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Mutants provide evidence of the importance of glycosydic chains in the activation of lipase 1 from *Candida rugosa*

STEFANIA BROCCA,[1] MATTIAS PERSSON,[2] ERNST WEHTJE,[2] PATRICK ADLERCREUTZ,[2] LILIA ALBERGHINA,[1] AND MARINA LOTTI[1]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy
[2]Department of Biotechnology, Lund University, Sweden

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Abstract

Sequence analysis of *Candida rugosa* lipase 1 (LIP1) predicts the presence of three N-linked glycosylation sites at asparagine 291, 314, 351. To investigate the relevance of sugar chains in the activation and stabilization of LIP1, we directed site mutagenesis to replace the above mentioned asparagine with glutamine residues. Comparison of the activity of mutants with that of the wild-type (wt) lipase indicates that both 314 and 351 Asn to Gln substitutions influence, although at a different extent, the enzyme activity both in hydrolysis and esterification reactions, but they do not alter the enzyme water activity profiles in organic solvents or temperature stability. Introduction of Gln to replace Asn351 is likely to disrupt a stabilizing interaction between the sugar chain and residues of the inner side of the lid in the enzyme active conformation. The effect of deglycosylation at position 314 is more difficult to explain and might suggest a more general role of the sugar moiety for the structural stability of lipase 1. Conversely, Asn291Gln substitution does not affect the lipolytic or the esterase activity of the mutant that behaves essentially as the wt enzyme. This observation supports the hypothesis that changes in activity of Asn314Gln and Asn351Gln mutants are specifically due to deglycosylation.

Keywords: *Candida rugosa*; glycosylation sites; *Pichia pastoris*; recombinant lipase; site-directed mutagenesis; synthetic gene; thermostability; water activity profiles

Asparagine-linked glycosylation is one of the more common post-translational modifications of eukaryotic proteins (Lehle & Tanner, 1995). N-glycans have been shown to play an important role for the biological function and the physico-chemical properties of many secreted and integral membrane proteins (Varki, 1993; Han & Lei, 1999). Furthermore, in several instances the glycosylation pattern has been proved to affect activity and thermostability of the biocatalyst (Longo & Combes, 1999).

In this work, we investigated the role of sugar chains for the biochemical and catalytic properties of the enzyme lipase 1 (LIP1) from the fungus *Candida rugosa*. The lipase isoenzymes of *C. rugosa* contain 4 to 7% sugars depending on the protein and on the culture conditions (Rua et al., 1992). Accordingly, the sequence of the genes encoding five lipase isoforms (LIP1–LIP5) characterized to date revealed the presence of one to three Asn-X-Ser/Thr consensus sequences in the correspondence of Asn291, Asn314, and Asn351 (Longhi et al., 1992; Lotti et al., 1993), out of which only position 351 is conserved within this enzyme family. LIP1 carries all three consensus sequences. Crystallographic analysis of native LIP1 showed that two ordered sugar residues, consisting of N-acetylglucosamine (NAG) units, are linked at position 351, one NAG is linked at position 314 (Grochulski et al., 1994b), whereas 291 is free.

Studies on structure–function relationships in CRL have been hampered by the use of CUG triplets to code for Ser instead of Leu in its genes (Kawaguchi et al., 1989; Alberghina & Lotti, 1997). Recently, we reported the successful expression in *Pichia pastoris* cells of a synthetic *C. rugosa LIP1* gene where CUG Ser codons are substituted with other triplets universally coding for Ser (Brocca et al., 1998).

The availability of a system for the functional expression of LIP1 makes feasible approaches aiming to clarify function–structure issues, to date inferred only on the basis of sequence and structure data analysis. In this frame, it was of particular interest to investigate the function of the carbohydrates linked to Asn351. This sugar chain is located at the LIP1 surface in the proximity of the structure called the *lid*, an amphipathic loop that masks the cata-

Reprint requests to: Marina Lotti, Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy; e-mail: marina.lotti@unimib.it.

Abbreviations: aw, water activity; CRL, *Candida rugosa* lipase; LIP, lipase protein; PCR, polymerase chain reaction; NAG, N-acetylglucosamine; PAGE, polyacrylamide gel electrophoresis; PDB, Protein Data Bank; pNP, p-nitrophenol; pNPL, p-nitrophenyl laurate; pNPP, p-nitrophenyl propionate; rLIP, recombinant lipase; *slip*, synthetic lipase gene; wt, wild-type.

lytic center until it is displaced during the activation of the enzyme in the presence of its substrate (Grochulski et al., 1993, 1994a, 1994b). A possible contribution of NAG units bound to Asn351 to the stabilization of the enzyme active conformation has been suggested (Grochulski et al., 1994a, 1994b).

Glycosylation has also been investigated in relation to the performance of enzymes in biotransformation processes, in particular in organic solvents. It is well known that the water content, as well as the solvent itself, influences the activity and water requirements of enzymes in organic solvents. In these media, lipases show marked water dependence. Some lipases have a constant increase in enzyme activity as the water concentration/activity (a_w) is increased, while some others (for instance *C. rugosa* lipase) have bell-shaped profiles. One explanation to the bell-shaped profiles is that the K_m value for the alcohol substrate in esterification is increased at higher water activities (Bovara et al., 1993; Wehtje & Adlercreutz, 1997). Another hypothesis, based on the observation that polyols and similar hydrophilic substances influence the activity of enzymes in organic media (Adlercreutz, 1993; Triantafyllou et al., 1995), is that the differences in water activity profiles for lipases could arise from differences in their glycosylation patterns.

In the present work, the enzymatic activity in different reactions, as well as the water activity profiles in an organic solvent, and the temperature stability in aqueous solution were investigated for LIP1 proteins mutated in the glycosylation sites.

Results

Construction and expression of mutant C. rugosa lipases

Functional mutants of recombinant lipase 1 from *C. rugosa* have been obtained based on the knowledge of its three-dimensional (3D) structure and on the availability of a suitable expression system. The wild-type recombinant *lip1* gene cloned in frame with the α-factor prepro-signal peptide from *Saccharomyces cerevisiae* (*pp-slip1*) (Brocca et al., 1998) was used as the template for PCR site-directed mutagenesis to replace Asn residues located at position 219, 314, and 351 by Gln (Fig. 1). The design of mutants was based on the predicted potential glycosylation sites and on the analysis of a refined 3D structure (Grochulski et al., 1993; PDB code 1CRL) of LIP1 in its open conformation, with the aid of the molecular graphics program INSIGHT (Molecular Simulation, San Diego, California). Mutagenesis was performed on a gene fragment defined by the two unique restriction sites for *Eco*RI (1,181) and *Xho*I (474) encompassing all three target positions. Sequencing confirmed that no random substitutions had been introduced. The recombinant lipase genes carrying each introduced mutation were cloned into the pPICαB expression vector under the control of the methanol-inducible alcohol oxidase (*AOX1)* promoter and expressed in *P. pastoris* X33 as described in Materials and methods. Lipase secreting transformants were selected on YPS-tributyrin plates by the formation of a clear halo surrounding positive clones. A suitable negative control (*P. pastoris* cells transformed with pPICZαB) never formed halos, even after several days of incubation.

Four clones for each lipase mutant were chosen to grow for 5 days in 250 mL Erlenmeyer flasks containing 50 mL BMMY medium. After 5 days cultivation, cells were separated by centrifugation and the resulting supernatant concentrated and further analyzed for activity.

The analysis by Western blotting of culture supernatants showed that the migration of mutant N291Q is identical to that of wild-type LIP1, whereas mutation on the other two positions causes a slight decrease in the protein size (Fig. 2). This finding suggested that N291 might be not recognized as a point of N-glycosylation, at least when the recombinant protein is expressed in *P. pastoris*.

Hydrolytic activity of the enzyme species

Wild-type and mutants of rLIP1 were tested in reactions for lipase activity measured as the hydrolysis of olive oil, for esterase activ-

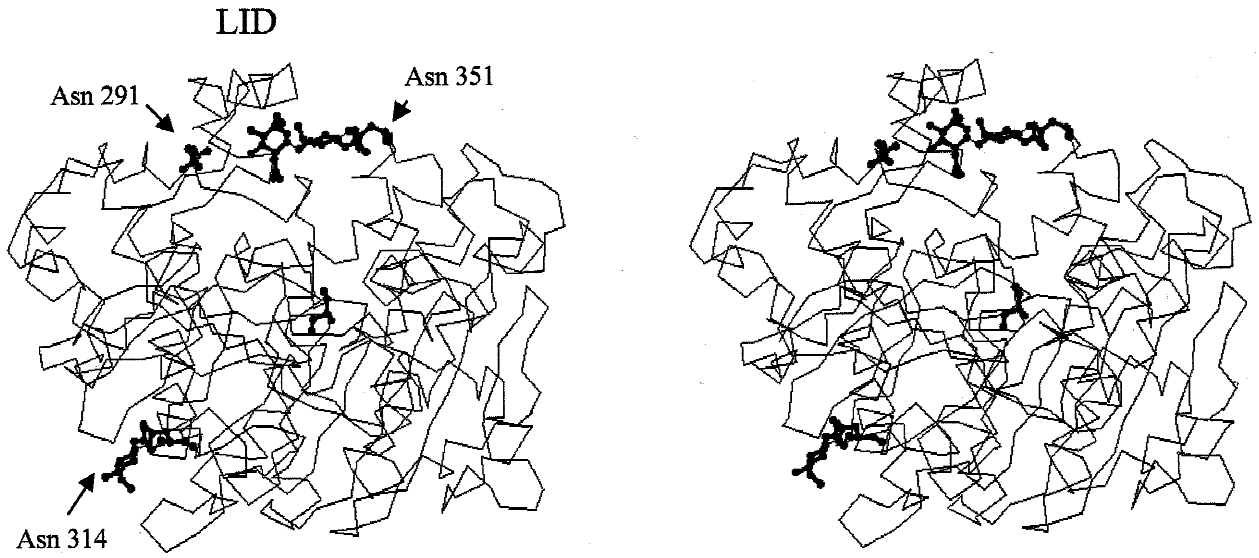

Fig. 1. Stereoview of the lipase 1 from *C. rugosa* (from PDB 1CRL). The glucan chains at Asp314 and Asp351 are shown in ball and stick representation; the active site is marked by the position of the catalytic serine at the molecule center. The position of the lid and points of N-glycosylation are marked.

Table 1A. *The specific activities for the different lipases from C. rugosa in hydrolysis reactions and in esterification*

Enzyme	pH-stat (μmol/min mg protein)	pNPP (μmol/min mg protein)	Esterification (μmol/min mg protein)	pNPL (μmol/min mg protein)
Lip 1	18	4.5	1.6	0.15
N291Q	32	4.7	1.0	0.25
N314Q	<1 n.d[a]	0.16	0.035	0.010
N351Q	<1 n.d[a]	0.03	0.009	0.013

[a]Not detected. The detection limit under the conditions used for the assay was determined to 1 (μmole/min mg protein).

ity, measured as the hydrolysis of p-nitrophenylesters and in esterification reactions in organic solvent.

The specific activities for LIP1 and N291Q were in the same order of magnitude in all the reactions studied (Table 1A). This result is in agreement with the hypothesis suggested by electrophoretic analysis (Fig. 2) that this potential site of glycosylation is not used. Mutant N351Q displayed the lowest activity in all reactions investigated except in the hydrolysis of pNPL where it shows a reaction rate similar to N314Q (Table 1A). These data indicate that the carbohydrate residues attached at Asn351 are important for the catalytic behavior of the enzyme and emphasize the importance of the two N-acetylglucosamines bound to this site in stabilizing the open conformation of the enzyme, as previously proposed by Grochulski et al. (1993).

The specific activity of N314Q mutant in ester hydrolysis and esterification reactions (Table 1A) was lower as compared to wt rLIP1 and N291Q, but higher than N351Q, thus evoking a different kind of effect for the glucan chains at position 314 and 351.

To determine if the various enzyme species displayed any difference in substrate specificity, the ratios of specific activities between different reactions were calculated (Table 1B). The ratio (pH-stat/pNPP) was higher for the N351Q mutant most probably due to the detection limit of our pH-stat equipment, which yields an overestimation of the ratio.

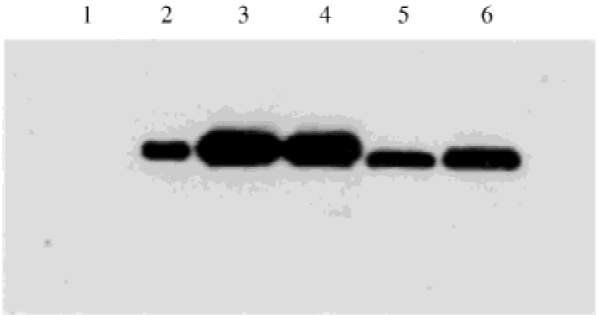

Fig. 2. Western blotting of recombinant lipase 1 expressed by *P. pastoris* cells transformed with pPic–*slip1* constructs. All cultures were performed in 50 mL rich, buffered medium, induced by addition of methanol to reach 1% v/v concentration on respect to the final culture volume (see Material and methods). Cultures supernatants were collected at the end of each fermentation (at 120 h) and clarified by centrifugation before sample preparation by 3 min heat denaturation and addition of SDS sample buffer. Lane 1: 20 μL wt Lip1 from not induced culture; Lane 2: ca. 0.2 μg of commercial lipase; Lane 3: 20 μL wt Lip1; Lane 4: 20 μL Lip1-N291Q; Lane 5: 20 μL Lip1-N314Q; Lane 6: 20 μL Lip1-N351Q.

The rations for the four different enzyme types were in the same order of magnitude indicating that the specificity of the mutant enzymes is unchanged.

Activity in low water media

The activity of the enzymes was tested in hexane with special focus put on their response to different water contents. All enzyme preparations had the same loading (mg protein/g carrier) in the esterification reaction, since it has been reported that the loading can affect the water activity profile for lipases (Valivety et al., 1994). The water activity profiles for LIP1 and the mutants N291Q, N314Q, N351Q were very similar with maximum initial rate at $a_w = 0.75$ (Fig. 3).

The main conclusion drawn from these experiments is that there is no effect on the water activity profiles when the glycosylation pattern of LIP1 is changed. Other factors affecting the enzyme preparation and the reaction rate are maybe more important for the water activity profiles, such as amount of protein on the carrier, mass transfer limitations, polarity of the substrate, and support material.

Temperature stability

The temperature stability for LIP1 and the mutants N291Q, N314Q, N351Q was investigated at two different temperatures 40 and 60 °C. The half-lives for the four different enzymes were similar at both temperatures. A marked decrease (from about 40 h to 3.5–4 h) was observed in half-lives for all enzyme types when the temperature was raised from 40 to 60 °C for all enzymes. These results clearly show that glycosylation does not affect the temperature stability of the enzyme under the conditions used.

Table 1B. *The ratios between specific activities for the different enzymes*[a]

Enzyme	pH stat/pNPP	Esterification/pNPP	pNPL/pNPP
LIP1	4.0	0.36	0.033
N291Q	6.8	0.21	0.053
N314Q	<6.3	0.22	0.063
N351Q	<33	0.30	0.43

[a]Experimental error was 5–8%.

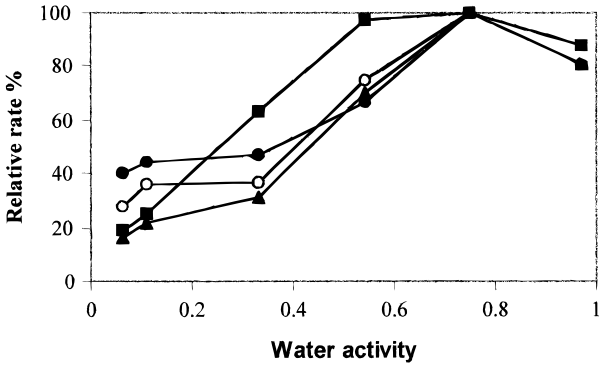

Fig. 3. Water activity profiles of different lipases in esterification reaction. The reactions were performed in hexane containing dodecanol (50 mM) and decanoic acid (100 mM). All lipases were immobilized on EP-100. Lipases LIP1(○), N351Q (▲), N314Q (●), and N291Q (■).

Discussion

In this paper, we approached the issue of the role of glycosylation in the catalytic behavior of *C. rugosa* lipase by the production of mutants on each of the three possible glycosylation sites in the isoenzyme LIP1.

The most prominent effect was observed when Asn351 was replaced. The glycan chain bound to this residue is located close to the protein lid. In LIP1, the lid is formed by a large amphipathic loop that, lying on the protein surface, undergoes conformational changes regulating the access to the active site. LIP1 in open conformation is characterized by the fact that the active site triad is accessible to the solvent and to the substrate molecules. The active site lies into the binding site conformed as a tunnel, into which the alkyl chain binds and that remains occluded from the solvent in the closed conformation (Grochulski et al., 1994a). It has been proposed that the loop interacts with the rest of the protein in completely different ways in the two states. Indeed during opening, the secondary structure of the lid does undergo significant changes by displacing and refolding, but maintaining its amphipathic character. In the closed form, the side facing the rest of the protein is hydrophobic and interacts with the hydrophobic residues surrounding the active site. The upper side is distinctly hydrophilic and forms part of the molecular surface exposed to the solvent. Grochulski et al. (1994b) showed that in LIP1 crystals the first NAG unit on Asn351 forms stacking interactions with Tyr299 in both enzyme conformation, whereas the second unit builds additional hydrogen bonds with Tyr69 and Glu70 when the lid is open, putting forth a contribution of sugars to the stabilization of the open conformation. The prominent loss of activity reported for the N351Q mutant in various reactions provides experimental support to this hypothesis.

A prominent functional role of this glycosylation site is further suggested by the observation that the glycosylation consensus sequence at N351 is not only the lone conserved among the *C. rugosa* lipases family (Lotti et al., 1993), but it is also present in the homologous *Geotrichum candidum* lipases at Asn364 (Bertolini et al., 1994).

The decrease in the enzyme activity caused by deglycosylation at position 314 is less straightforward to explain, since it removes a sugar chain located far away from significant structural and/or functional elements. The decrease in activity of N314Q mutant could rather point to a possible role of sugars in the stabilization of the enzyme structure or to a reduced folding ability of the mutated protein. Expression of mutated proteins in host cells has been show to be able to evoke unexpected cell responses eventually leading to an impaired folding and/or secretion of the mutant (Sagt et al., 1998).

Removal of the potential glycosylation site at Asn291 does not produce remarkable effects neither in the size nor in the activity of LIP1. In the protein primary structure, N291 is enclosed in the sequence NNTP (Longhi et al., 1992). Gavel and Von Heijne (1990) proposed that glycosylation is strongly inhibited by proline residues both in positions X and Y in the consensus Asn-X-Thr/Ser-Y. However, whereas a site with X = Pro (Pro +1) is nonglycosylated in at least 90% of all cases, for Y = Pro (Pro +3) this value is ~50%. Therefore, it was rather difficult to predict the experimental result based on sequence analysis. The observations reported are all consistent with this site being not involved in glycosylation and suggest that in *P. pastoris* expression system, LIP1 undergoes the same glycosylation pathway than in *C. rugosa*.

Materials and methods

Strains, plasmids, and growth conditions

Escherichia coli strain JM101 (Promega Co., Madison, Wisconsin) was used as host for plasmid amplification and *P. pastoris* X-33 (Cregg et al., 1993; Invitrogen Co., San Diego, California) for the expression of recombinant lipases. The plasmid used for cloning and expression was pPICαB (Invitrogen). *E. coli* was grown at 37 °C in low salt Luria–Bertani medium (LB) containing 25 μg/mL zeocin (Invitrogen) for selection of clones carrying pPICZαB. *P. pastoris* was grown in shaking flasks at 30 °C in YEP (1% Yeast Extract, 2% Peptone) buffered medium containing 1% glycerol and 0.1 M phosphate buffer pH 6.0 (BMGY).

Liquid or solid YEPD medium (1% Yeast Extract, 2% Peptone, 2% Glucose) was used to maintain yeast cultures; YEPDS (YEPD + 2% Sorbitol) + 1% tributyrin plates containing zeocin (100 μg/mL) were used to select *P. pastoris* transformants.

Enzymes and chemicals

Restriction enzymes used in this study were obtained from New England Biolabs (Beverly, Massachusetts). Methanol and hexane were of p.a. grade and purchased from Merck (Darmstadt, Germany). p-Nitrophenyl laurate (pNPL), p-nitrophenyl propionate (pNPP), gum arabic, glycerol (99%), and olive oil were obtained from Sigma Chemical Co. (St. Louis, Missouri). Polypropylene powder EP-100 (200–400 μm) was a gift from Akzo (Obernburg, Germany). Dye reagent and bovine serum albumin (BSA) were from Bio-Rad (New York, New York).

DNA mutagenesis

Template plasmid DNA was purified using either the alkaline lysis for mini preparations (Birnboim & Doly, 1979) or the Qiagen plasmid kits for mini- or maxi-preparations (Qiagen, Hilden, Germany). Standard DNA manipulations were carried out according to Sambrook et al. (1989).

Site-directed mutagenesis was performed by overlap extention by the polymerase chain reaction (PCR) technique (Ho et al.,

1989) using 10–50 ng of pPIC-*slip1* plasmid DNA as the template and pairs of mutagenic complementary 18–31 mers (50 pmol) as primers. Reactions, in a total volume of 100 μL, were catalyzed by 2,5 units of *Pfu* TurboTM Polymerase (Stratagene, GmbH, Heidelberg, Germany). Samples were subjected to 25 cycles of denaturation (1 min, 94 °C), annealing (1 min, 77 °C), and extention (1 min, 72 °C), and a final step of extention of 10 min at 72 °C, using a DNA Thermal Cycler (Perkin Elmer, Foster City, California). The reaction products were analyzed on an agarose gel (0,8%) and treated for 2 h at 37 °C with *Dpn*I endonucleases, and then used directly in a subsequent overlap extention reaction. Ten microliters from the two PCR mixtures containing the overlapping fragments were mixed and subjected to PCR amplification using the external oligonucleotide primers. Second step PCR products were treated for 2–3 h at 37 °C with *Dpn*I and subjected to gel purification (Qiagen gel extraction kit, Hilden, Germany). Each mutant gene fragment was cloned into the vector pPIC-*slip1* replacing the corresponding wild-type sequence. Recombinant plasmids thus obtained were introduced by transformation into *E. coli* cells. Sequencing was employed to check fragments of mutated genes generated by PCR.

Transformation of P. pastoris cells, plate assay of lipase activity, and expression of wt and mutant LIP1

P. pastoris X-33 cells were transformed with 10 μg of *Sac*I-linearized plasmid DNA according to Klebe et al. (1983). Positive transformants were checked for lipase activity by transferring colonies onto YPS + 1% tributyrin plates. Activity was detected after overnight incubation at 30 °C by the formation of a clear halo.

Fifty microliter cultures were grown in 250 mL flasks at 30 °C, in a rich buffered medium (BMMY) at pH 6.0 for 5 days, reaching an OD_{600} of 60–80. The cultures were maintained at constant pH by adding 1 M phosphate buffer pH 6.0 and fed by addition of 250 μL 100% methanol twice a day; shaking rate was 250 rpm.

The wild-type and mutant enzymes were identified by electrophoresis on 10% SDS-polyacrylamide gel (Laemmli, 1970), with the wild-type LIP1 as a molecular mass reference. Culture supernatant samples and rough cellular extracts were subjected to Western blotting analysis with a polyclonal antibody raised against *G. candidum* lipase (GCL) and kindly provided by Chris Davis (Unilever, Uinted Kingdom).

Native LIP1 (0.13 mg/mL concentrated) and mutant forms of LIP1 from *C. rugosa* (N291Q, N314Q, and N351Q, protein concentrations 0.11, 0.13, and 0.24 mg/mL, respectively) were obtained from the culture supernatants of *P. pastoris* after concentration with a Minitan system (Millipore Co., Bedford, Massachusetts) by tangential flow filtration.

The protein concentration was determined according to Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used a standard.

Preparation of immobilized enzyme by adsorption

All lipases were immobilized by adsorption (room temperature, o.n.) onto solid support EP-100 (polypropylene) previously wetted with ethanol (3 mL/g EP-100). For all preparations the loading was adjusted to be 5 (mg protein/g carrier). The preparation was filtered and washed with water. Finally, phosphate buffer (200 mM pH 7.0, 1 mL/g support) was added and the preparation was dried overnight under reduced pressure.

Esterification in organic solvent

Enzyme preparations and the substrate solutions (50 mM dodecanol and 100 mM decanoic acid in hexane) were equilibrated (at least 16 h) over saturated salt solutions at 25 °C to obtain a defined initial water activity. Salts used were LiBr ($a_w = 0.06$), LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.54$), NaCl ($a_w = 0.75$), and K_2SO_4 ($a_w = 0.97$).

Reactions were performed in 4 mL containers with Teflon-lined septa and started by mixing enzyme preparation and 2.0 mL substrate solution. The reactions were performed at 25 °C and shaken on a horizontal shaker, 175 rpm. Fifty microliter samples were withdrawn at different stages of conversion.

The alcohol substrate and the ester were analyzed using a Shimadzu gas chromatograph (GC-14 A) equipped with a flame ionization detector (FID) and a column packed with GP10% SP-216-PS on Supelcoport. Helium was used as a carrier gas. The temperature of the injector was 200 °C and for the detector 240 °C. The temperature of the column was 175 °C. The compounds were quantified from the relative peak areas, and response factors were obtained from standard curves.

Hydrolysis of pNPL and pNPP

Activity was determined by following the formation of p-nitrophenol (pNP) in the hydrolysis of p-nitrophenol laurate (pNPL) or p-nitrophenol propionate (pNPP). Substrate solution (10 μL of 100 mM ester in methanol) was well mixed with 2 mL phosphate buffer (200 mM pH 7.0) in a 3 mL cuvette. The reaction was started by addition of enzyme solution. The absorbance increase at 405 nm was monitored on a Shimadzu UV-120-02 spectrophotometer. One unit of lipase activity was defined as the amount of enzyme that releases 1.0 μmol (pNP) per min.

Hydrolysis of olive oil

Gum arabic (0.6 g), glycerol (54 mL), NaCl (1.79 g), and K_2HPO_4 (0.04 g) were mixed with distilled water to a final volume of 100 mL. The emulsion was prepared by mixing this emulsion reagent (10 mL), with olive oil (3 mL), and distilled water (47 mL) in an Ultra-Turrax.

Reactions were started by adding enzyme solution to 5 mL emulsion. The fatty acids released were titrated with 10 mM NaOH at 25 °C at pH 8.0. One unit was defined as 1 μmol fatty acid released per minute from olive oil. The detection limit under the conditions used for the pH-stat assay was 1 (μmol/min mg protein).

Temperature stability

Enzyme solution (2 mL) was incubated at 40 or 60 °C. Samples (100 μL) were withdrawn at different times to follow the deactivation of enzyme. The residual activity was measured by following the formation of pNP in the hydrolysis of pNPP. Half-lives were calculated from first order exponential decay of the activity.

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