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Design, total synthesis, and functional overexpression of the *Candida rugosa lip1* gene coding for a major industrial lipase

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Abstract

The dimorphic yeast *Candida rugosa* has an unusual codon usage that hampers the functional expression of genes derived from this yeast in a conventional heterologous host. Commercial samples of *C. rugosa* lipase (CRL) are widely used in industry, but contain several different isoforms encoded by the *lip* gene family, among which the isoform encoded by the gene *lip1* is the most prominent. In a first laborious attempt, the *lip1* gene was systematically modified by site-directed mutagenesis to gain functional expression in *Saccharomyces cerevisiae*. As alternative approach, the gene (1647 bp) was completely synthesized with an optimized nucleotide sequence in terms of heterologous expression in yeast and simplified genetic manipulation. The synthetic gene was functionally expressed in both hosts *S. cerevisiae* and *Pichia pastoris*, and the effect of heterologous leader sequences on expression and secretion was investigated. In particular, using *P. pastoris* cells, the synthetic gene was functionally overexpressed, allowing for the first time to produce recombinant Lip1 of high purity at a level of 150 U/mL culture medium. The physicochemical and catalytic properties of the recombinant lipase were compared with those of a commercial, nonrecombinant *C. rugosa* lipase preparation containing lipase isoforms.

Keywords: *Candida rugosa*; leader sequence; *Pichia pastoris*; overexpression; recombinant lipase; *Saccharomyces cerevesiae*, substrate specificity; synthetic gene

Lipases (triacylglycerol lipase EC 3.1.1.3) catalyze the hydrolysis of triacylglycerides at water/oil interfaces. In vitro, however, they are versatile enzymes because of their ability to catalyze the hydrolysis and synthesis of a great variety of esters, or the resolution of racemic mixtures (Boland et al., 1991).

Candida rugosa lipases (CRLs) are among the commercial lipases most often employed in hydrolysis and synthesis of a wide range of esters of commercial interest (Kötting & Eibl, 1994; Vulfson, 1994). In most biocatalytic applications crude enzyme preparations, obtained by TCA precipitation of the culture supernatant, are applied (Weber et al., 1995).

A high-sequence homology (between 60 and 70%) was revealed among several lipase genes that have been cloned from *C. rugosa* (Longhi et al., 1992; Lotti et al., 1993a); differences in glycosylation of these lipases are thought to contribute to this heterogeneity (Rúa et al., 1993). In line with this finding, several lipase isoforms have been isolated from a commercial enzyme preparation, and it has been shown that the gene product of *lip1* is the major constituent.

Because the isoforms might differ in their catalytic performances (Rúa et al., 1993), and properties of the isoforms may even be affected by the purification procedures used (Hernaiz et al., 1994), cloning and expression of the *lip1* gene was thought to be the most suitable approach for the production and characterization of pure *C. rugosa* lipase with optimized properties for biocatalytic applications.

Unfortunately, the dimorphic yeast *C. rugosa* obeys a noncanonical codon usage in which the triplet CUG, a universal codon for leucine, is read as serine (Kawaguchi et al., 1989). In most phylogenetically related *Candida species*, CUG is extremely rare, with the notable exception of *C. rugosa*, where it accounts for about 40% of the total serine codons (Longhi et al., 1992; Lotti et al., 1993b).

In the *lip1* gene, 20 out of its 47 serine residues, including the catalytic Ser_{209}, are encoded by CUG triplets. As a consequence, the heterologous expression of Lip1 in *Saccharomyces cerevisiae* resulted in an inactive lipase (Fusetti et al., 1996). Hence, it was

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Abbreviations: CRL, *C. rugosa* lipase; N.D., not determined; MW, molecular weight; pH_{opt}, pH optimum of activity; pH_{stab}, pH stability; pNPP, *p*-nitrophenyl palmitate; T_{opt}, temperature optimum of activity; T_{stab}, temperature stability after 30 min of incubation.

concluded that the exchange of several or even most CUG codons by universal serine triplets (UCN, AGY) is required for the expression of a functional Lip1 protein in heterologous hosts.

In this paper we report on two approaches toward the expression of functional Lip1 protein: (1) the mutagenesis of a natural *lip1* gene and its expression in *S. cerevisiae*, and (2) the chemical-enzymatic synthesis of the same gene. Three recombinant synthetic genes (*slip1*) were constructed differing in the leader sequence put in frame with the gene. The recombinant genes were expressed in *S. cerevisiae* and *Pichia pastoris*. This latter is a methylotrophic yeast that has become increasingly attractive as a host for industrial-scale production of heterologous proteins (Tschopp et al., 1987). As in *S. cerevisiae*, the secretion by *Pichia* cells requires the presence of a signal sequence fused to the expressed protein. Several different secretion signals, including the native secretion signals of some heterologous proteins (Paifer et al., 1994), were found to be effective. Because, on the other hand, the secretion signal sequence from the *S. cerevisiae* α-factor prepro-peptide has been used with most success (Lotti et al., 1993a), we compared the effectiveness of the natural *lip1* leader sequence with the α-factor prepro-sequence and with the α-factor pre-signal sequence alone. In addition, physical-chemical and catalytic properties of the recombinant lipase produced by *P. pastoris* were compared with commercial CRLs, revealing that they are nearly identical.

In summary, the use of a synthetic gene and a suitable heterologous host allows for the first time to produce this important enzyme in high yields and purity, and to alter it by mutagenesis for further industrial applications as well as for studies on structure–function relationships.

Results and discussion

Site-directed mutagenesis of C. rugosa lipase gene

Five members belonging to the multigene family coding for lipases in *C. rugosa* have been cloned and sequenced (Lotti et al., 1993a). The present study has been carried out using the *lip1* sequence, because it codes for the major lipase isoform, which is the best characterized and whose crystallographic structure has been solved (Grochulski et al., 1993). The genomic sequence of *lip1* contains a unique ORF of 1,647 bp, corresponding to a protein of 549 residues with an N-terminal stretch of 15 hydrophobic residues, encoding a signal peptide. The mature Lip1 protein contains 46 Ser residues, 19 of them encoded by CTG triplets (Longhi et al., 1992).

Our first attempt to overcome the problems occurring in the heterologous expression of the natural *lip1* gene was the replacement, by site-directed mutagenesis of the codons CTG by other universal codons for serine. Because it may be a tremendous task to change all 20 CTG codons, we tried to define a hierarchy of the functionally and structurally most important Ser residues to be mutated. Based on the alignment with other serine hydrolases and on the 3D structure of the enzyme, we selected a group of eight Ser as the target for the mutagenesis. We produced four mutant genes containing an increasing number of restored Ser residues (2, 3, 5, 8) (Table 1), which either were highly conserved within the lipase/esterase family (Cygler et al., 1993) or considered of importance to maintain protein structure and function.

Because it was previously demonstrated by Fusetti et al. that the natural *lip1* gene is efficiently expressed in *S. cerevisiae* as inactive, intracellular protein only if the endogenous leader sequence is replaced by the signal peptide of the *Kluyveromyces lactis* killer toxin (Fusetti et al., 1996), we used the same system for the expression of the four Ser mutants. The mutant genes were cloned into pEMBLyex4, a shuttle expression vector for *S. cerevisiae* cells containing the inducible *UAS GAL* sequence. Recombinant yeast cells grown under inducing conditions, accumulated intracellular inactive gene product of *lip1* at a level of about 10–20 mg/L culture. The same result was obtained for all mutants, independently of the number of Ser residues restored.

Although analysis of recombinant proteins glycosylation provided evidence for N-glycosylation, as in the case of the wild-type Lip1, implying a correct targeting of the proteins to the ER, their secretion failed, suggesting difficulties at the posttranscriptional level. It is feasible that the mutations still present in the protein might affect the correct folding and, consequently, its correct processing through the secretory pathway.

Construction of a synthetic, codon-optimized C. rugosa lipase gene

As a second approach for the functional expression of *lip1* in conventional yeasts, a synthetic gene (*slip1*) was completely synthesized by the method of mutually priming long overlapping oligonucleotides (Ausubel et al., 1994).

The nucleotide sequence of the *lip1* gene was subdivided into four separately synthesized segments of ca. 400 bp (Fig. 1). Three kinds of changes were introduced into the synthetic version: (1) All CTGs codons were exchanged with universal serine codons; (2) to facilitate the expression in conventional yeasts like *S. cerevisiae* and *P. pastoris*, several codons poorly represented in *S. cerevisiae* coding sequences were exchanged to highly used codons (Sharp et al., 1988), thereby decreasing the general G + C content from 63 to 46%; and (3) unique restriction sites were strategically positioned throughout the gene to facilitate (a) the analysis of primary subclones containing the four blocks and the construction of the gene by their assembly, and (b) future work on site-directed mu-

Table 1. *Characteristics of mutant C. rugosa lipase genes produced by site-directed mutagenesis*

Mutant	Number of mutated CUG Ser residues	Position of mutated CUG Ser residues
1	2	Ser^{209}, Ser^{241}
2	3	Ser^{209}, Ser^{241}, Ser^{282}
3	5	Ser^{209}, Ser^{241}, Ser^{282}, Ser^{348}, Ser^{349}
4	8	Ser^{209},Ser^{241},Ser^{282},Ser^{348},Ser^{349},Ser^{365},Ser^{436},Ser^{440}

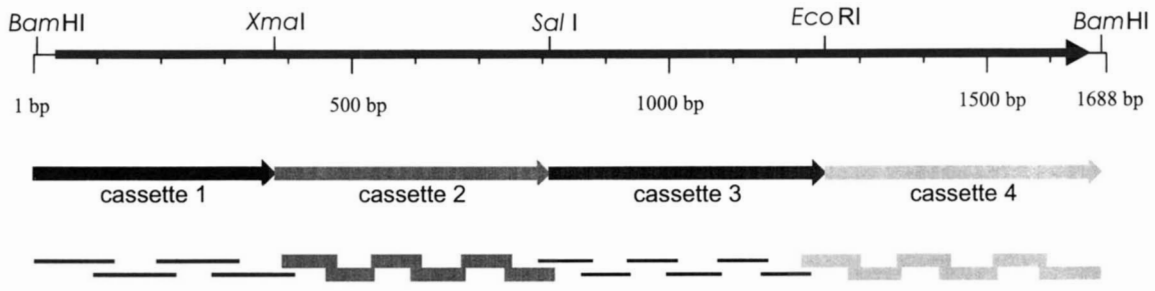

Fig. 1. Design of the synthetic gene. Positions and lengths of oligonucleotides covering the 1,688 bp of the synthetic fragment, including the *LIP1* gene (1,647 bp) indicated as an arrow. Each cassette was synthesized using two or three couples of long overlapping oligonucleotides, represented in the third row as solid bars. Each pair of oligonucleotides included a 20 bp duplex segment to promote the annealing stability during the mutually primed synthesis. Additional residues were added beyond sites used for cloning at 5′- and 3′-terminus of the oligonucleotides, to allow efficient enzyme cleavage. In the case of cassette I and cassette IV, the synthesis of starting (5′-terminus in cassette I) and terminal (3′-terminus in cassette IV) extremities were extended beyond their normal end point to generate ends containing convenient cloning sites also present in pUC19 vector polylinker. Separately synthesized gene cassettes were cloned into the pUC19 vector and ligated to assemble the entire gene.

tagenesis. With the same purpose, some restriction sites were removed without changing the coding region. The final codon-optimized version of the *slip1* gene has 77% of nucleotide sequence identity with the natural gene.

Expression of recombinant C. rugosa lipase in P. pastoris and S. cerevisiae

The synthetic gene, containing its natural leader sequence (*nl-slip1*), was modified via PCR and two other hybrid versions of the recombinant *slip1* gene were obtained by fusion of the nucleotide sequence coding for the mature Lip1 protein with the *S. cerevisiae* α-factor prepro-signal peptide (*pp-slip1*) or with its 19 aa α-factor pre-signal sequence (*p-slip1*). The three recombinant lipase genes were inserted into the vectors pPICZαB and pYES2, generating two series of plasmids (pPIC-nl-slip1, pPIC-pp-slip1, pPIC-p-slip1, and pY-nl-slip1, pY-pp-slip1, pY-p-slip1), for expression in *P. pastoris* and *S. cerevisiae*, respectively.

Expression of Lip1 synthetic genes in P. pastoris

Pichia pastoris is a yeast capable to metabolize methanol as its sole carbon source. The enzyme alcohol oxidase (AOX) is involved in the metabolism of methanol and its transcription tightly regulated and strongly induced by methanol (more than 30% of the total soluble protein in cells grown with methanol as a carbon source). The three *slip1* genes were each inserted into pPICZαB, a shuttle expression vector, under the control of the strong *AOX1* promoter (Brake et al., 1984).

Lipase secretion of *P. pastoris* transformants was evidenced on minimal tributyrin-methanol plates by the formation of a clear halo surrounding positive clones. All transformants obtained with pPIC-pp-slip1 and pPIC-p-slip1 displayed, to different extent, lipasic activity after overnight incubation on minimal tributyrin plates. However, a longer incubation time of 48–72 h was necessary for clones harboring pPIC-nl-slip1 where the synthetic gene is preceded by its natural leader sequence, indicating a significantly reduced lipase secretion or production level. A suitable negative control (*Pichia* cells transformed with pPICZαB) never formed halos, even after several days of incubation under inducing conditions.

Recombinant *P. pastoris* transformants (10 clones of each vector) producing lipase were selected on minimal tributyrin-methanol plates by their formation of a clear halo. They were cultivated using methanol as an inducer, in small volume cultures (20 mL) for 48 h. Different clones varied largely as to their lipolytic activity toward *p*-nitrophenyl palmitate in the culture supernatant (1 to 20 U/mL). This may be due to a gene dosage effect caused by a multiple, genomic insertion of the foreign gene (by a mechanism of in vivo circularization of transforming DNA) also reported by other authors (Clare et al., 1991a, 1991b).

Highly productive clones were grown in 2-L Erlenmeyer flasks containing 200 mL BMMY medium. After five days induction, the lipase activity of the supernatants reached 85 U/mL for the pPIC-pp-slip1 and pPIC-p-slip1 transformants and 3 U/mL for the pPIC-nl-slip21 transformant. Culture supernatants were directly subjected to SDS-PAGE. A single band at 60 kDa corresponding to the expected molecular weight for Lip1 was detected, demonstrating that no other proteins were secreted by recombinant *P. pastoris* (Fig. 2). The same band was visualized after Western blotting analysis using lipase-specific antibodies for immunodetection.

The identity of Lip1 expressed by *Pichia* cells harboring pPIC-pp-slip1 plasmid was further confirmed by amino terminal sequencing of the secreted protein, indicating the presence of two species. The major product of prepro-α-*lip1* was obtained by a cleavage between Arg and Glu in the sequence:

$$\text{Glu Lys Arg} * \text{Glu Ala Glu Ala} \,..\, \text{Ala Pro} \qquad (1)$$

where $*$ is the site of the cleavage and .. is the joining point between the signal sequence and the mature protein. The remainder sequence begins with an Ala residue at position +1.

The processing of the α-factor mating signal sequence in pPICZα is reported to occur usually in two steps (Brake et al., 1984): first, the preliminary cleavage by the *kex2* product between Arg and Glu, and second, the cleavage by the *ste13* gene product of Glu-Ala repeats. The efficiency of this process is related to the amino acid sequence of the expressed protein. In our case, it can be assumed that, in analogy with Kex2 behavior, also the cleavage efficiency of Ste13 is affected by the presence of a Pro residue at position +2 in the expressed protein.

Presence of intracellular accumulated recombinant lipase was analyzed by Western blotting using lipase-specific antibodies for

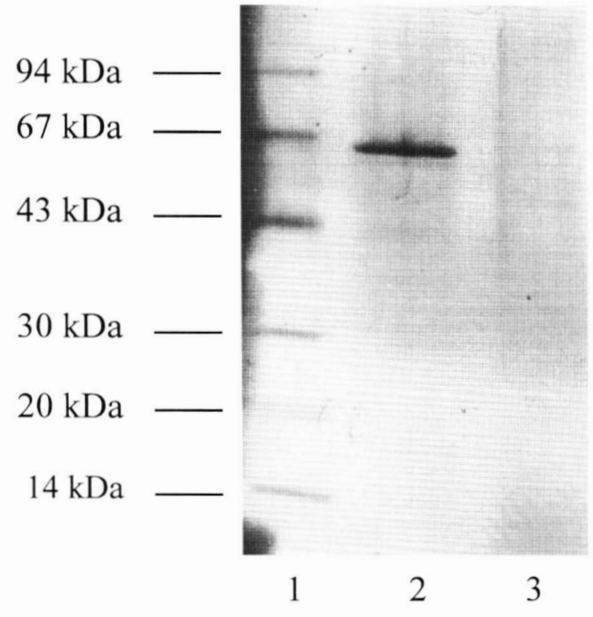

Fig. 2. SDS-PAGE analysis of recombinant *C. rugosa* lipase. Lane 1: molecular weight standard in kDa; lane 2: lipase from 0.5-mL culture supernatant withdrawn after five days induction; lane 3: 0.5 mL of supernatant withdrawn after five days without induction. Proteins are stained with silver.

immunodetection. The synthetic lipase was in small extent intracellular accumulated in recombinant *P. pastoris* cells when fused to either the α-factor pre- or prepro-signal sequence. However, no intra-cellular accumulation was observed with the low-level expressed synthetic lipase preceded by its natural signal sequence. This suggests that the natural leader sequence functions as secretory signal sequence in *P. pastoris*, but hampers expression of recombinant lipase in this yeast due to either transcriptional and/or translational hindrance or protein instability within the host cells. On the other hand, the α-factor pre-signal sequence is efficient for both secretion and expression in *P. pastoris* as the α-factor prepro-signal sequence, also observed for secretion and expression of heterologous proteins in *S. cerevisiae* (Payne et al., 1995).

The clone with the highest level of lipase secretion, selected among the *P. pastoris* clones harboring the plasmid pPIC-pp-slip1, was subjected to fermentation in a 1-L bioreactor, using rich standard medium (BMMY) at pH 6.0. Lipolytic activity in the supernatant was 125 U/mL after 208 h (Fig. 3A). To further improve the expression level in the of Lip1, we carried out a high cell density fermentation of the same clone. Lipase activity increased to 150 U/mL after 280 h fermentation in 1-L culture (Fig. 3B), but the productivity remained in the same range of 500–600 U/L*h. Thus, we are able to achieve about twofold productivity compared to the production from *C. rugosa* cells of native lipase, which has been reported at a level of 250 U/L*h in the presence of 8 g/L oleic acid as a carbon source and inducer after 60 h of fermentation (Del Rio et al., 1990).

Expression of Lip1 synthetic genes in S. cerevisiae

All *S. cerevisiae* transformants obtained with pY-nl-slip1, pY-pp-lip1, and pY-p-slip1 displayed, in different extent, lipasic activity on minimal tributyrin-galactose plates after 24 h at 30°C, but not the transformants obtained with pYES2.

For further investigations the best clones for the lipase production were chosen and cultivated in a 2-L Erlenmeyer flask containing 200 mL minimal-glucose medium until they reached an OD_{600} of 2–3. After five days of induction in minimal-galactose medium, the activity of the supernatants was 5–7 U/mL for the pY-pp-slip1 and pY-p-slip1 transformants as well for the pY-nl-slip1 transformants. Thus, the lipase expression levels achieved in *S. cerevisiae* are 12- to 17-fold lower than those obtained in *P. pastoris* in comparable small-scale cultivations. These findings are in agreement with general 10- to 100-fold higher heterologous protein expression levels observed in *P. pastoris* in comparison to *S. cerevisiae* (Buckholz & Gleeson, 1991).

Even the synthetic codon-optimized *lip1* gene preceded by its natural leader sequence (*nl-slip1*) was expressed and secreted in

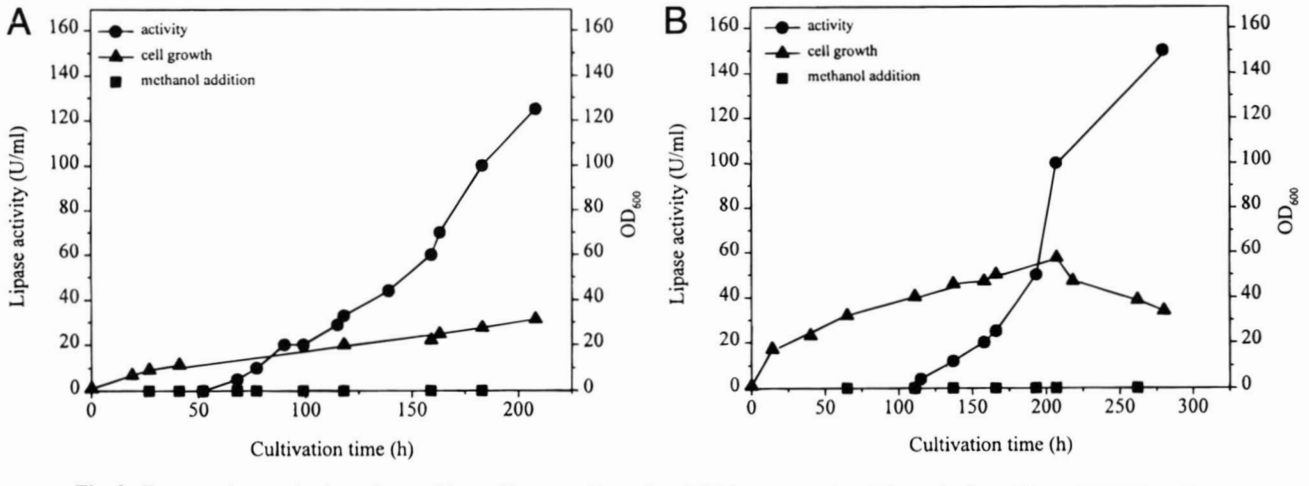

Fig. 3. Fermentative production of recombinant *C. rugosa* lipase in a 1-L bioreactor using (**A**) standard conditions: BMMY medium, 30 °C and pH 6.0, and (**B**) high cell density conditions as reported by Payne et al. at 30 °C and pH 6.0. The lipase activity was measured with a pH-stat, using tributyrin as substrate, at 30 °C and pH 7.2.

S. cerevisiae, thus demonstrating the function of this 15 aa stretch as secretory signal also in *S. cerevisiae* cells.

In contrast, expression of the natural and mutated *lip1* gene in *S. cerevisiae* failed, and neither secretion nor intracellular accumulation of lipase could be detected before substitution of the natural leader sequence with the *K. lactis* killer toxin (Fusetti et al., 1996). In this perspective, the nucleotide modifications of the natural *lip1* gene leader sequence seems to be of critical importance for the expression in *S. cerevisiae*. Indeed, the effectiveness of the natural leader sequence in driving the secretion from *S. cerevisiae*, in our experiments could be due to the genetic code optimization of *slip1* gene, as well of its signal sequence.

Characterization of the recombinant lipase

SDS-PAGE analysis of Lip1 produced by *P. pastoris* harboring the plasmid pPIC-p-slip1, directly taken from the culture supernatant, showed a single protein band of 60 kDa molecular weight (Fig. 2), corresponding to the molecular size reported for the native Lip1 and its isoforms (Longhi et al., 1992; Rúa et al., 1993).

The deduced amino acid sequence of Lip1 contains three potential *N*-glycosylation sites at position 291, 314, and 351. After deglycosylation, the molecular weight decreased by 3 kDa, suggesting a carbohydrate content of 5% for the recombinant protein. A carbohydrate content between 3.6–8% has been reported for various isoforms of nonrecombinant *C. rugosa* lipases (CRL) (Rúa et al., 1993). Lip1 was deglycosylated before and after denaturation; identical molecular weights were observed, but deglycosylation in the native state required almost the double incubation time.

As shown in Table 2, commercial *C. rugosa* lipase preparation (CRL) and Lip1 were most active at 30–40 °C. The optimum pH was at pH 7.0 and decreased to 60% at pH 8.0. Isoelectric focusing of Lip1 revealed two bands with slightly different pI of 3.9 and 4.0, possibly due to different glycosylation forms. Rúa et al. (1993) found pI values between 4.8 and 5.04 for the four components of lipase B, whose N-terminal sequence coincides with Lip1. Thus, recombinant Lip1 expressed in *P. pastoris* shows an isoelectric point slightly lower than expected.

To compare the substrate specificity of the recombinant Lip1 to a commercial preparation of *C. rugosa* lipase, activity assays with various triacylglycerides differing in acyl group chain length were carried out. Both lipases showed higher lipolytic activity toward tricaproin (C8) and tricaprin (C10), rather than for triolein (C18:1), but high hydrolytic activity was also found with long-chain triacylglycerides when a natural oil, such as cocoa butter was used (Fig. 4A). These data are in good agreement with literature data concerning CRL (Soumanou et al., 1997). Additionally, the substrate specificity of CRL and Lip1 where compared using various methyl esters differing in acyl group chain length (C6–C22) in an equimolar substrate mixture. After lipolysis, the produced fatty acids were quantified by GC analysis (Fig. 4B). Again, both lipases showed maximum activity toward middle-chain methyl esters (C8 and C10), rather than toward long-chain methyl esters. No activity was found on respect to C18–C22.

Because the physicochemical and the catalytic properties of Lip1 and CRL coincide, we conclude that our procedure to express the synthetic gene *lip1* in *P. pastoris*, in fact, leads to a functionally active form of Lip1. Moreover, we produced a further indirect proof to the hypothesis that *lip1* encodes the major isoform present in the commercial CRL preparation.

Table 2. *Comparison of properties of commercial and recombinant lipase from C. rugosa*

Properties	Commercial CRL	Recombinant CRL
MW (Da)	60,000	60,000
pI	N.D.	3.9–4.0
T_{opt} (°C)	30–40	30–40
pH_{opt}	6.5–7.5	6.5–7.5
T_{stab} (°C)	50	50
pH_{stab}	8.0–8.5	8.0–8.5
Specific activity (U/mg)		
pNPP	15	5.4
Tributyrin	27	10

Experimental protocol

Strains, plasmids, and media

E. coli DH5a was used as host for plasmid amplification and *P. pastoris* GS115 (Invitrogen), *S. cerevisiae* INVSC2 (Invitrogen), and *S. cerevisiae* X4004.3A (MATa, lys5, met2, ura3, trp1) for the expression of recombinant lipase. Plasmids used for cloning were pUC19, PCYTEXP1 (Belev et al., 1991), pEMBLyex4 (Fusetti et al., 1996), pYES (Invitrogen), and pPICZαB (Invitrogen) for expression. *E. coli* was grown at 37°C in Luria-Bertani medium (LB) containing 100 μg/mL ampicillin or 25 μg/mL zeocin for selection of clones transformed with pUC19, pCYTEXP1, or pPICZαB, respectively. *P. pastoris* was grown in shaking flasks at 30 °C in a rich standard medium containing 1% glycerol (BMGY) before induction, or 0.5% methanol (BMMY) for the induction as suggested by Invitrogen. Batch growth of *S. cerevisiae* was at 30 °C in 0.67% (w/v) Yeast Nitrogen Base (Difco, UK) minimal medium supplemented with the appropriate amino acid at 50 mg/L and either 2% glucose or 2% galactose as the carbon source.

For maintaining yeast cultures and plates YEPD medium was used (1% Yeast Extract, 2% Peptone, 2% Dextrose), and for selection of *P. pastoris* transformants YEPS (YEP + 2% Sorbitol) plates containing zeocin (100 mg/mL) were used.

The procedure for culture of the transformed *S. cerevisiae* cells, protein extraction, and detection have been described elsewhere (Fusetti et al., 1996).

Gene design, cassette synthesis, and assembly of the final codon-optimized gene

The gene was divided into four fragments of ca. 400 bp each, which were separately cloned. The four resulting subconstructs were as follows: cassette I (4 oligos, average length = 112 mer), cassette II to cassette IV (6 oligos, average length = 92 mer) (Fig. 1). The four cassettes were each synthesized in one step, by using the mutually priming long oligonucleotides in a PCR (Ausubel et al., 1994). The obtained ca. 400 bp fragments were directly blunt-end cloned into pUC19 digested with *SmaI*. Although the mutation rate observed following DNA sequencing was rather low (2/400 nt), it was necessary to remove undesired mutations. A number of restriction sites present in the synthetic gene and in the

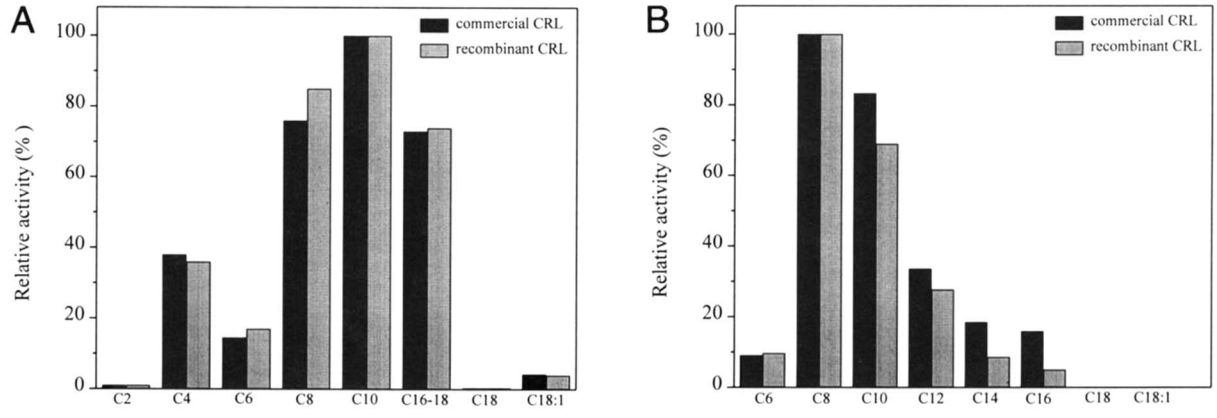

Fig. 4. Substrate specificity of recombinant and commercial *C. rugosa* lipase. Activity toward various triacylglycerides (**A**) and fatty acid methyl esters (**B**) of different chain length of the acyl group (C2–C18). Cocoa butter (C16–18) contains predominantly palmitic acid (C16) in the *sn*-1, oleic acid (C18:1) in the *sn*-2 and stearic acid (C18) or palmitic acid (C16) in *the sn*-3 position of the triacylglycerol. Relative activities were determined at pH 7.2 and 30 °C. Activity toward triacylglycerols was measured by pH-stat assay. Lipolysis of fatty acid methyl esters was carried out in a pH-stat until titration of 6.6 mM free fatty acids. And after derivatization to the respective silyl ether, released fatty acids were determined by GC-analysis.

cloning vector pUC19 were used to combine in one-copy error-free, different sequences coming from distinct clones of the same cassette. The four error-free cassettes, cloned into pUC19, were designated as pUC-I to pUC-IV.

The complete codon-optimized synthetic lipase gene was assembled into pUC19. The *Xma*I/blunt-*Sal*I fragment from pUC-II was ligated into pUC-III previously linearized with *Pst*I/blunt and *Sal*I and giving pUC-(II-III). Subsequently, the *Xma*I-*Eco*RI fragment from pUC-(II-III) was inserted into pUC-I digested with *Xma*I and *Eco*RI, resulting in pUC-(I-III). Finally, the *Eco*RI-*Eco*RI fragment from pUC-IV was cloned into pUC-(I-III) cut with *Eco*RI.

The correctly assembled lipase cloning vector, pUC-(I-IV), was identified and characterized by restriction enzymes analysis, and the entire sequence was reconfirmed by automated sequencing.

Construction of Lip1 expression vectors

The synthetic *lip1* (*slip1*) gene was subcloned into the pCYTEXP1 vector as an intermediate step for the in-frame fusion of the gene with the *S. cerevisiae* α-factor leader sequence. The *Bam*HI fragment from pUC(I-IV) was ligated in a *Bam*HI linearized pCYTEXP1 vector, giving pCY-*slip1*. To fuse the sequence encoding the mature lipase directly with the α-factor prepro-signal sequence present in the *P. pastoris* expression vector pPICZαB, a PCR was performed with one primer complementary to the pPICZαB sequence, including the *Pme*I site and the second complementary to the 3′ end of the α-factor signal sequence, including the 5′ sequence of the mature lipase along with a *Bgl*I site. The obtained PCR product was cloned in *Sph*I/blunt linearized pCYTEXP1, resulting in pCYprepro-α-factor. The *Bgl*I fragment from pCY-slip1, containing all the mature form of the lipase synthetic gene, was inserted into *Bgl*I digested plasmid pCyprepro-α-factor, thereby restoring the ampicillin resistance gene. The obtained plasmid pCYpp-slip1, containing the mature synthetic lipase gene, fused to the prepro-α-factor leader sequence, was *Bam*HI/blunt-*Hind*III digested, and the resulting fragment ligated into pPICZαB linearized with *Xba*I/blunt-*Hind*III, giving pPIC-pp-slip1.

As the expression vector in *S. cerevisiae* cells pYES2, a 2-μm-based vector containing the strong galactose inducible GAL1–GAL10 promoter, was used.

pPIC-nl-slip1 was *Bam*HI digested and ligated into pYES2 linearized with the same enzyme and dephosphorylated, giving pY-nl-slip1. pY-nl-slip1 was used in turn for the cloning into pYES2 vector of the synthetic lipase gene preceded by α-factor prepro- and pre-signal sequences. The *Hind*III-*Bste*II fragments containing the recombinant genes were isolated from pPIC-pp-slip1 and pPIC-p-slip1 and inserted in pYES2 digested with the same enzymes. The plasmids thus obtained were designed as pY-pp-slip1 and pY-p-slip1, respectively.

Expression of Lip1 in P. pastoris

Pichia pastoris GS115 cells were transformed with pPIC-pp-slip1 by electroporation and transformations were plated onto solid selective medium (YEPD containing zeocin). Positive transformants were checked for lipase activity by transferring colonies onto minimal tributyrin-methanol plates. The tributyrin-methanol plates were incubated at 30 °C for 48 h with 0.1 mL of methanol added to the lid of each plate every 24 h. Lipase-producing clones formed a clear halo compared to the opaque tributyrin emulsion. All transformants obtained with pPIC-pp-slip1 displayed, to different extent, lipasic activity after overnight incubation. A suitable negative control (*Pichia* cells transformed with pPICZαB) never formed halos, even after several days of incubation in inducing conditions.

Expression of Lip1 in S. cerevisiae

S. cerevisiae Invsc2 cells were transformed with pY-nl-slip1, pY-pp-slip1, and pYpre*LIP*, by electroporation. Transformations were plated onto solid minimal medium containing tributyrin and galactose. Transformants, selected on the basis of their ability to grow in the absence of leucine, were directly screened for lipase production and secretion by formation of transparent halos on tributyrin containing minimal plates.

Fermentations

The fermentation was carried out in a 1-L bioreactor (Braun) at 30 °C, in a rich standard medium (BMMY) at pH 6.0. For the high cell density fermentation, conditions reported by Payne et al. (1995) at pH 7.5 were used. The bioreactors were inoculated with 50-mL of a flask culture grown overnight to an OD_{600} of 2–3 in BMGY medium and maintained at constant pH by adding 2 M HCl and 2 M NaOH. The stirring rate was 350 rpm, and the aeration rate was 1 L/min. The lipolytic activity of the supernatants and the cell wet weight were monitored throughout the fermentation. Five milliliters of methanol were added daily to the bioreactor. In the case of the high cell density fermentation, methanol was added after the cell culture reached 75 mg/mL of cell wet weight.

Physicochemical and catalytic characterization

All the measurements for the physicochemical, as well for the catalytic characterization, were carried out directly with the culture supernatant, without any kind of further purification. Lipase activity was routinely measured with tributyrin as substrate in a pH-stat assay at 30 °C and pH 7.2, as described by Schmidt-Dannert et al. (1996). Fast determination of lipolytic activity in the culture supernatant of small scale cultures was performed with a spectrophotometric assay using *p*-nitrophenyl palmitate as a substrate at 30 °C, as reported earlier (Schmidt-Dannert et al., 1996). For the determination of the substrate specificity using triacylglycerols as substrates, 20 mM of triacylglycerols, 5% (w/v) of cocoa butter were each emulsified in distilled water, containing gum arabic (20 mg/mL), and used as a substrate solution in the pH-stat assay at 30 °C an pH 7.2. The tristearin emulsion was obtained by adding 5% (v/v) aceton, being verified that, in the same conditions, the lipases retained 47% of their full activity on tributyrin. For the determination of substrate specificity using fatty acid methyl esters as substrates, 110 mM of a fatty acid methyl ester mixture (10 mM of each ester) in 5 mM Tris-buffer pH 7.5 was prepared. Lipolysis was carried out in a pH-stat at 30 °C and pH 7.2 until titration of 6.6 mM of free fatty acids. Subsequently, a 2 mL reaction mixture was withdrawn and lipolytic reaction stopped by the addition of 0.1 mL orthophosphoric acid. Next, free fatty acids were three times extracted with diethylether:*n*-hexane (1:1) and 50 μL of extracted fatty acids dried under nitrogen. By addition of 50 μL MSHFBA (*N*-methyl-*N*-trimethyl-heptafluorobutyramid, Fluka) and incubation for 15 min at room temperature the free fatty acids were derivated to the silyl ethers and determined by GC after further addition of 50 μL ethanol and 200 μL *n*-hexane (gas chromatograph: Fison 800, column: Optima 5 (25 m × 0.25 mm) (Macherey & Nagel), temperature program: 40 °C 2 min, 4 °C/min 250 °C 15 min, injector 350 °C, flame ionization detector 360 °C, 75 kPa).

The effect of pH and temperature on enzyme activity was determined at 30 °C and pH 7.2 and various temperatures, respectively. Enzyme stability was analyzed by determining the residual lipase activity after incubation of aliquots of lipase solution for 20 h at 4 °C in 0.1 M phosphate buffers at different pHs and for 30 min in 25 mM Tris-buffer, pH 7.5 at various temperatures, respectively.

Analytical methods

N-Amino terminal sequence analysis was performed with a gas-phase sequencer 470A (Applied Biosystems). Concentrated lipase containing culture supernatant was subjected to SDS-PAGE. After blotting on a PVDF membrane, the lipase band was cut out and used for N-terminal sequence determination. Immunoblot analysis was performed with polyclonal antibodies specific for the homologous lipase from *Geotrichum candidum*. For signal development, the washed PVDF membrane was incubated with POD-conjugated anti-rabbit immunoglobulin (Boehringer Mannheim) as described by the supplier. Deglycosylation of protein samples was performed for 12 h with endo-β-*N*-acetylglucosaminidase H (25 mU/mg protein) at 37 °C in a 50-mM potassium acetate buffer, pH 5.5, containing 0.5 mM PMSF. For the deglycosylation of denatured samples, the protein was first incubated with 0.01% (m/v) SDS at 95 °C for 3 min, and the reaction was carried out for 24 h.

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