LIP2 from *P. pastoris*

One unit of enzyme will hydrolyse 1.0 μmol of *p*-nitrophenyl butyrate to *p*-nitrophenol and butyric acid per min at 37 °C and pH 7.0.

Purification step	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Ultrafiltration	2.89	1095	1.00	100
Octyl-Sepharose	1.18	1705	1.56	64
Q-Sepharose	0.38	1818	1.66	22

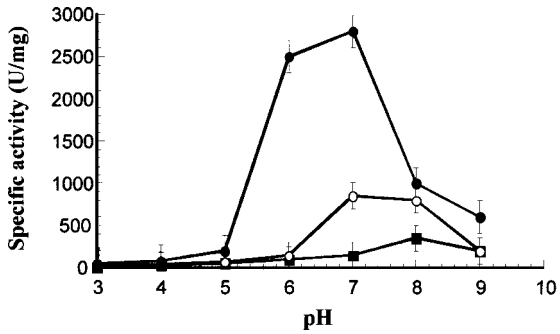

Figure 2 pH-dependence of recombinant LIP2, LIP4 and CL

The specific activities of recombinant LIP2 (●), LIP4 (○) and CL (■) were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 37 °C. Values are means ± S.D. from three independent experiments.

Characterization of recombinant CRL

The pH dependence of LIP2, LIP4 and a commercial lipase (CL) were studied using *p*-nitrophenyl butyrate at 37 °C (Figure 2). The optimal pH for LIP2 was 7.0. However, 90 % of enzyme activity was retained at pH 6.0. The optimal pH values for LIP4 and CL were pH 7–8 and 8 respectively. In comparison with LIP4 and CL, lipase LIP2 showed much higher specific activity at all pH levels tested. The clearest difference was observed at pH 6.0. Thus the relative specific activities of LIP2, LIP4 and CL were 100:4:3 at pH 6.0, whereas they were 100:80:25 at pH 8.0. Obviously, applications of LIP2 are especially advantageous at slightly acidic and neutral pH values.

The effects of temperature on *C. rugosa* LIP2, LIP4 and CL are depicted in Figure 3. The optimal temperatures for LIP2, LIP4 and CL were 40–50 °C, 40 °C and 37 °C respectively. Thus LIP2 demonstrated a broad temperature optimum range (30–50 °C), and much higher activity compared with LIP4 and CL throughout the temperature range. It is also advantageous that LIP2 exhibited relatively high activity at low temperatures. Its specific activity at 10 °C was 1000 units/mg, approx. 50 % of the highest enzyme activity. The data suggest that this enzyme can be effectively applied for the bioconversion of both labile substances and low-boiling-point compounds at a low temperature.

Table 3 Substrate specificity of recombinant lipases and CL in the hydrolysis of *p*-nitrophenyl (*p*-NP) esters containing fatty acids of various chain lengths

One unit of esterase activity is the amount of enzyme that hydrolyses 1.0 μmol of *p*-nitrophenyl ester per min at 37 °C and pH 7.0. Values are means ± S.D. from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Substrate	No. of carbon atoms in fatty acid chain	Specific activity (units/mg)		
		LIP2	LIP4	CL
p-NP acetate	C_2	11 ± 1 (0.4%)	10 ± 1 (0.7%)	16 ± 2 (3.1%)
p-NP butyrate	C_4	1986 ± 30 (72%)	899 ± 20 (63%)	359 ± 42 (72%)
p-NP caproate	C_6	108 ± 15 (4%)	151 ± 13 (11%)	72 ± 5 (14%)
p-NP caprylate	C_8	978 ± 126 (35%)	504 ± 24 (35%)	498 ± 67 (100%)
p-NP caprate	C_{10}	1453 ± 210 (53%)	1295 ± 179 (91%)	395 ± 19 (79%)
p-NP laurate	C_{12}	2567 ± 277 (93%)	867 ± 41 (61%)	269 ± 44 (54%)
p-NP myristate	C_{14}	2567 ± 277 (93%)	1140 ± 41 (80%)	372 ± 5 (75%)
p-NP palmitate	C_{16}	2766 ± 4 (100%)	1429 ± 127 (100%)	317 ± 5 (64%)
p-NP stearate	C_{18}	1580 ± 21 (57%)	580 ± 21 (41%)	67 ± 1 (13%)

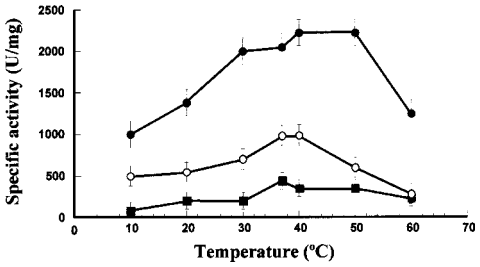

Figure 3 Effect of temperature on the activities of recombinant LIP2, LIP4 and CL

The activities of recombinant LIP2 (●), LIP4 (○) and CL (■) were measured by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate at pH 7.0. Values are means ± S.D. from three independent experiments.

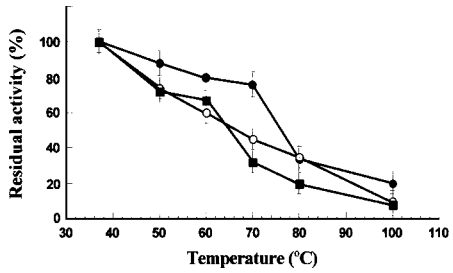

Figure 4 Thermal stability of recombinant LIP2, LIP4 and CL

The residual activities of recombinant LIP2 (●), LIP4 (○), and CL (■) after 10 min of heating at different temperatures were measured spectrophotometrically using *p*-nitrophenyl caprylate as substrate at 37 °C and pH 7.0. The percentages represent relative activities, taking that in the assay at 37 °C as 100%. The residual activities of LIP2, LIP4 and CL in the 37 °C assay were 933, 1132 and 482 units/mg respectively. Values are means ± S.D. from three independent experiments.

The residual activities of LIP2, LIP4 and CL were assayed after heating at various temperatures for 10 min. Comparison of enzyme thermostability in the 50–70 °C range showed that LIP2 was more stable than LIP4 or CL (Figure 4). In particular, the residual activities of LIP2, LIP4 and CL at 70 °C were 80%, 50% and 35% respectively.

Substrate specificities of recombinant CRLs

Hydrolysis of *p*-nitrophenyl esters containing fatty acids of various chain lengths by LIP2, LIP4 and CL demonstrated different preferences for the ester substrates (Table 3). The most favourable substrate for LIP2 and LIP4 lipases was *p*-nitrophenyl palmitate. CL demonstrated a preference for *p*-nitrophenyl caprylate. Both LIP2 and LIP4 showed much higher activity towards medium- and long-chain fatty acid esters (C_{12}–C_{18}), but LIP2 exhibited 2–3 times higher activity than LIP4. Specific activities assayed with most of the *p*-nitrophenyl esters, including *p*-nitrophenyl butyrate, caprylate, caprate, laurate, myristate, palmitate and stearate, were ranged in the order LIP2 > LIP4 > CL.

The lipolytic activity of LIP2 was compared with the activities of LIP4 and CL. Table 4 shows the different preferences of LIP2, LIP4 and CL for triacylglycerols containing fatty acids of various chain lengths. Both LIP4 and CL displayed the highest activity for hydrolysis of the medium-chain triacylglycerol (tricaprylin); however, LIP2 was more active towards a short-chain triacylglycerol (tributyrin). Among the 10 triacylglycerols tested, LIP2 displayed higher activities towards C_2, C_4, C_6, C_{12}, C_{18} and $C_{18:1}$ than LIP4 or CL; LIP4 showed better substrate specificities for C_8 and C_{16} than LIP2 or CL; while CL hydrolysed C_{10} and C_{14} more efficiently than LIP2 or LIP4. Therefore different LIP isoforms should be used for different industrial applications in triacylglycerol hydrolysis.

The comparative cholesterol esterase activities of LIP2, LIP4 and CL were studied with three different esters, as shown in Table 5. With these substrates, LIP2 demonstrated much higher specific activity than LIP4 or CL. Cholesteryl laurate was the preferred substrate for all enzymes tested. However, LIP2 surpassed both LIP4 and CL in its activity towards cholesteryl laurate: the ratio of specific activities for LIP2, LIP4 and CL was approx. 29:7:1.

Equimolar mixtures of different alcohols were employed for evaluating the substrate specificities of LIP2, LIP4 and CL in the esterification of myristic acid. Table 6 shows that the activity of LIP2 was higher than those of LIP4 or CL in the synthesis of hexadecyl and octadecyl myristates. Thus long-chain alcohols were preferred by LIP2 in myristic ester synthesis. In contrast, CL demonstrated better performance in the synthesis of hexyl, octyl and dodecyl myristate, suggesting a preference for medium- and short-chain alcohols.

Elucidation of substrate specificity in the esterification of n-propanol was also carried out using equimolar mixtures

Table 4 Substrate specificity of recombinant lipases and CL in the hydrolysis of triacylglycerols containing fatty acids of various chain lengths

One unit of lipolytic activity is the amount of enzyme necessary to produce 1.0 μmol of acid per min at 37 °C. Values are means ± S.D. from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Substrate	No. of carbon atoms in fatty acid chain	Specific activity (units/mg)		
		LIP2	LIP4	CL
Triacetin	C_2	39 ± 1 (2%)	10 ± 1 (0%)	11 ± 1 (0%)
Tributyrin	C_4	2540 ± 60 (100%)	1138 ± 10 (28%)	1029 ± 64 (33%)
Tricaproin	C_6	599 ± 37 (24%)	167 ± 7 (4%)	358 ± 14 (11%)
Tricaprylin	C_8	1239 ± 31 (49%)	4082 ± 298 (100%)	3118 ± 190 (100%)
Tricaprin	C_{10}	1399 ± 176 (55%)	628 ± 11 (15%)	2160 ± 75 (69%)
Trilaurin	C_{12}	1743 ± 110 (69%)	389 ± 4 (10%)	1502 ± 8 (48%)
Trimyristin	C_{14}	504 ± 33 (20%)	375 ± 33 (9%)	915 ± 26 (29%)
Tripalmitin	C_{16}	54 ± 6 (2%)	151 ± 10 (4%)	137 ± 12 (4%)
Tristearin	C_{18}	422 ± 9 (17%)	348 ± 38 (9%)	39 ± 2 (1%)
Triolein	$C_{18:1}$	513 ± 4 (20%)	352 ± 5 (9%)	303 ± 24 (10%)

Table 5 Substrate specificity of recombinant lipases and CL in the hydrolysis of cholesteryl esters containing fatty acids of various chain lengths

One unit of esterase activity is the amount of enzyme necessary to hydrolyse 1.0 μmol of acid per min at 37 °C. Values are means ± S.D. from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Substrate	No. of carbon atoms in fatty acid chain	$10^2 \times$ Specific activity (units/mg)		
		LIP2	LIP4	CL
Cholesteryl butyrate	C_4	127.1 ± 1.4 (32%)	24.7 ± 1.3 (25%)	7.3 ± 0.1 (53%)
Cholesteryl laurate	C_{12}	402.0 ± 35.5 (100%)	98.2 ± 3.2 (100%)	13.8 ± 0.5 (100%)
Cholesteryl stearate	C_{18}	127.1 ± 1.4 (32%)	45.0 ± 1.8 (46%)	6.5 ± 0.7 (47%)

Table 6 Substrate specificity of recombinant lipases and CL in the esterification of myristic acid with alcohols of various chain lengths

The initial rate was determined for the esterification of myristic acid with an equimolar mixture of alcohols of various chain lengths at 30 °C. Values are means ± S.D. from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Ester	$10^{-3} \times$ Initial rate of esterification (μmol · h^{-1} · mg^{-1})		
	LIP2	LIP4	CL
n-Hexyl myristate	1.86 ± 0.17 (57%)	2.35 ± 0.24 (100%)	3.89 ± 0.47 (100%)
n-Octyl myristate	2.28 ± 0.27 (70%)	1.55 ± 0.16 (66%)	2.73 ± 0.30 (70%)
n-Dodecyl myristate	1.16 ± 0.11 (35%)	0.66 ± 0.07 (28%)	1.79 ± 0.20 (46%)
n-Hexadecyl myristate	2.33 ± 0.22 (72%)	1.04 ± 0.08 (44%)	1.13 ± 0.13 (29%)
n-Octadecyl myristate	3.26 ± 0.33 (100%)	1.71 ± 0.11 (72%)	1.19 ± 0.07 (31%)

Table 7 Substrate specificity of recombinant lipases and CL in the esterification of n-propanol with fatty acids of various chain lengths

The initial rate of esterification was determined for the esterification of n-propanol with an equimolar mixture of fatty acids of various chain lengths at 30 °C. Values are means ± S.D. from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Ester	$10^{-3} \times$ Initial rate of esterification (μmol · h^{-1} · mg^{-1})		
	LIP2	LIP4	CL
n-Propyl butyrate	9.96 ± 1.04 (100%)	5.19 ± 0.49 (100%)	0.95 ± 0.05 (99%)
n-Propyl octanoate	2.41 ± 0.24 (24%)	2.35 ± 0.11 (45%)	0.68 ± 0.05 (70%)
n-Propyl dodecanoate	1.10 ± 0.13 (11%)	1.35 ± 0.06 (26%)	0.43 ± 0.05 (45%)
n-Propyl hexadecanoate	0.68 ± 0.07 (7%)	1.56 ± 0.21 (30%)	0.96 ± 0.12 (100%)
n-Propyl octadecanoate	0.77 ± 0.02 (8%)	1.01 ± 0.06 (19%)	0.94 ± 0.12 (98%)

of fatty acids of various chain lengths. Table 7 shows that LIP2 possessed much higher activity in the synthesis of propyl butyrate than LIP4 or CL, suggesting that it favours short-chain fatty acids in the esterification of n-propanol. In contrast, LIP4 was best in the synthesis of n-propyl dodecanoate, hexadecanoate and octadecanoate, suggesting that long-chain fatty acids are preferred in the synthesis of propyl esters.

Molecular modelling of LIP2 and LIP4

In order to understand the molecular basis of substrate specificity, we compared the geometry and properties of LIP2 and LIP4. 3-D structures of the LIP2 and LIP4 isoenzymes were obtained from the web-based SWISS-MODEL server, which utilizes the comparative modelling approach [39]. After pairwise superimposition, the overall modelled structures were very similar to those of the template structures. A total of 534 Cα atoms superimpose with root-mean-square deviations of less than 0.1 Å (1 Å = 0.1 nm). Figure 5 shows the comparative 3-D topography of substrate-binding sites for *C. rugosa* LIP2 and LIP4 lipases. The results allowed analysis of the cholesterol linoleat-binding site (Figure 5A) and the acyl-binding tunnel (Figure 5B). Most of the residues located in close contact with the cholesterol linoleate molecule and in place of the acyl chain (within the 4 Å cut-off distance) were identical and shared the same conformations as those of the template residues. Some of the substrate-binding residues showed differences between LIP2 and LIP4. Only one definite difference was found at the acyl-binding sites: Val-296 in LIP2 was replaced by Ala in LIP4. At the alcohol-binding sites,

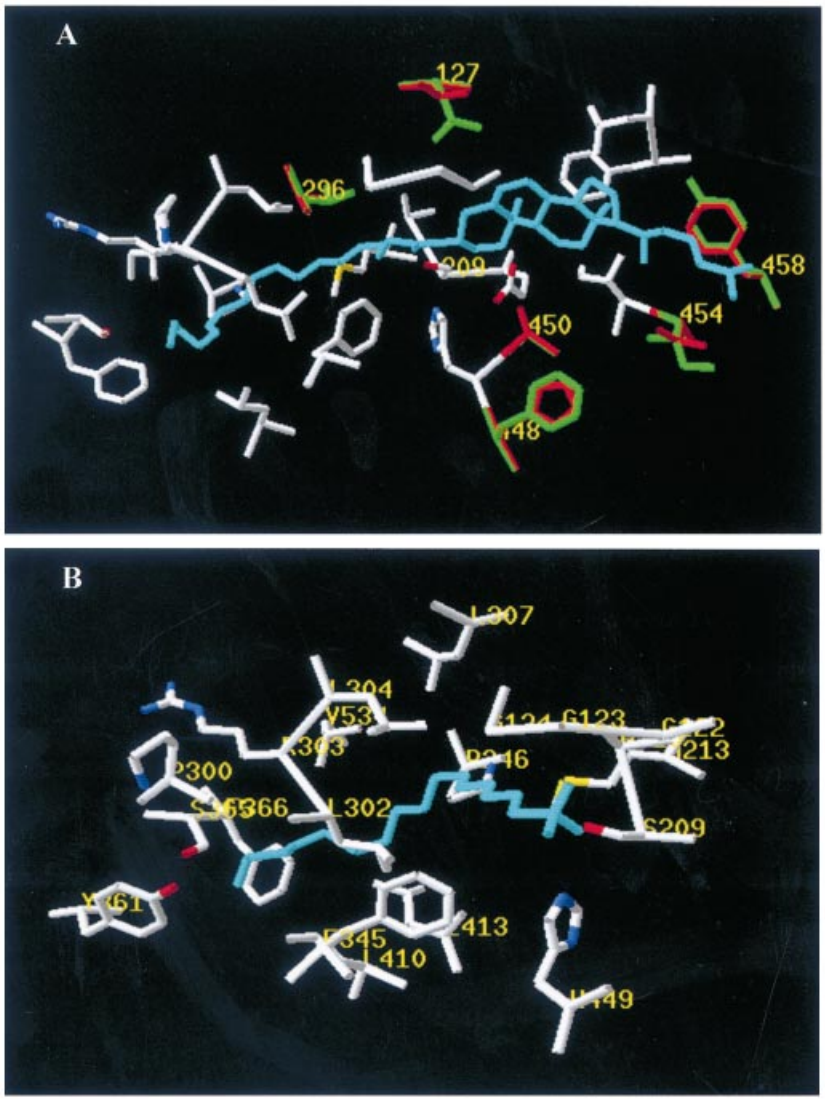

Figure 5 3-D topography of substrate-binding sites in *C. rugosa* LIP2 and LIP4 lipases

(**A**) Cholesterol linoleate-binding site. The residues located in close contact to the cholesterol linoleate molecule (coloured blue) are shown. The residues that are different between LIP2 and LIP4 are coloured green in LIP2 and red in LIP4, and are indicated by residue numbers. The residues that are the same in LIP2 and LIP4 are coloured white. (**B**) Acyl-binding tunnel. The residues located in close contact to the acyl chain of a hexadecanesulphonate inhibitor (coloured blue) are shown. The residues are coloured as in (**A**).

Leu-127, Phe-448, Gly-450, Ile-454 and Tyr-458 of LIP2 were replaced by Val, His, Ala, Val and Phe respectively in LIP4.

DISCUSSION

The difficulty in the heterologous expression of lipase *LIP2* genes stems from *C. rugosa*'s non-universal codon usage, in which the triplet CTG, the universal codon for leucine, is read as serine [19]. The recombinant LIP1 isoenzyme has been functionally expressed in *S. cerevisiae* and *P. pastoris* by completely synthesizing the *lip1* gene with an optimized nucleotide sequence [40]. Previously we engineered and functionally expressed the synthetic gene of LIP4 [29,30]. In the present work we performed functional expression of the codon-optimized gene for the LIP2 isoform in *P. pastoris*. The transformant cells were highly stable, and it is possible to increase LIP2 production with a higher-density fermentation scale [41].

We have demonstrated that expression of LIP1 and LIP3, the major CRL isoforms, is high and constitutive. In contrast, the amount of native LIP2 in commercial CRL is very low, and its expression varies depending on culture conditions [18]. It is, therefore, very difficult to purify an amount of LIP2 that is sufficient for biochemical analysis or industrial applications. The successful functional expression of recombinant LIP2 in *P. pastoris* by multiple mutagenesis of non-universal serine codons is an important breakthrough, opening the way to the large-scale industrial preparation of recombinant LIP2 and to the further engineering of catalytic properties desired for applications of the enzyme.

Recombinant LIP2 overexpressed in *P. pastoris* has a similar molecular mass to the native LIP2 isoenzyme, which had been purified from a *C. rugosa* fermentation [31]. These two enzymes showed similar substrate specificities for the hydrolysis of long-chain *p*-nitrophenyl esters. However, the recombinant enzyme

Figure 6 Amino acid sequence identity of the substrate-binding sites in the various CRL isoenzymes

The acyl-binding sites are shaded grey, and the alcohol-binding sites are not shaded. Residues forming the oxyanion hole (Gly-123, Gly-124 and Ala-210) and the catalytic site (Ser-209 and His-449) are shaded black. Residues identical with those of LIP1 are represented by dots. The residue numbers are indicated above the alignment.

displays higher specific activity than the native enzyme towards all *p*-nitrophenyl esters tested. In particular, the chain-length preference for triacylglycerols was quite different between these two enzymes. Recombinant LIP2 preferred to hydrolyse the short-chain triacylglycerol (tributyrin), whereas the native LIP2 was more active on the long-chain triacylglycerol (triolein). These variations may be due to differences in glycosylation, the additional N-terminal peptide or the amino acid substitutions between the recombinant and native LIP2. These points need to be investigated further.

Since CRLs have been widely used in biotechnological applications, and almost all the applications use crude commercial enzymes, comparison of pure recombinant LIP2 and LIP4 lipases with a crude preparation will provide an evaluation of the potential uses of pure recombinant enzymes in biotechnological applications. The recombinant LIP2 possesses unique and remarkable catalytic properties, which essentially differ from those of LIP4 and CL. For example, the high specific activity of LIP2 towards cholesteryl esters (Table 5) might be very useful for the determination of cholesterol in clinical and food analyses. Since approx. 70–80 % of serum cholesterol is esterified by various fatty acids [42], if coupled to cholesterol oxidase and peroxidase, LIP2 could be useful as an enzymic sensor of serum cholesterol. LIP2 showed high activity towards long-chain alcohols in esterification with myristic acid (Table 6). This enzyme could be used in the industrial production of wax esters, such as hexadecyl myristate and octadecyl myristate, used in lubricants and cosmetics. In the esterification of propanol with various fatty acids, LIP2 preferred short-chain fatty acids such as butyric acid (Table 7) and, therefore, might be useful in the industrial production of flavour esters.

We are also attempting to resolve the structures of the recombinant lipases to reveal detailed conformational features near the catalytic centre. X-ray crystallography, computer-assisted modelling and protein engineering have been widely used for the structural investigation of lipases [43]. The structures of complexes of substrates or substrate analogues with LIP1 or LIP3 have been published [26–28], which have helped to identify the binding site. Since the 3-D structures of LIP2 and LIP4 are not available, comparative or homology protein modelling [44] can be used to calculate useful 3-D topography. Comparative modelling uses experimentally determined protein structures (templates) to predict the conformation of another protein with a similar amino acid sequence. This is possible because a small change in the sequence usually results in a small change in the 3-D structure [45].

3-D structures of the substrate-binding sites of *C. rugosa* LIP2 and LIP4 lipases were obtained through comparative modelling. As shown in Figure 6, six out of ten residues at the alcohol-binding site and two out of 15 residues at the acyl-binding site were not identical among the four *C. rugosa* isoenzymes considered in the present study. It is suggested that the alcohol-binding site shows lower conservation than the acyl-binding tunnel and, therefore, might play an important role in substrate specificity. For example, some replacements obviously changed the hydrophobicity in the alcohol-binding site, reflected in the higher activity of LIP2 than of LIP4 in the hydrolysis of cholesterol esters (Table 5) and in the synthesis of myristate esters (Table 6). Other biochemical properties are more difficult to explain. Our preliminary results suggest that the flap domain could greatly affect the enzyme specificity and other biochemical properties (G.-C. Lee, S.-J. Tang and J.-F. Shaw, unpublished work). Crystallization of recombinant LIP2 and LIP4 for X-ray diffraction analysis is currently under way, in order to elucidate the structural basis of enzyme specificity.

This work was supported by grant NSC-90-2313-B-001-023 from the National Science Council, Republic of China.

REFERENCES

1 Wang, Y. J., Sheu, J. Y., Wang, F. F. and Shaw, J. F. (1988) The lipase catalyzed oil hydrolysis in the absence of added emulsifier. Biotechnol. Bioeng. **31**, 628–633
2 Klibanov, A. M. (1990) Asymmetric transformations catalyzed in organic solvents. Acc. Chem. Res. **23**, 114–120
3 Shaw, J. F., Chang, R. C., Wang, F. F. and Wang, Y. J. (1990) Lipolytic activities of lipase immobilized on six support materials. Biotechnol. Bioeng. **35**, 132–137
4 Valivety, R. H., Halling, P. J., Peilow, A. D. and Macrae, A. R. (1994) Relationship between water activity and catalytic activity of lipases in organic media. Effects of supports, loading and enzyme preparation. Eur. J. Biochem. **222**, 461–466
5 Vulfson, E. N. (1994) Industrial applications of lipases. In Lipases – Their Structure, Biochemistry and Application (Woolley, P. and Petersen, S. B., eds.), pp. 271–288, Cambridge University Press, Cambridge, U.K.
6 Gandhi, N. N. (1997) Applications of lipase. J. Am. Oil Chem. Soc. **74**, 621–634
7 Benjamin, S. and Pandey, A. (1998) *Candida rugosa* lipases: Molecular biology and versatility in biotechnology. Yeast **14**, 1069–1087
8 Barton, M. J., Hamman, J. P., Fichter, K. C. and Calton, G. J. (1990) Enzymatic resolution of (R,S)-2-(4-hydroxyphenoxy) propionic acid. Enzyme Microb. Technol. **12**, 577–583
9 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
10 Rúa, M. L., Diaz Maurino, T., Fernandez, V. M., Otero, C. and Ballesteros, A. (1993) Purification and characterization of two distinct lipases from *Candida cylindracea*. Biochim. Biophys. Acta **1156**, 181–189
11 Kaiser, R., Erman, M., Duax, W. L., Ghosh, D. and Joernvall, H. (1994) Monomeric and dimeric forms of cholesterol esterase from *Candida cylindracea*: Primary structure, identity in peptide patterns, and additional microheterogeneity. FEBS Lett. **337**, 123–127
12 Diczfalusy, M. A., Hellman, U. and Alexson, S. E. H. (1997) Isolation of carboxylester lipase (CEL) isoenzymes from *Candida rugosa* and identification of the corresponding genes. Arch. Biochem. Biophys. **348**, 1–8
13 Ferrer, P., Montesinos, J. L., Valero, F. and Sola, C. (2001) Production of native and recombinant lipases by *Candida rugosa* - A review. Appl. Biochem. Biotechnol. **95**, 221–255
14 Linko, Y. Y. and Wu, X. Y. (1996) Biocatalytic production of useful esters by two forms of lipase from *Candida rugosa*. J. Chem. Technol. Biotechnol. **65**, 163–170
15 Hernaiz, M. J., Sanchez, M. J. M. and Sinisterra, J. V. (1997) New differences between isoenzymes A and B from *Candida rugosa* lipase. Biotechnol. Lett. **19**, 303–306
16 Lopez, C., Guerra, N. P. and Rúa, M. L. (2000) Purification and characterization of two isoforms from *Candida rugosa* lipase B. Biotechnol. Lett. **22**, 1291–1294
17 Chang, R. C., Chou, S. J. and Shaw, J. F. (1994) Multiple forms and functions of *Candida rugosa* lipase. Biotechnol. Appl. Biochem. **19**, 93–97
18 Lee, G. C., Tang, S. J., Sun, K. H. and Shaw, J. F. (1999) Analysis of the gene family encoding lipases in *Candida rugosa* by competitive reverse transcription-PCR. Appl. Environ. Microbiol. **65**, 3888–3895
19 Kawaguchi, Y., Honda, H., Taniguchi Morimura, J. and Iwasaki, S. (1989) The codon CUG is read as serine in an asporogenic yeast *Candida cylindracea*. Nature (London) **341**, 164–166
20 Longhi, S., Fusetti, F., Grandori, R., Lotti, M., Vanoni, M. and Alberghina, L. (1992) Cloning and nucleotide sequences of two lipase genes from *Candida cylindracea*. Biochim. Biophys. Acta **1131**, 227–232

21 Lotti, M., Grandori, R., Fusetti, F., Longhi, S., Brocca, S., Tramontano, A. and Alberghina, L. (1993) Cloning and analysis of *Candida cylindracea* lipase sequences. Gene **124**, 45–55

22 Brocca, S., Grandori, R., Breviario, D. and Lotti, M. (1995) Localization of lipase genes on *Candida rugosa* chromosomes. Curr. Genet. **28**, 454–457

23 Lotti, M., Tramontano, A., Longhi, S., Fusetti, F., Brocca, S., Pizzi, E. and Alberghina, L. (1994) Variability within the *Candida rugosa* lipases family. Protein Eng. **7**, 531–535

24 Hernaiz, M. J., Rúa, M., Celda, B., Medina, P., Sinisterra, J. V. and Sanchez, M. J. M. (1994) Contribution to the study of the alteration of lipase activity of *Candida rugosa* by ions and buffers. Appl. Biochem. Biotechnol. **44**, 213–229

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

26 Ghosh, D., Wawrzak, Z., Pletnev, V. Z., Li, N., Kaiser, R., Pangborn, W., Jornvall, H., Erman, M. and Duax, W. L. (1995) Structure of uncomplexed and linoleate-bound *Candida cylindracea* cholesterol esterase. Structure **3**, 279–288

27 Cygler, M., Grochulski, P., Kazlauskas, R. J., Schrag, J. D., Bouthillier, F., Rubin, B., Serreqi, A. N. and Gupta, A. K. (1994) A structural basis for the chiral preferences of lipases. J. Am. Chem. Soc. **116**, 3180–3186

28 Grochulski, P., Bouthillier, F., Kazlauskas, R. J., Serreqi, A. N., Schrag, J. D., Ziomek, E. and Cygler, M. (1994) Analogs of reaction intermediates identify a unique substrate binding site in *Candida rugosa* lipase. Biochemistry **33**, 3494–3500

29 Tang, S. J., Sun, K. H., Sun, G. H., Chang, T. Y. and Lee, G. C. (2000) Recombinant expression of the *Candida rugosa* lip4 lipase in *Escherichia coli*. Protein Expression Purif. **20**, 308–313

30 Tang, S. J., Shaw, J. F., Sun, K. H., Sun, G. H., Chang, T. Y., Lin, C. K., Lo, Y. C. and Lee, G. C. (2001) Recombinant expression and characterization of the *Candida rugosa* lip4 lipase in *Pichia pastoris*: Comparison of glycosylation, activity, and stability. Arch. Biochem. Biophys. **387**, 93–98

31 Pernas, M. A., Lopez, C., Pastrana, L. and Rúa, M. L. (2000) Purification and characterization of Lip2 and Lip3 isoenzymes from a *Candida rugosa* pilot-plant scale fed-batch fermentation. J. Biotechnol. **84**, 163–174

32 Ge, L. and Rudolph, P. (1997) Simultaneous introduction of multiple mutations using overlap extension PCR. Biotechniques **22**, 28–30

33 Redondo, O., Herrero, A., Bello, J. F., Roig, M. G., Calvo, M. V., Plou, F. J. and Burguillo, F. J. (1995) Comparative kinetic study of lipases A and B from *Candida rugosa* in the hydrolysis of lipid p-nitrophenyl esters in mixed micelles with Triton X-100. Biochim. Biophys. Acta **1243**, 15–24

34 Siedel, J., Rollinger, W., Roschlau, P. and Ziegenhorn, J. (1985) Total cholesterol, end point and kinetic method. In Methods of Enzymatic Analysis (3rd edn), pp. 139–148, VCH, Weinhein

35 Tiss, A., Carriere, F. and Verger, R. (2001) Effects of gum arabic on lipase interfacial binding and activity. Anal. Biochem. **294**, 36–43

36 Bairoch, A. and Apweiler, R. (2000) The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. **28**, 45–48

37 Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank. Nucleic Acids Res. **28**, 235–242

38 Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V. and Cregg, J. M. (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene **186**, 37–44

39 Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis **18**, 2714–2723

40 Brocca, S., Schmidt, D. C., Lotti, M., Alberghina, L. and Schmid, R. D. (1998) Design, total synthesis, and functional overexpression of the *Candida rugosa lip1* gene coding for a major industrial lipase. Protein Sci. **7**, 1415–1422

41 Cereghino, J. L. and Cregg, J. M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. **24**, 45–66

42 Roschlau, P., Bernt, E. and Gruber, W. (1974) Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. Z. Klin. Chem. Klin. Biochem. **12**, 403–407

43 Svendsen, A. (2000) Lipase protein engineering. Biochim. Biophys. Acta **1543**, 223–238

44 Sanchez, R. and Sali, A. (1997) Advances in comparative protein-structure modelling. Curr. Opin. Struct. Biol. **7**, 206–214

45 Lesk, A. M. and Chothia, C. (1986) The response of protein structure to amino-acid sequence changes. Philos. Trans. R. Soc. London, Ser. B **317**, 345–356

Received 13 March 2002/15 May 2002; accepted 21 May 2002
Published as BJ Immediate Publication 21 May 2002, DOI 10.1042/BJ20020404