

The Use of Rational Mutagenesis to Modify the Chain Length Specificity of a *Rhizopus delemar* Lipase

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INTRODUCTION

Due to the occurrence of the carboxylic acid ester bond in numerous compounds of pharmaceutical, medical, and industrial importance, there is widespread interest in the characterization of lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) and their use as applied catalysts. Within the past ten years, lipase-encoding genes from several organisms have been cloned. Some of these have been expressed in their new genetic backgrounds. Within the past six years, X-ray diffraction studies have produced models of the three-dimensional structures of several lipases. Also within this time frame, biochemical and molecular genetic data have been gathered that allow the identification, in some lipases, of those amino acids that play crucial roles in enzyme activity. These areas of research have converged to the extent that a coordinated approach, using computer-assisted molecular modeling and recombinant DNA technology, to examine structure-function relationships in these enzymes is now possible. Our laboratory has recently completed such a study¹ and the results of that study will form the basis of this review.

Lipases can be classified according to their abilities to hydrolyze the ester bonds of triglycerides. Two main groups of enzymes have been identified: (a) those hydrolyzing all three esters of the substrate and (b) those hydrolyzing only the terminal [1,3-] positions. Other groupings can also be made, for example, based on whether an enzyme displays specificity with regard to the degree of substitution of the glyceride or with regard to chain length or degree and location of unsaturation in the fatty acyl portions of the glyceride. It is axiomatic that the origins of these specificities lie in the amino acid sequences of the polypeptide chains of the enzymes. However, detailed information regarding the structural determinants of these specificities is largely unknown.

The class of fungal lipases exhibiting 1,3-positional specificity toward the substrate contains enzymes from several organisms. These share varying degrees of genetic homology, often quite high, and have similar three-dimensional structures and active-site geometries. This group contains lipases from *Rhizomucor miehei* (Rm),² *Rhizopus delemar* (Rd),³ *Rhizopus niveus*,⁴ *Humicola lanuginosa* (Hl),⁵ and *Trichothium camembertii* (Pc).⁶ Several enzymes in this group are commercially available and are among the lipases most commonly used as applied catalysts. The

work of Derewenda and others has resulted in 3-D structures for a number of these enzymes⁷⁻⁹ and in delineation of their probable active sites⁷ and substrate-binding domains.¹⁰

The enzymes of this family are composed of single polypeptide chains, with molecular masses between 30,000 and 35,000, and all adopt a similar three dimensional structure (FIGURE 1). It is likely that all members of the family are initially synthesized as preproenzymes, which then undergo proteolytic maturation. The catalytic center consists of a triad of amino acids: serine, histidine, and aspartic acid. The serine is responsible for a nucleophilic attack on the carbonyl carbon of the scissile ester bond. This serine lies within a consensus sequence, Gly-X-Ser-X-Gly which is sufficiently conserved among lipases and esterases as to be diagnostic of the enzyme family. The catalytic triad is buried beneath the solvent-accessible surface of the protein. One loop of the peptide chain (the "lid") is capable of lying over the active-site cleft. The phenomenon of interfacial activation, whereby lipases become catalytically active only in the presence of a solvent-substrate interface, appears to result from a stabilization of the open-lid conformation, exposing the active site to substrate. Near the active site, there is a shallow surface trough that appears to accommodate the acyl chain of the scissile fatty acid (FIGURE 2). Based on insights provided by examinations of the three-dimensional structures of the *Rh. delemar* and *Rm. miehei* lipases, we have conducted directed mutagenesis of the gene for the *Rh. delemar* enzyme, focusing on sites near the active center, within the lid, and in the substrate-binding domains. We have then examined the effects of such mutagenesis on the activities and chain length specificities of the resulting enzymes.

MATERIALS AND METHODS

Molecular Modeling

Initial studies employed the crystal coordinates of the structure of the *Rm* lipase (generously provided by Z. Derewenda), which is highly homologous to the *Rd* enzyme¹¹ and for which crystallographic data indicate the site of binding of active-site directed inhibitors.¹⁰ Subsequent work utilized the coordinates of the *Rd* enzyme.¹ Molecular modeling was performed on an Indigo2 computer (Silicon Graphics Incorporated, Mountain View, California) using Sybyl software (Tripos, Incorporated, St. Louis, Missouri).

The main experimental approach involved visual inspection of the models to identify those amino acid residues with apparent potential for physical interference with the fatty acyl chain of bound substrate. These residues were then subjected to site-directed mutagenesis. Other mutagenesis experiments involved changing amino acids that might play a role in enzyme activation or the catalytic event.

DNA Manipulations

Vector Construction

Plasmid pUC8.2-14 contains a cDNA encoding the entire preprolipase of *Rh. delemar* under the control of the lactose operon regulatory elements.⁵ By introducing

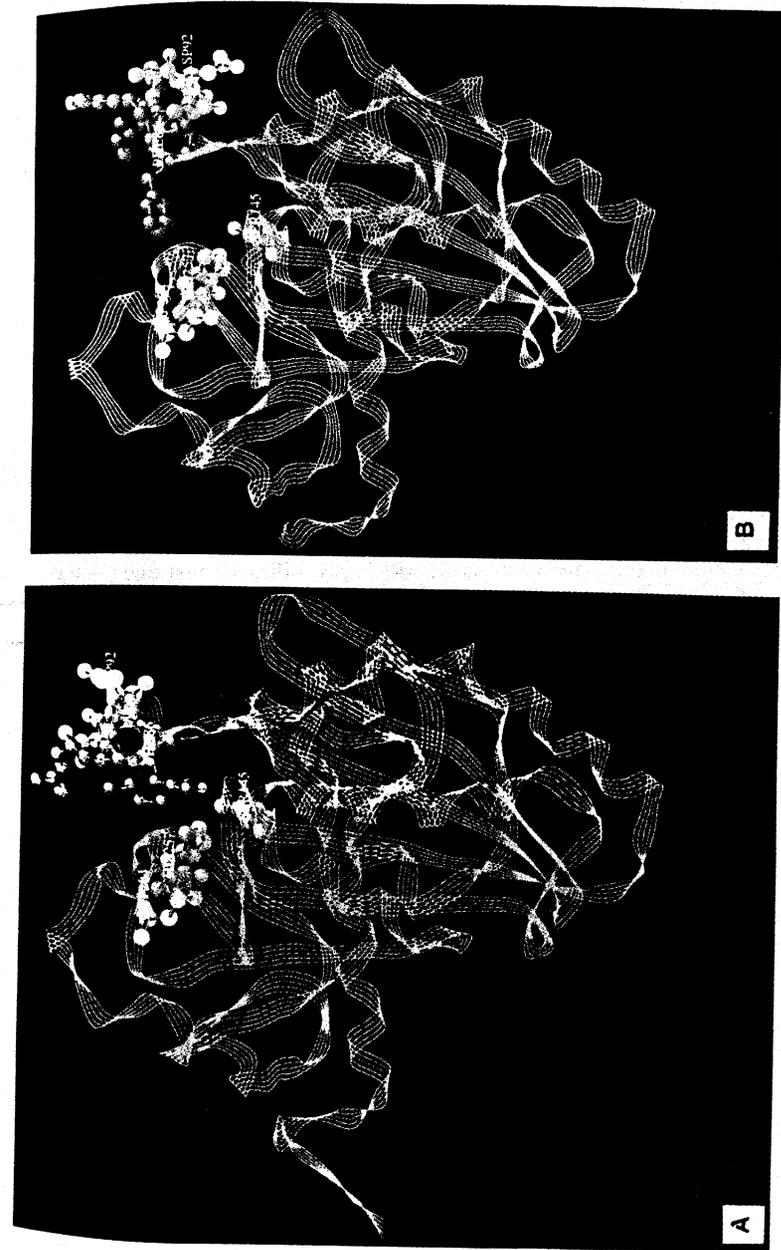


FIGURE 1. Three-dimensional structure of the *Rhizopus delemar* lipase, based on crystallographic coordinates.^{8,9} In panel A, the "lid", residues 86-92, is in the closed conformation. In panel B, the lid is partially opened, exposing the active-site residues: ser145, asp204, and his257.

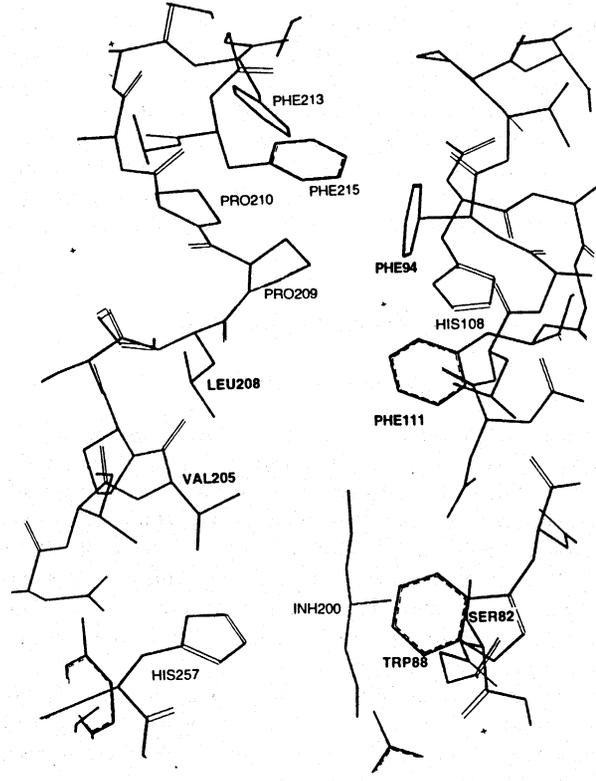


FIGURE 2. Structure of a portion of the *Rhizomucor miehei* (Rm) lipase with the inhibitor *n*-hexylphosphonate ethyl ester (INH200) bound in the active site and probable substrate-binding region (coordinates kindly provided by Z. Derewenda).¹⁰ Residues that were the focus of the present study are visible. These include ser82 (thr83 in *Rhizopus delemar* lipase), which stabilizes the oxyanion catalytic intermediate; trp88 (ala89 in *Rh. delemar*), which is in the lid of the enzyme; and residues lining the substrate-binding region. The corresponding binding region residues in the *Rh. delemar* lipase are phe95 in place of Rm phe94, his109 (Rm his108), phe112 (phe111), val206 (val205), val209 (leu208), pro210 (pro209), pro211 (pro210), phe214 (phe213), and phe216 (phe215).

*Nco*I sites at appropriate locations in this plasmid, it was possible to isolate *Nco*I-*Bam*HI fragments encoding either the prolipase or the mature lipase. These were subcloned into plasmid pET11-d, creating plasmids pET11-d-431 (produces mature lipase) and pET11-d-1231s (produces prolipase), which were proficient at lipase production (FIGURE 3).¹² These vectors allowed the isolation of relatively large amounts of enzyme. For the experiments described here, the origin of phage fl was introduced into these plasmids, producing pET11-d-fl-431 and pET11-d-fl-1231s.¹ This modification allowed the production of single-stranded circular DNA for use in site-directed mutagenesis without reducing the suitabilities of the plasmids for the production of enzyme.

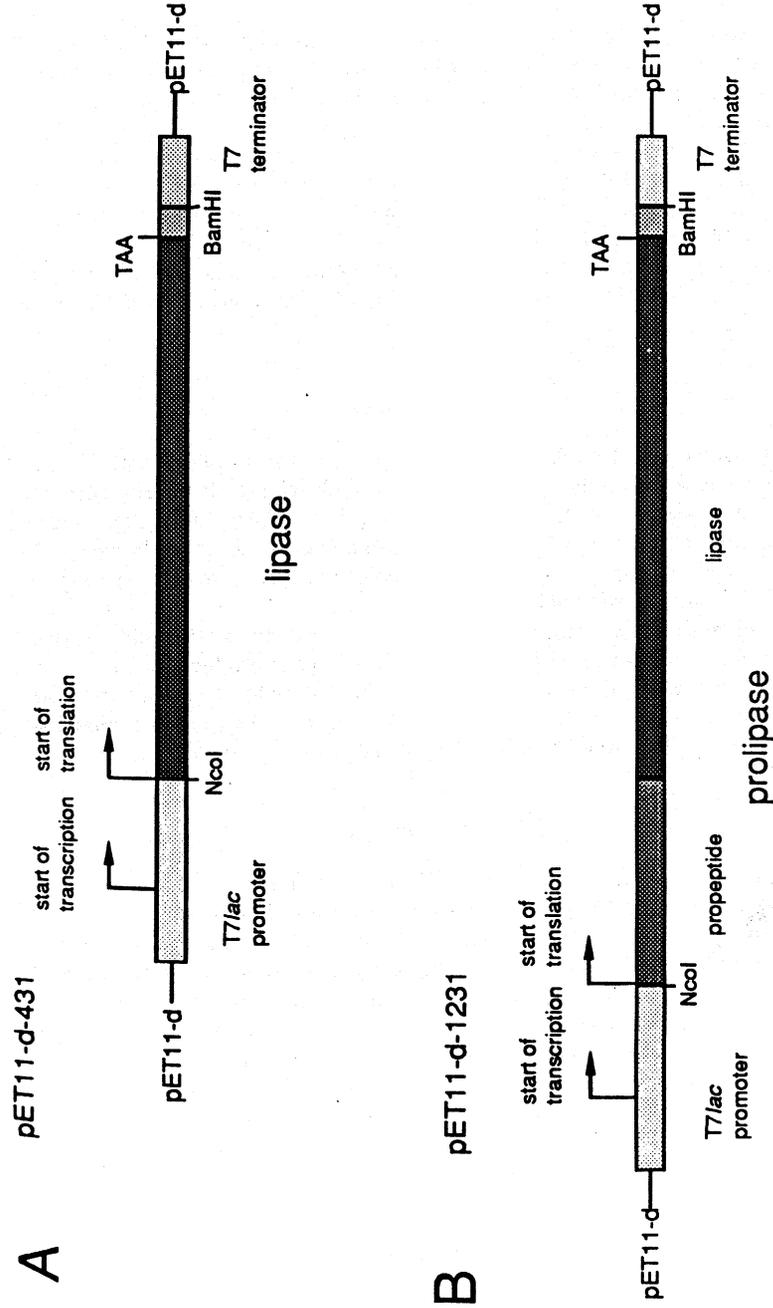


FIGURE 3. Schematic representation of the (A) lipase- and (B) prolipase-encoding DNA segments inserted into the expression plasmid pET11-d. These constructs¹² were the basis of those created here, by the insertion of the phage fl origin of replication, for regulated lipase gene expression and the production of single-stranded circular DNAs. The termination codon for lipase and prolipase is indicated by the nucleotide triplet TAA.

Oligonucleotide-directed Mutagenesis

Single-stranded DNA for use in site-directed mutagenesis was obtained by infecting plasmid-containing *E. coli* JM101 cells with helper phage M13KO7 as described.¹² Mutagenesis was carried out by the method of Kunkel¹³ using the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad Laboratories, Hercules, California). Mutagenic primers were designed to change both the amino acid sequence at a desired single site and the restriction enzyme fragmentation pattern of the DNA. This facilitated the detection of mutated plasmid DNA. Primers were so constructed that all possible amino acids were inserted at the site of interest.

DNA sequences were determined by the method of Sanger *et al.*¹⁴ using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, Ohio).

Mutant Screening

For the detection of modified prolipases produced from plasmid pET11-d-fl-1231s, screening relied on the ability of free fatty acids released from triglycerides by lipase to cause a distinctive change in the fluorescence spectrum and intensity of the dye rhodamine B included in solid growth medium containing single homogeneous triglycerides.¹⁵ DNA sequencing was used to confirm the presence of the appropriate mutation in plasmids identified in this manner.

Such a phenotypic screening method could not be used to detect modified mature lipases because it was not possible to recover viable cells producing active mature enzyme. Therefore, to introduce mutations into the mature lipase, mutations created in the prolipase gene were transferred into the mature lipase gene by recombinant DNA techniques. DNA sequencing verified the acquisition of the mutations.

Enzyme Production

Late log phase cultures of *E. coli* BL21 (DE3) harboring wild-type or mutant plasmids were induced for lipase production by the addition of isopropyl- β -D-thiogalactoside and were harvested after 3 to 4 hours. Cells producing prolipase were broken by sonication. This lysate was used directly as the source of active prolipase.

All versions of the mature lipase, wild type and mutant, required refolding of the enzyme, initially present in inclusion bodies, before determination of enzyme activity.¹¹ To do this, the cells were broken by lysozyme-detergent treatment and their inclusion bodies were recovered by centrifugation. These were then solubilized in 8 M urea and the lipase within them was refolded by dilution in the presence of cystine and cysteine. Refolded mature lipase was purified to homogeneity by affinity and ion exchange chromatographies.

Enzyme Activity Measurements

Lipase activities were measured titrimetrically by a pH-stat method¹⁶ at 25 °C and pH 7.5 using 0.1 N NaOH as the titrant. The substrates were 200 mM tributyrin (TB,

a triglyceride containing 4-carbon fatty acids), tricaprylin (TC, a homoglyceride containing 8-carbon fatty acids), or olive oil (OO, predominantly 18-carbon fatty acids) emulsified in aqueous 4.2% gum arabic solutions containing 14 mM CaCl₂. A unit (U) of activity released one micromole of fatty acid per minute.

Substrate selectivities were measured by incubation of the purified enzyme (in the case of the mature lipase) or total cell lysate (in the case of prolipase) at pH 7.5 for 2 h at 30 °C in solutions containing 0.4 M each of tributyrin, tricaprylin, and triolein. The degree of hydrolysis of each substrate was then determined by gas chromatography.

RESULTS AND DISCUSSION

Mutagenesis Experiments

In our experience, the production of active mature lipase is toxic to *E. coli*, whereas prolipase synthesis is tolerated. Therefore, when a phenotypic assay was to be used to assess the effects of site-directed mutagenesis on enzyme activity, the mutations were first created in the prolipase gene. This was then expressed in *E. coli*. After using rhodamine media to identify mutant prolipases of interest, these mutations were transferred into the mature lipase gene. This gene was expressed at high levels, leading to the accumulation of inclusion bodies containing inactive mutant mature lipases. These were isolated and the lipase within them was solubilized and renatured to yield active enzyme.

To examine the effects on activity of particular amino acid substitutions within the lid or active site of the lipase, the desired mutations were introduced into the gene for the mature enzyme. The mutated lipases encoded by these genes were then purified from inclusion bodies and characterized.

The introduction of the origin of replication of phage f1 into the lipase and prolipase expression plasmids, as first described for the transcription termination factor, rho,¹⁷ created plasmids that could be used not only for lipase production, but also for production of the single-stranded DNAs required for site-directed mutagenesis.

Role of Threonine (thr) 83 in Facilitating Catalysis

In the Rm lipase, serine (ser) 82 lies very close to ser144, the active-site serine. It has been postulated that ser82 assists catalysis by stabilizing the tetrahedral oxyanion catalytic intermediate.¹⁰ The analogous residue in the Rd lipase is a threonine (thr) located at position 83. The mutagenic conversion of thr83 to an alanine (ala) decreased the specific activity by almost three orders of magnitude (TABLE 1). When thr83 was mutagenically converted to a serine, the specific activities on various substrates were between one-fifth and one-half of that of the wild-type enzyme (TABLE 1). Alanine is unable to supply a hydroxyl group to stabilize the transition state intermediate, whereas it is feasible that serine could provide this stabilization. Thus, these data support the postulated role for ser82/thr83 in the catalytic event. Similar results have been reported for the *Penicillium camembertii* monoglyceride and diglyceride lipase.¹⁸

In this family of enzymes, a short stretch of the peptide chain (residues 86 to 92 in the Rd lipase) occludes the active site in the absence of substrate.⁷ It has been postulated, and demonstrated using inhibitors,^{10,19} that this lid is displaced when substrate analogues bind to the active site. This is thought to be the structural basis of the biochemical phenomenon termed "interfacial activation," the prerequisite that a substrate-water interface be present before a lipase will be catalytically active. Except for the Rd lipase, all known members of this family contain a trp in the lid, at a site corresponding to the alanine in position 89 of the Rd lipase. The presence of such a highly conserved residue suggests that the trp may play a crucial role in interfacial activation. To investigate this theory, ala89 in the Rd lipase was mutagenically converted to trp and the effects of this change on enzyme activity were determined. There was no increase in enzyme activity. Instead, there was a 22–42% reduction in specific activity relative to that of the purified wild-type enzyme (TABLE 1). However, compared to the wild-type enzyme, there were no profound changes in the relative activity toward various substrates tested (TABLE 1, FIGURE 4). Further work is required to precisely identify the importance and role of the trp in the lids of these enzymes.

Directed Mutagenesis of Sites within the Substrate-binding Groove

Studies with the Rm lipase employing the active-site-directed covalent inhibitor *n*-hexylphosphonate ethyl ester have identified a groove on the surface of the enzyme that appears to accommodate the fatty acyl chain of the scissile ester of the substrate (FIGURE 2).¹⁰ Our goal was to determine whether alteration of residues within this groove would affect activity or substrate preference. In the Rd lipase, residues lying in positions 95 (phe), 112 (phe), 206 (val), and 209 (val) (corresponding to Rm residues 94, 111, 205, and 208, respectively) form part of this groove and are highly conserved in actuality or functionality in the Rd, Rm, Hl, and Pc enzymes.⁹ These sites were individually subjected to site-directed mutagenesis using primers that would introduce all possible amino acid substitutions at these locations. The resulting prolipase DNAs were introduced into bacteria by transformation or electroporation and expressed.

TABLE 1. Titrimetric Analysis (Single-Substrate Assay) of Lipolytic Activity of Refolded and Purified Recombinant Mature Lipases

Mutation ^a	Specific Activity (U/mL) ^b		
	Olive Oil	Tricaprylin	Tributylin
wild type	3149	8539	2944
thr83 → ala	4	7	1
thr83 → ser	1664	3350	442
ala89 → trp	2106	6628	1702

^aAmino acids numbered as in reference 5.

^bMicromoles of fatty acid released per minute per mg of protein. The values are averages of two experimental determinations.

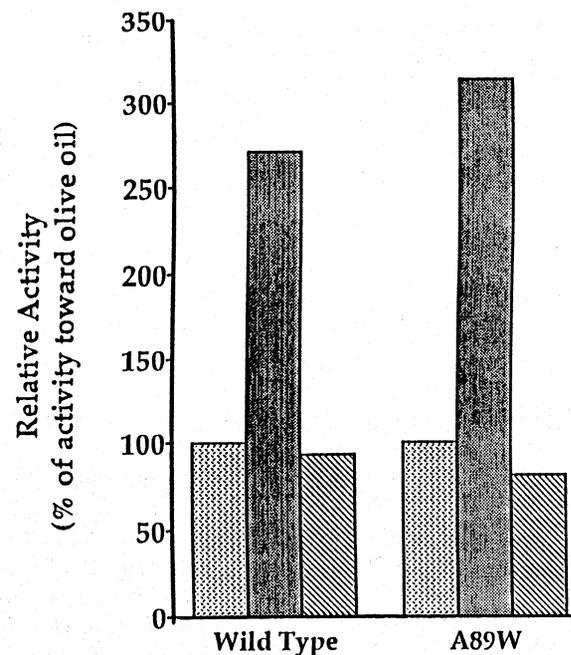


FIGURE 4. Relative activities calculated from the results of titrimetric assays of wild-type and mutant A89W lipase on olive oil (dotted bars), tricaprylin (dark bars), and tributyrin (hatched bars). Raw data are listed in TABLE 1. The activity on olive oil was set at 100%.

Mutated Prolipases

Bacteria bearing mutated prolipase DNAs were grown on rhodamine plates containing either olive oil or tricaprylin. This allowed visual identification of colonies that differed from wild type in their lipolytic activities toward these substrates. The majority of the cells showed no change in phenotype on these media, suggesting that, even though the residues at positions 95, 112, 206, and 209 of the Rd lipase are highly conserved, a number of replacements are tolerated without major changes in enzyme activity. There were also some mutants that exhibited a lipase-negative phenotype, presumably due to deficiencies in folding, substrate binding, or catalysis.

Five mutant prolipases were identified that exhibited altered activities on rhodamine media compared with the wild-type prolipase (TABLE 2). In these enzymes, chosen on the basis of an altered phenotype, there was no obvious pattern to the identity or type of amino acid introduced by mutation: in some cases, a large aromatic side chain was replaced by a small charged one (F95D); in others, a small nonpolar group was replaced by a large aromatic (V209W), a small polar (V206T), or a small nonpolar residue (V209G).

TABLE 2 shows the lipolytic activities of these enzymes toward the individual substrates OO, TC, and TB. In virtually all cases, there was a reduction in activity

TABLE 2. Lipolytic Activity (Single-Substrate Assay) in Lysates of Prolipase-producing *E. coli* BL21 (DE3)

Mutation	Activity (U/mL) ^a		
	Olive Oil	Tricaprylin	Tributylin
wild type	158	270	65
phe95 → asp	35	198	29
phe112 → trp	100	59	65
val209 → trp	16	99	93
val206 → thr	64	234	41
val209 → gly	54	114	50

^aMicromoles of fatty acid released per minute per mg of protein. The values are averages of two experimental determinations.

compared with wild-type enzyme. In FIGURE 5A, these activities are expressed relative to a value of 100% for the activity toward triolein.

Another measure of the fatty acid specificity of an enzyme is gained by assaying it in the presence of a mixture of substrates and then determining the preference for the various fatty acids. These data are shown in TABLE 3 and FIGURE 5B.

The wild-type prolipase slightly prefers TC over OO, with TB being a somewhat poorer substrate than OO (FIGURE 5). For the mutant prolipases, an increase in the relative activity toward TC was the most common observation (four of the five isolates), with increases of as much as 3.6-fold in the assay of single substrates (FIGURE 5A). In mixed-substrate assays, this trait was much less pronounced, although still apparent (FIGURE 5B).

The replacement of phe95 with an aspartic acid caused a marked increase in the relative activity toward middle-chain-length substrates. This effect is most pronounced in the case of single substrates (TABLE 2, FIGURE 5A), but is also seen in mixed-substrate assays (TABLE 3, FIGURE 5B).

Replacement of phe112 by trp caused a nearly 3-fold reduction in the relative activity toward TC with both single and multiple substrates, changing C8 from the most to the least favored of the chain lengths examined (FIGURE 5). The relative activity against TB increased slightly in single-substrate assays and over 6-fold in mixed substrates (FIGURE 5). Thus, the mutagenic replacement of a large side chain by an even bulkier one reduced the relative activity toward medium-chain fatty acids and increased the activity toward short-chain ones.

A somewhat similar situation was seen in the activity toward mixed substrates when a bulky hydrophobic trp replaced val209, which lies across the substrate-binding trough from phe112. Compared with wild type, val209trp prolipase displayed a greatly increased (nearly 7-fold) relative activity toward short-chain substrates (TB) in the mixed-substrate assay (FIGURE 5B). Relative activity toward TC was also increased. In contrast to the F112W mutation, however, V209W retained its high relative activity toward TB, and its relative activity toward TC was even further enhanced, in single-substrate assays (FIGURE 5A).

The mutations V206T and V209G both yielded prolipases with increased relative activities toward middle-chain-length substrates (TC) in the single-substrate assay (FIGURE 5A). However, these did not persist in the mixed-substrate assay, where

these enzymes displayed activities very similar to wild-type prolipase (TABLE 3, FIGURE 5B). Perhaps the natures of the substrate emulsions are sufficiently different in the single- versus multiple-substrate cases to account for these differences in specificity.

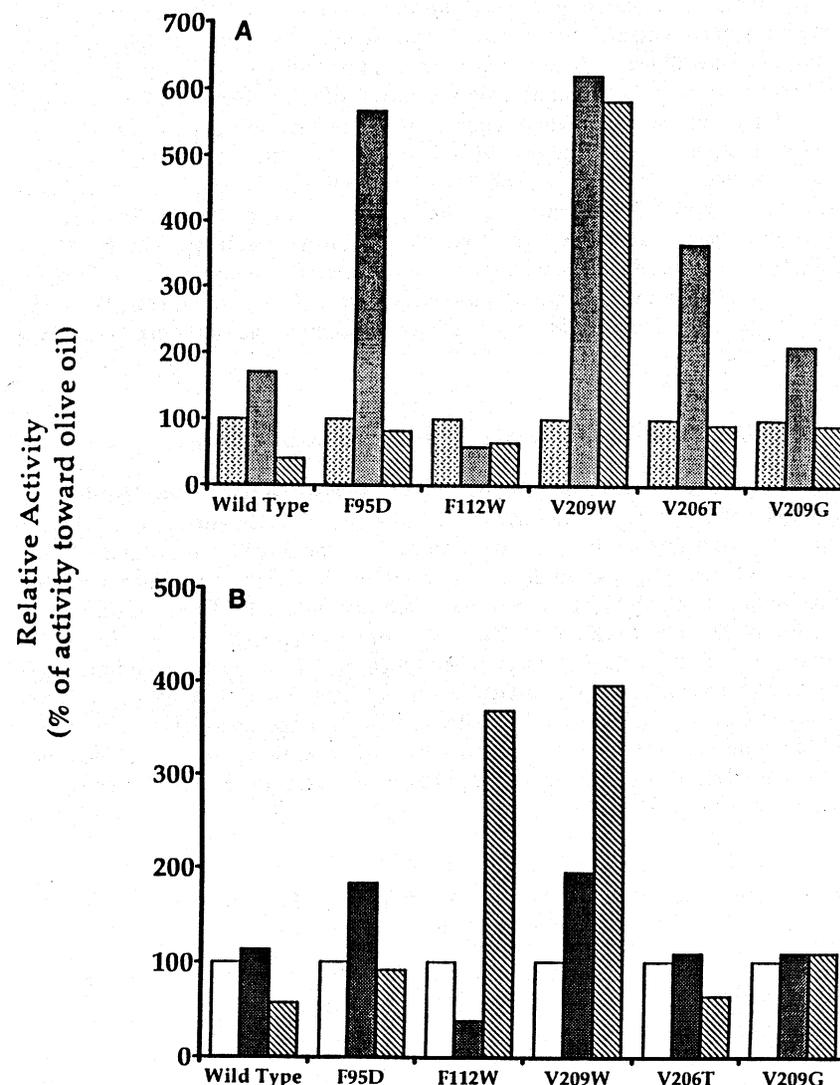


FIGURE 5. The relative lipolytic activities of crude wild-type and mutant *Rh. delemar* prolipases against (A) the single substrates olive oil (dotted bars, set at 100%), tricaprylin (dark bars), and tributyrin (hatched bars) and (B) mixtures of the substrates triolein (light bars, set at 100%), tricaprylin (dark bars), and tributyrin (hatched bars). Data are from TABLES 2 and 3.

TABLE 3. Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin, and Tributyrin by Prolipase in Cell Lysates

Mutation	% Hydrolysis		
	Triolein	Tricaprylin	Tributyrin
wild type	66.8	75.8	39.0
phe95 → asp	36.9	67.7	34.1
phe112 → trp	18.4	7.2	67.7
val209 → trp	18.2	35.4	72.0
val206 → thr	66.1	72.4	43.0
val209 → gly	54.8	60.0	60.0

TABLE 4. Titrimetric Analysis (Single Substrates) of Lipolytic Activity of Refolded, Purified Recombinant Mature Lipases

Mutation	Specific Activity (U/mL) ^a		
	Olive Oil	Tricaprylin	Tributyrin
wild type	3149	8539	2944
phe95 → asp	821	2978	404
phe112 → trp	1279	1127	1800
val209 → trp	281	1398	3896
val206 → thr	187	1079	281
val209 → gly	187	466	296

^aMicromoles of fatty acid released per minute per mg of protein. The values are averages of two experimental determinations.

Mutated Mature Lipases

It is not possible to make conclusions about the effects of mutation on the specific activities of these proenzymes because these data were collected using crude lysates with unknown enzyme concentrations. To address this issue, the mutations introduced into the prolipase gene were transferred into the mature lipase gene by recombinant DNA techniques. The resulting mutant mature lipase genes were expressed; the lipases were recovered from inclusion bodies, refolded, and purified to homogeneity; and their activities were determined. The specific activities of these

TABLE 5. Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin, and Tributyrin by Purified, Refolded Mutant Mature Lipases

Mutation	% Hydrolysis ^a		
	Triolein	Tricaprylin	Tributyrin
wild type	80.1	83.9	42.4
phe95 → asp	59.3	79.5	55.3
phe112 → trp	40.3	32.1	58.7
val209 → trp	34.3	42.5	66.4
val206 → thr	15.6	21.3	8.9
val209 → gly	47.1	46.7	47.6

^aValues are averages of two assays. Five μg of protein was added to each reaction tube, except for wild-type enzyme (2.5 μg assayed).

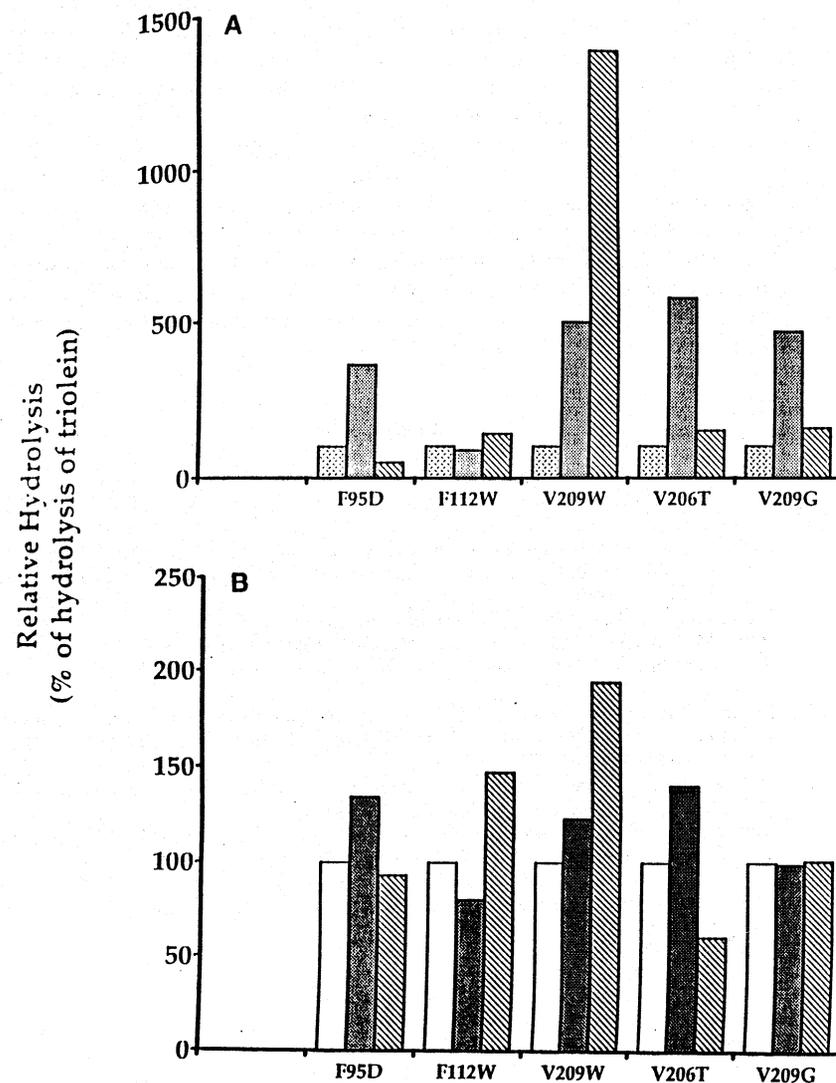


FIGURE 6. The relative lipolytic activities of purified mature wild-type and mutant *Rh. delemar* lipases: (A) single substrates olive oil (dotted bars, set at 100%), tricaprylin (dark bars), and tributyrin (hatched bars); (B) mixed substrates triolein (light bars, set at 100%), tricaprylin (dark bars), and tributyrin (hatched bars). Data are from TABLES 4 and 5.

enzymes against single (TABLE 4) and mixed (TABLE 5) substrates were determined and expressed relative to the activities toward oleic acid (FIGURE 6). It is notable that, relative to the wild-type lipase, all the mutations caused a substantial reduction in the specific activity of the resulting enzyme (TABLE 4), a phenomenon whose cause remains to be understood.

In general, the mutations caused the same phenotype in the mature enzymes as had been observed in the prolipases (above). [This validates the approach of expressing and screening mutant prolipases, which are produced in active forms, rather than focusing on the mature forms of the lipases, which require refolding before they can be assayed.] An exception to this is that the mature V209W lipase had more than twice the relative activity of its prolipase analogue toward TB (FIGURES 5 and 6). It is not known if this difference is due to an interaction of the propeptide portion of the enzyme with the substrate-binding region of the lipase or to the differences in purity between the prolipase and mature lipase preparations.

These studies have initiated an exploration of the relationship between structure and substrate specificity in this class of lipolytic enzymes. Additional work now under way seeks to further define the relationship between primary sequence and substrate specificity in the *Rhizopus delemar* lipase.

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Production, Characterization, and Molecular Modeling of Lipases for Esterification

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INTRODUCTION

Lipases (EC 3.1.1.3, triacylglycerol acyl hydrolases) are currently among the most studied enzymes owing to their great potential in a number of applications.^{1,2} They catalyze a wide variety of hydrolysis, esterification, transesterification, and polyesterification reactions. The lipase from *Candida rugosa* (ex. *C. cylindracea*) Diddens and Lodder (ATCC 14830; CBS 6330) is one of the most investigated and cost-efficient.³ Nevertheless, relatively little has been published on the production of *C. rugosa* lipase since the early work of the 1960s. *Rhizomucor miehei* lipase, on the other hand, has been shown to be an excellent biocatalyst in polyester synthesis largely because of the 3-D structure of its active center. The X-ray crystallographic structures of both lipases in "open" conformation have been recently determined,^{4,5} which allows the investigation of lipase-catalyzed reactions by also molecular modeling. The present report discusses some strategies for the production and purification of *C. rugosa* lipase, the relationships between the hydrolytic and synthetic activities of lipases, and molecular modeling of the enzyme-substrate complexes of both *C. rugosa* and *R. miehei* lipases with implications to their performance as biocatalysts.

MATERIALS AND METHODS

Chemicals

Oleic acid (99%) and olive oil emulsion (50%) were purchased from Sigma (St. Louis, Missouri); 1-butanol (*p.a.*) was from E. Merck (Darmstadt, Germany); and yeast and meat extracts and peptone were from Difco (Detroit, Michigan). Refined,

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