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A Monolayer and Bulk Study on the Kinetic Behavior of *Pseudomonas glumae* Lipase Using Synthetic Pseudoglycerides†

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ABSTRACT: A heat-stable lipase from *Pseudomonas glumae* was purified to homogeneity. Its positional and stereospecific properties were investigated and compared with those of the well-known porcine pancreatic lipase. The kinetic properties of both enzymes were determined by use of six isomeric synthetic pseudoglycerides all composed of a single hydrolyzable fatty acyl ester bond and two lipase-resistant groups: one acylamino and one ether function. Two enzyme assay techniques were applied: a detergent-free system, the monomolecular surface film technique, and the pH-stat technique using clear micellar solutions of substrate in the presence of Triton X-100. Regarding the cleavage of primary ester bonds, *P. glumae* lipase possesses no stereopreference. In contrast, a large stereopreference in favor of the R-isomer is found for the hydrolysis of secondary ester bonds. Secondary ester bonds are efficiently cleaved by the lipase, which makes it of potential interest for enzymatic synthetic purposes. For the hydrolysis of this R-isomer a correlation between the experimental catalytic turnover rate and the binding constant for micelles was observed. The kinetic data of *P. glumae* lipase have been analyzed in terms of the scooting and hopping models for the action of lipolytic enzymes [Upreti, G. C., & Jain, M. K. (1980) *J. Membr. Biol. 55*, 113–121]. The results presented in this study are best explained by assuming that *glumae* lipase leaves the interface after a limited number of catalytic cycles.

Lipases are known as extremely versatile enzymes. Their properties allow widespread application in industry, ranging from (stereo)specific synthesis of compounds (e.g., of interest to the chemical and pharmaceutical business) to the improvement of detergency in laundry washing systems. For optimal application of lipases it is important, however, to understand their basic properties, e.g., the recognition of lipid–water interfaces. This understanding can be used to adjust the properties of the applied lipase with genetic engineering techniques. Structural information of lipases is generally rather poor. Recently, the results of X-ray crystallographic studies on three lipases have appeared in the literature (Brady et al., 1990; Winkler et al., 1990; Schrag et al., 1991) and the structure of a lipase–inhibitor complex has been published (Brzozowski et al., 1991). Only the Cα coordinates of the *Rhizomucor miehei* lipase have recently been deposited in the protein databank. Their homology at the level of amino acid sequence is rather poor, which is a general property of the lipase family.

We have chosen to investigate lipases from *Pseudomonas* species, with one reason being their preferred properties in several application areas like laundry systems and in the transesterification of edible fats, as well as extensive sequence information of *Pseudomonas* lipases that are at least 90% homologous. The lipase from *Pseudomonas glumae* is a 32-kDa protein that consists of a single chain. The enzyme is not glycosylated and contains one disulfide bridge between residues 190 and 269. The existence of one consensus peptide is found in the amino acid sequence surrounding serine 87: G-X-S-X-G. This sequence is encountered in many other lipases and proteases (Brenner et al., 1988) and is shown to be present at the active site of these enzymes.

The determination of the positional and stereospecific preference of lipases acting on natural or synthetic triglycerides is subject to several problems. In theory, enantiomeric triglycerides containing three different acyl groups seem to be ideal substrates for the investigation of the properties of lipases. The enzymatically released free fatty acids can be separated

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and quantified by gas–liquid chromatography. These substrates, however, are not commercially available and have to be prepared by chemical synthesis. A more serious difficulty is the fatty acyl migration in the resulting di- and monoglycerides. These partial glycerides are further hydrolyzed by many lipases. A complicating factor in this technique might be the preference of some lipases for a certain chain length or degree of unsaturation (Jensen, 1974; Chau & Tai, 1981; Wang et al., 1982). This latter problem can be avoided by using as substrate triglycerides containing three identical acyl ester bonds: enzymatic hydrolysis is stopped before the formation of monoglycerides occurs and the free hydroxyl groups in the resulting diglycerides are blocked with an optically active reagent. Subsequent HPLC analysis of the positional isomeric and/or diastereoisomeric reaction products provides the information on the initial attack of the lipase (Rogalska et al., 1990; Laakso & Christie, 1990). If a chirally discriminating HPLC column is available, the OH-blocking agent does not need to be optically active (Itabashi et al., 1990).

In our opinion, however, acyl migration could still complicate this analysis method, in particular if the lipase is able to hydrolyze both primary and secondary ester bonds. Therefore, in this study we wanted to investigate whether synthetic triglyceride analogues containing a single hydrolyzable acyl ester bond might be useful to rapidly screen positional specificity of various lipases without problems of acyl migration. In order to be able to analyze simultaneously the stereospecific properties of the enzymes, chirally pure enantiomeric substrates were required. Moreover, the substrate properties of the synthetic analogues should be analyzable in both the presence and absence of detergents.

These constraints led us to the preparation of six isomeric synthetic pseudoglycerides all composed of one single hydrolyzable *n*-docanoyl ester bond and two lipase-resistant groups (one *n*-decanoylamino and one methyl ether). The kinetic behavior of one lipase from mammalian [porcine pancreatic lipase (PPL), MW 50 000] and one lipase from microbial origin [*P. glumae* lipase (PGL), MW 32 000] was investigated.

Materials and Methods

(*R*)- and (*S*)-*O*-serine benzyl ether were purchased from Bachem. Inc. (*R*)- and (*S*)-glycidol and their tosyl derivatives were from Aldrich and Janssen Pharmaceutica, respectively. Thionyl chloride, triethylamine, *n*-decanoyl chloride, and silicic acid were from Merck. Trityl chloride and boron trifluoride etherate were from Aldrich; $NaBH_4$, NaH, and CH_3I were from Janssen Pharmaceutica. All other chemicals were of analytical quality.

Thin-layer chromatography was done on HPTLC plates from Merck. Optical rotations ($[\alpha]_D^{20}$) were determined with a Perkin Elmer polarimeter, Model 241 MC.

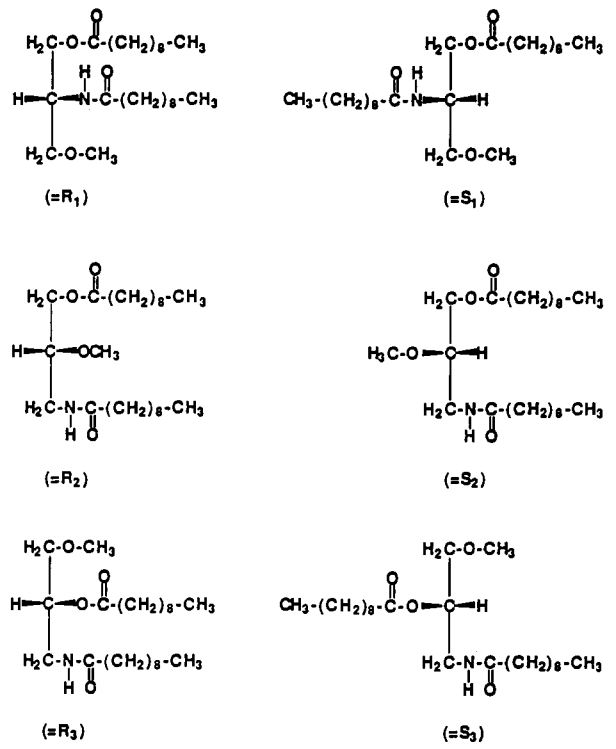
^1H NMR measurements were carried out on a 360-MHz Bruker machine. Melting points (mp) were determined on a Leitz melting points microscope and are uncorrected.

Synthesis of the Six (Stereo)isomeric Triglyceride Analogues (Scheme I). R_1: (*S*)-*O*-Serine benzyl ether was esterified with MeOH and thionyl chloride as described by Guttmann and Boissonnas (1958): yield 97%; R_f = 0.83 in $CHCl_3$–MeOH–H_2O (65/25/4 v/v); mp 158–160 °C; $[\alpha]_D^{20}$ = −1.73° (c, 16 in EtOH). The amino function was acylated with *n*-decanoyl chloride in dry chloroform and triethylamine: yield 100%, R_f = 0.87 in $CHCl_3$–MeOH (95/5 v/v); $[\alpha]_D^{20}$ = 1.20° (c, 11.5 in $CHCl_3$). Subsequently, the methyl ester was reduced with $NaBH_4$ in tetrahedrofurane. The resulting crude alcohol was purified by SiO_2 chromatography using chloroform–methanol (98/2 v/v) as eluent: yield 56%; R_f = 0.45 in $CHCl_3$–MeOH (95/5 v/v); mp 37–38 °C; $[\alpha]_D^{20}$ = +5.83° (c, 18 in EtOH). The primary alcohol function was converted to the corresponding methyl ether with use of NaH and CH_3I in dimethylformamide as described by Brimacombe et al. (1966). After column chromatography on SiO_2 using chloroform–methanol (99/1 v/v) as eluent, the pure methyl ether was obtained in a yield of 96%: R_f = 0.65 in ether–hexane (9/1 v/v); $[\alpha]_D^{20}$ = +0.79° (c, 12 in EtOH). The benzyl ether function was removed by catalytic hydrogenolysis with use of Pd as catalyst: yield 100%; R_f = 0.26 in chloroform–methanol (98/2 v/v); $[\alpha]_D^{20}$ = −2.48° (c, 14.5 in EtOH). Finally, the primary alcohol group was acylated with *n*-decanoyl chloride in dry chloroform and triethylamine. The end product was purified by silicic acid chromatography using ether–hexane (1/1 v/v) as eluent. The described compound, R_1, a colorless oil, was obtained in a yield of 85%: R_f = 0.28 in ether–hexane (1/1 v/v); $[\alpha]_D^{20}$ = −0.28° (in substance); ^1H NMR ($CDCl_3$–CD_3OD = 1/1) δ 0.85 (6 H, CH_3), 1.25 (24 H, alkyl), 1.65 (4 H, βCH_2), 2.30 (2 H, αCH_2CON), 2.40 (2 H, αCH_2COO), 3.35 (3 H, OCH_3), 3.5 (2 H, CH_2OCO), 4.25 (2 H, CH_2O), 4.3 (H, CH).

S_1: This stereoisomer was prepared as described above starting from (*R*)-O-serine benzyl ether. $[\alpha]_D^{20}$ = +0.30°. Chromatographic and NMR characteristics were identical with those of the R_1 enantiomer.

S_2: (*S*)-Glycidol was converted into (*S*)-1-amino-1-deoxyglycerol with aqueous ammonia at 4 °C as described by Sowden and Fisher (1942): yield 97%. The free amino group was acylated with *n*-decanoyl chloride in dry dimethylformamide and triethylamine: yield 84%; mp 92–93 °C; R_f = 0.73 in $CHCl_3$-MeOH-H_2O (65/35/8 v/v); $[\alpha]_D^{20}$ = +2.62° (c, 10.3 in $CHCl_3$–MeOH = 1/1). Subsequently, the primary hydroxyl function was protected by reaction with trityl chloride

Scheme I: Structural Formulas and Stereoconfiguration of the Six Isomeric Triglyceride Analogues Used as Substrates[a]



[a] R_1 stands for (*R*)-1,2-didecanoyl-2-deoxyamino-3-*O*-methylglycerol; R_2 stands for (*R*)-1,3-didecanoyl-3-deoxyamino-2-*O*-methylglycerol; R_3 stands for (*R*)-2,3-didecanoyl-3-deoxyamino-1-*O*-methylglycerol. S_1, S_2, and S_3 are the optical antipodes of R_1, R_2, and R_3, respectively.

Table I: Purification of *P. glumae* Lipase

purification step	total protein (mg)	total act.[a] (units)	sp act. (units/mg)	yield (%)	purification factor
DEAE-Trisacryl-M–phenyl-Sepharose	1163	500 000	430	100	1
alcohol–acetone precipitation	725	435 000	600	87	1.4
DEAE-cellulose, pH 8.4	110.1	216 000	1962	43	4.6
2nd DEAE-cellulose, pH 8.4	52.3	162 000	3096	32.4	7.2

[a] Standard pH-stat assay at pH 7.0 on the mixed micellar solution of tributyrin and Triton X-100.

in dry chloroform and triethylamine. After silicic acid chromatography using ether–hexane (1/1 v/v) as eluent, the pure *O*-trityl derivative was obtained in a yield of 75%: R_f = 0.4 in ether–hexane (1/1 v/v); $[\alpha]_D^{20}$ = +12.61° (c, 20.3 in $CHCl_3$). The secondary hydroxyl group was converted into the methyl ether using NaH and methyl iodide in dry dimethylformamide. A small sample of the reaction mixture was purified by silicic acid chromatography: R_f = 0.5 in ether–hexane (75/25 v/v); $[\alpha]_D^{20}$ = +13.36° (c, 17 in $CHCl_3$). The bulk of the crude material was detritylated in chloroform using BF_3–MeOH and purified by silicic acid chromatography: yield, calculated on 1-*O*-trityl-3-(decanoylamino)-3-deoxyglycerol, 47%; R_f = 0.36 in $CHCl_3$–MeOH (95/5 v/v); $[\alpha]_D^{20}$ = −7.17° (c, 50 in $CHCl_3$). Finally, pure S_2 was obtained by acylation of the primary hydroxyl group with *n*-decanoyl chloride in dry chloroform and triethylamine: yield 95%, colorless oil; R_f = 0.82 in $CHCl_3$–MeOH (95/5 v/v); $[\alpha]_D^{20}$ = +1.07° (c, 10 in $CHCl_3$) and +6.8° (in substance); ^{1}H NMR ($CDCl_3$–CD_3OD = 1:1) δ 0.90 (6 H, CH_3), 1.25 (24 H, alkyl), 1.60 (4 H, βCH_2), 2.35 (2 H, αCH_2CON and CH_2COO), 3.3 (H, CH), 3.4 (3 H, OCH_3), 3.6 (2 H, CH_2N), 4.00 and 4.25 (2 H, CH_2O).

R_2: This stereoisomer was prepared as described above starting from (*R*)-glycidol. $[\alpha]_D^{20}$ = −0.95° (c, 10 in chloroform) and −6.7° (in substance). Chromatographic and NMR characteristics were identical with those of the S_2 enantiomer.

S_2: Alternative Synthesis Starting from (R)-Glycidyl Tosylate. (*S*)-Benzylglycidol was prepared from (*R*)-glycidyl tosylate as described by Byun and Bittman (1989): yield 87%; R_f = 0.86 in $CHCl_3$–MeOH (95/5 v/v); $[\alpha]_D^{20}$ = +5.4° (c, 5 in C_6H_6). Subsequent reaction of (*S*)-benzyl glycidol with aqueous ammonia (25%), overnight at 4 °C, produced (*R*)-1-benzyl-3-amino-3-deoxyglycerol in quantitative yield. The latter compound was specifically aminoacylated in dry chloroform and triethylamine with use of 0.9 mol of *n*-decanoyl chloride to prevent O-acylation. The reaction product was purified by crystallization from ether–hexane (1/1 v/v) yield (*S*)-1-benzyl-3-*N*-decanoyl-3-deoxyglycerol: mp 50–52 °C; R_f = 0.42 in $CHCl_3$–MeOH (95/5 v/v); yield 41%. The secondary hydroxyl group was converted into the corresponding methyl ether function by reaction with 3 mol of NaH and 3 mol of CH_3I: yield quantitative; R_f = 0.75 in $CHCl_3$–MeOH (95/5 v/v). After removal of the benzyl ether function by catalytic hydrogenolysis (Pd as catalyst, acetic acid as solvent), the reaction product was purified on silicic acid with use of ether–hexane (9/1 v/v) as eluent: yield 95%; R_f = 0.43 in $CHCl_3$–MeOH (95/5 v/v).

Finally, reacylation of the primary group with *n*-decanoyl chloride in dry chloroform and triethylamine and column chromatographic purification on silicic acid pure S_2 was obtained: yield 77%; R_f = 0.24 in ether–hexane (1/1 v/v); $[\alpha]_D^{20}$ = +7.2 (in substance). Chromatographic and NMR characteristics were indistinguishable from those of S_2 prepared as described above by a different pathway.

R_3: (*S*)-Glycidyl tosylate was converted into 1-*O*-tosyl-3-*O*-methyl-glycerol using methanol in dry chloroform as described by Guivisdalsky and Bittman (1989): yield quantitative. The *O*-tosyl function was replaced by an amino group with use of liquid ammonia in a pressure bottle according to Sowden and Fischer (1942): yield, after silicic acid chromatography, 40%; R_f = 0.34 in $CHCl_3$–MeOH–H_2O (65/25/4 v/v); $[\alpha]_D^{20}$ = +7.36° (c, 59 in $CHCl_3$). Simultaneous acylation of the amino group and the secondary alcohol function was performed with *n*-decanoyl chloride in dry chloroform and triethylamine. After silicic acid chromatography using ether–hexane (1/1 v/v) as eluent, pure R_3 was obtained as a colorless wax in a yield of 90%: mp 32.5 °C; R_f = 0.33 in ether–hexane (75/25 v/v); $[\alpha]_D^{20}$ = +2.28° (c, 10 in $CHCl_3$); ^{1}H NMR ($CDCl_3$–CD_3OD = 1/1) δ 0.90 (6 H, CH_3), 1.30 (24 H, alkyl), 1.60 (4 H, βCH_2), 2.25 (2 H, αCH_2CON), 2.35 (2 H, αCH_2COO), 3.35 (3 H, OCH_3), 3.45 (2 H, CH_2N), 3.5 (2 H, CH_2N), 5.05 (1 H, CHO).

S_3: This stereoisomer was prepared as described above starting from (*R*)-glycidol: mp 32.5 °C; $[\alpha]_D^{20}$ = −2.3° (c, 10 in $CHCl_3$). Chromatographic and NMR characteristics were identical with those of the R_3 enantiomer.

Enzymes. Crude lipase from *P. glumae* (PGL; sp act. = 20 units/mg) was obtained from Unilever Research Laboratory, Vlaardingen, and purified as shown in Table I. All purification steps were carried out at 4 °C.

The lipase solution (30 mM Tris-HCl, 200 mM NaCl, pH 7.2) was first passed over a DEAE-Trisacryl-M (IBF-LKB) column. This step removed most of the color while lipase itself does not bind to the column material under these conditions. The eluate was adjusted to pH 8.4 and at a final NaCl concentration of 2 M was loaded onto a phenyl-Sepharose column. The column was rinsed with ten times its total volume 30 mM Tris, 2 M NaCl at pH 8.4. The active lipase eluted from the column when distilled water was applied to it. After dialysis and lyophilization a completely stable crude lipase preparation was obtained. The lipase was dissolved as 1 mg/mL in 5 mM Tris-HCl, 40 mM NaCl at pH 8.0, and an equal volume of ethanol was added stepwise. Precipitated proteins were removed by centrifugation, and all lipase activity was recovered in the supernatant. Upon addition of 4 volumes of acetone the enzyme precipitated. The precipitate was dissolved in 5 mM Tris-HCl, pH 8.4, and subjected to ion-exchange chromatography on a DEAE-cellulose column (20 cm, 45 mL) equilibrated with the same buffer. Lipase eluted when a linear NaCl gradient (0–0.15 M) of 8 bed volumes was applied. After dialysis, DEAE-cellulose chromatography was repeated under the same conditions. From the second column the lipase eluted as a symmetrical peak with constant specific activity in all fractions. Units were determined according the standard bulk assay of Triton X-100–tributylrin stabilized mixed micelles as described below. The purified enzyme was stored as 0.5–1.5 mg/mL samples at −20 °C in a buffer composed of 5 mM Tris-HCl, 10 mM $CaCl_2$, and 50 mM NaCl at pH 7.5. No decrease in specific activity was observed upon storage for three months. SDS–PAGE electrophoresis showed a single protein band with a molecular mass of 32 kDa in perfect agreement with the cDNA derived amino acid sequence (Batenburg et al., 1991).

Porcine pancreatic lipase (PPL) was purified according to Verger et al. (1969), in a form devoid of colipase. Protein concentrations were determined by absorbance using $E_{280nm}^{1\%}$

of 10.0 and 13.0 for PGL and PPL, respectively.

Enzyme Assays. (*1*) *pH-stat Measurements.* Standard activity measurements of the microbial lipase (PGL) were carried out with use of mixed micellar solutions of tributyrin in Triton X-100. The reaction mixture (2.5 mL) consisted of 67 mM tributyrin and 134 mM Triton X-100 in 5 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM NaCl, pH 7.0. The free fatty acid released by hydrolysis of the substrate as determined by a Radiometer titration set (PHM-84 pH meter, a TTT-80 titrator, an ABU-80 autoburet, a TTT-60 titration assembly, and a Rec-80 servograph). Assays were performed under nitrogen with 10 mM sodium hydroxide at 25 °C.

For the longer chain synthetic substrates (see Scheme I), mixed micelles with Triton X-100 (5 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM NaCl, 8 mM substrate, and 200 mM Triton X-100) are effectively hydrolyzed at pH 8 by the microbial lipase. Various amounts of this mixture were used in the assays to vary the substrate concentration. In these experiments the molar fraction of substrate ([substrate]/{[substrate] + [Triton X-100]}) is constant. Initial velocities were measured; for the higher substrate concentrations the curves were linear up to 20% of substrate hydrolyzed. Apparent K_m and V_m values were obtained from these data by nonlinear regression analysis (Simplex method; Nelder et al., 1965). To change the substrate concentration *in* the interface, similar measurements were performed at different molar fractions of substrate (Dennis, 1973).

For the determination of positional preference and stereospecificity of porcine pancreatic lipase gum arabic stabilized suspensions were prepared with a Waring Blender: 5 mM pseudotriglyceride was mixed for 5 min in 5 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM NaCl, 1% gum arabic at pH 8.

(*2*) *Monolayer Measurements.* Before each experiment the Teflon trough was thoroughly washed as follows: First it was washed with tap water and then gently brushed in the presence of distilled alcohol and subsequently with tap water again. Finally, it was completely rinsed with distilled water. Unless quoted elsewhere the aqueous subphase was composed of 10 mM Tris-HCl, 20 mM $CaCl_2$, 100 mM NaCl at pH 8.0. Residual surface active impurities were removed before each experiment by sweeping and suction of the surface.

The monolayer experiments were performed on a KSV 5000 barostat (KSV Instruments, Helsinki) controlled by a Sperry PC/Microl T. For surface pressure/mean molecular area isotherms a rectangular trough with an area of 32250 mm^2 (235 × 150 mm) was used. The "zero-order trough" with two compartments (Verger & De Haas, 1973), a thermostated reaction compartment (165 mL, 97 cm^2), and a 330-mL reservoir compartment connected by a small surface channel was used for kinetic experiments. The surface pressure was determined by a Wilhelmy plate attached to an electromicrobalance connected in turn to a microcomputer controlling the movement of the barrier.

Surface pressure versus mean molecular area isotherms were measured for all synthetic compounds. Stock solutions of individual lipids were prepared in distilled $CHCl_3$ just before use. Fifty microliters of a lipid solution with an accurately known concentration was spread on the aqueous buffer. The monolayer was allowed to stabilize for 5 min before it was compressed with a continuous increase in surface pressure per minute [linear compression of 1 mN/(m·min)]. Every 3 s, data were collected and analyzed with proprietary software from KSV Instruments (Helsinki).

The enzymatic determinations were performed on the "zero-order trough" described above. The reaction compartment thermostated at 23.5 ± 0.5 °C was magnetically stirred. A lipid film was spread from a chloroform solution and covered both compartments. The film was compressed to a predetermined surface pressure with a barrier speed of 15 cm^2/min. The monolayer was allowed to stabilize for several minutes before enzyme was injected under the film of the reaction compartment. Constant surface pressure was maintained by (computer-controlled) compensatory barrier movement. Data were sampled every 10 s. At each surface pressure, three different kinetic measurements were made. Activity was shown to be proportional to the amount of protein added. Kinetic data were analyzed according to a previous model (Verger et al., 1973; Ransac et al., 1990a) and fitted by nonlinear regression analysis (Simplex method; Nelder et al., 1965).

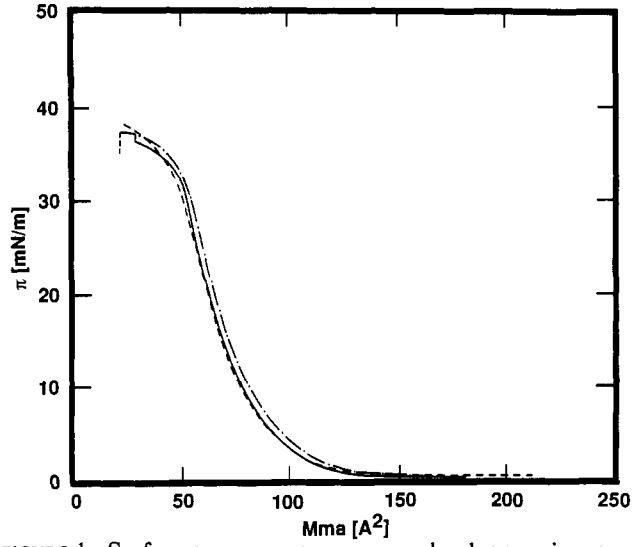


FIGURE 1: Surface pressure versus mean molecular area in monomolecular films of (*R*)-1,2-didecanoyl-2-deoxyamino-3-*O*-methylglycerol (---), (*R*)-1,3-didecanoyl-3-deoxyamino-2-*O*-methylglycerol (–·–), and (*R*)-2,3-didecanoyl-3-deoxyamino-1-*O*-methylglycerol (–). For further details see Materials and Methods.

RESULTS

Monomolecular Film Properties of the Chiral Pseudoglycerides. In Figure 1 the surface pressure/mean molecular area isotherms of the three isomeric pseudotriglycerides (R_1, R_2, and R_3 in Scheme I) are represented. The isotherms of the enantiomeric compounds S_1, S_2, and S_3 exactly coincide with those of R_1, R_2, and R_3, respectively, and indicate a high chemical purity of the synthetic triglyceride analogues. These pseudotriglycerides form stable monomolecular films at the air–water interface up to 32 mN/m. The enzymatically produced reaction products, *n*-decanoic acid and monomethoxy(decanoylamino)deoxyglycerol, are highly water soluble at pH ≥ 6 and diffuse immediately into the aqueous bulk phase. For all three positional isomers a collapse of 36 mN/m was found.

It is evident that the force/area curves of R_1 and R_3 can hardly be differentiated, whereas the more symmetric chemical structure of R_2 results in a slightly larger molecular area. A similar difference has been found for isomeric 1,2- and 1,3-dioleoylglycerol (Dr. R. Demel, personal communication).

Positional Preference and Stereospecificity of Porcine Pancreatic Lipase. Porcine pancreatic lipase (PPL) has been studied extensively. This enzyme has a 1,3 positional specificity and lacks stereospecifity when acting upon chiral triglycerides (Tattrie et al., 1958; Morley et al., 1974). Using triglyceride analogues (containing one alkyl and two acyl functions), Ransac et al. (1990b) confirmed the lack of ste-

Table II: Enzymatic Velocities of Porcine Pancreatic Lipase Measured with the Surface Barostat Technique and the pH-stat Technique for All Six Pseudoglycerides[a]

	surface barostat[b] V at 25 mN/m in 10^{13} molecules/(cm^2·min)	pH-stat[c] V_{max}^{app} (units/mg)
R_1	3.6	13
S_1	2.8	12
R_1/S_1	1.3	1.1
R_2	2.8	7
S_2	2.9	10
R_2/S_2	1	0.7
R_3	0	0
S_3	0	0

[a] Full names and stereoconfigurations are given in Scheme I. For standard monolayer conditions see Materials and Methods. [b] Deviations in the measured activities are within 10% of the presented value. [c] Bulk velocities were obtained from an emulsion that consisted of 5 mM pseudoglyceride in 5 mM Tris-HCl–10 mM $CaCl_2$–50 mM NaCl–1% gum arabic at pH 8 and 25 °C (the emulsion was prepared by 5 min of mixing in a Waring Blender). Standard deviations less than 5% were calculated.

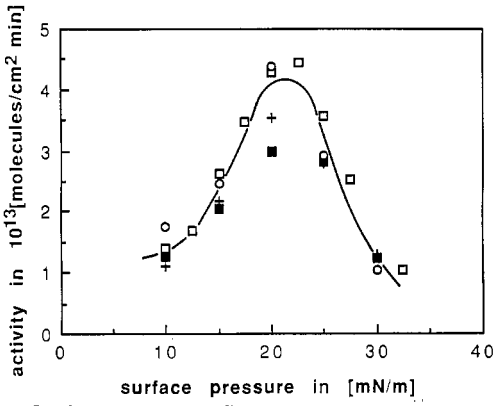


FIGURE 2: Surface pressure profiles of porcine pancreas lipase on the triglyceride analogues (□) R_1, (■) S_1, (○) R_2, and (+) S_2. Final lipase concentration in the reaction compartment was 100 ng/mL. No barrier movement was measured with use of compounds R_3 and S_3 (see Scheme I). For further details see Materials and Methods.

reospecificity of PPL. In contrast, the latter authors reported stereoselectivity for the R-isomer if diglyceride analogues (containing one acyl and one aminoacyl function) were used as substrate. For reasons of comparison our initial experiments with the synthetic triglyceride analogues (Scheme I) were performed with use of porcine pancreatic lipase (PPL). The kinetics were determined by the pH-stat and the monomolecular surface film technique. Several problems were encountered with bulk pH-stat titration. Detergents need to be present in the assay in order to solubilize the longer chain synthetic substrates. The mammalian enzyme, however, is strongly inhibited by Triton X-100. The positional preference and stereospecificity of the pancreatic lipase could not be determined on mixed micelles or a solution of substrate and this detergent. PPL shows only 0.3% activity on mixed micelles consisting of tributyrin and Triton X-100 (compared to the activity measured on pure tributyrin emulsions). Several neutral and positively and negatively charged detergents were tested as well. With these also, nonlinear kinetics were observed. To overcome these problems, gum arabic stabilized suspensions were used. Table II shows the hydrolysis rates of the six isomeric triglyceride analogues by porcine pancreatic lipase. It is clear that PPL displays hardly any stereopreference for the 1 or 3 ester position (couples R_1/S_1 and R_2/S_2). This results supports the conclusion from Ransac et al. (1990b) that this enzyme shows no stereopreference on synthetic triglycerides. Furthermore, PPL is unable to cleave secondary

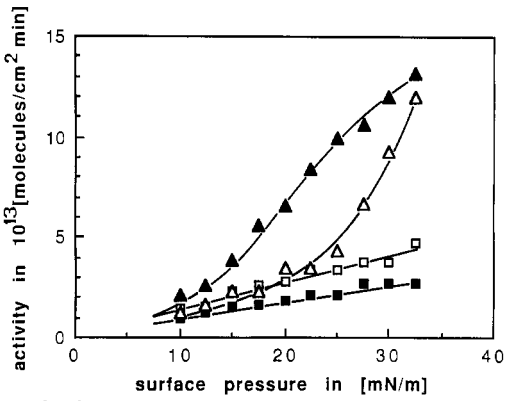


FIGURE 3: Surface pressure profiles of *P. glumae* lipase acting on the triglyceride analogues containing a primary ester bond (for the explanation of the short-hand notation, see Scheme I): (□) R_1, (■) S_1, (▲) R_2, (△) S_2. Final lipase concentration in the reaction compartment was 5 ng/mL. For further details see Materials and Methods.

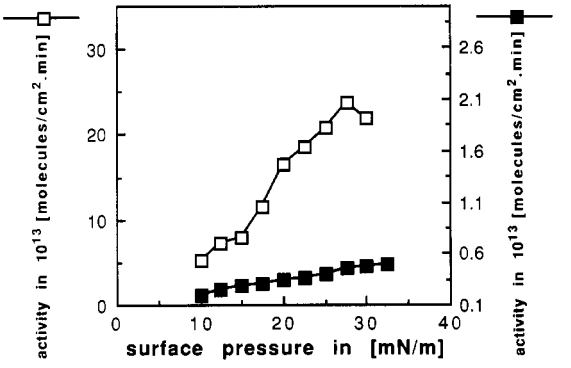


FIGURE 4: Surface pressure profiles of *P. glumae* lipase acting on the enantiomeric triglyceride analogues R_3 and S_3. Final lipase concentration was 5 ng/mL. Note the difference in ordinate scaling. For further details see Materials and Methods.

ester bonds. The activity of the mammalian enzyme toward these triglyceride analogues were also tested by means of the monomolecular surface film technique. In Figure 2 the hydrolysis rate of the four PPL-degradable isomers is shown as a function of the surface pressure. The bell-shaped activity profile with an optimum around 20–23 mN/m is well-known for this enzyme acting on electrically neutral substrate films (Rietsch et al., 1977). In agreement with the results given in Table II, the four isomeric triglyceride analogues are hydrolyzed with similar rates independent of substrate packing density in the film. Despite the fact that extremely low activities can be determined by the monolayer technique, compounds R_3 and S_3 containing a secondary ester function were found to be fully resistant to PPL over the whole surface pressure range.

Positional Preference and Stereospecificity of P. glumae Lipase Determined by the Surface Barostat Technique. For the kinetic characterization of the microbial lipase from *P. glumae* (PGL), the activity–surface pressure profiles of this enzyme on the triglyceride analogues were measured. The results are shown in Figure 3 for the enantiomeric couples R_1/S_1 and R_2/S_2 and in Figure 4 for R_3/S_3. In contrast with the bell-shaped activity profiles of the pancreatic lipase, the activity profiles of the microbial enzyme are continuously increasing with the surface pressure of the substrate film. For all three sets of (stereo)isomers this is observed from 10 to 30 mN/m. In this pressure range the R-conformation is always preferentially hydrolyzed. The hydrolysis rates of the R_1/S_1 and R_2/S_2 substrate couples are rather similar, which indicates that the enzyme shows a weak stereopreference for the hy-

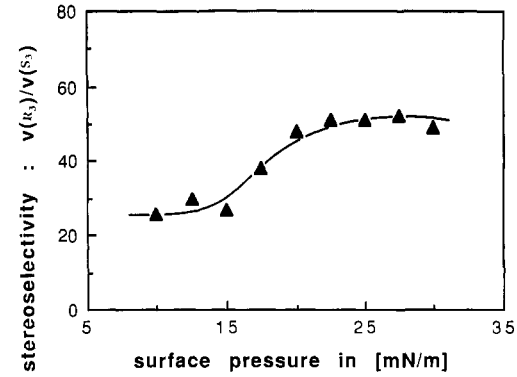


FIGURE 5: Stereoselectivity profile as a function of the surface pressure for *P. glumae* lipase acting on two enantiomeric pseudotriglycerides containing a secondary ester bond. The stereoselectivity index is the ratio between steady-state hydrolysis rates of compound R_3 and S_3 and was calculated from the data presented in Figure 4.

drolysis of a primary ester function. A striking stereoselectivity, however, is observed for the R_3/S_3 couple containing a secondary ester bond. Note the 50-fold difference in the ordinate scaling in Figure 4. The microbial lipase shows a strong stereopreference for the R-isomer. The ratio of the activities presented in Figure 4 is given in Figure 5. The pronounced preference of PGL for the R_3-enantiomer, as compared to its optical antipode, appeared to be surface pressure dependent. Increasing the surface pressure from 10 to 30 mN/m results in an increase in the stereoselectivity index from 25 to 50.

Positional Preference and Stereospecificity of P. glumae Lipase Determined by the pH-stat Technique. The kinetic behavior of the microbial lipase was also studied on Triton X-100 stabilized micelles of the six (isomeric) pseudoglycerides. As proposed by Verger and de Haas (1976), two steps are believed to be important for the action of lipolytic enzymes at interfaces. The first step is binding to the interface. This is controlled by the total amount of micelles present in the assay. By increasing this micellar concentration, all enzyme can be directed to the interface. The second step is binding of a substrate monomer to the active site of the enzyme. This step is regulated by the interfacial substrate concentration (expressed in molar fraction of substrate). Due to the stability of the mixed micelles a limited range between 0.005 and 0.04 molar fraction of substrate was investigated. The maximal activity determined on all isomers was found to be directly proportional to the mole fraction of substrate (data not shown). This indicates that the affinity for the substrate, in this range of interfacial substrate concentrations, is very low and can not be quantified. The apparent K_m values of R_3 and S_3 as a function of the interfacial substrate concentration need some comment. The apparent K_m for isomer S_3 is constant over the mole fraction range from 0.005 to 0.04 whereas for isomer R_3 the K_m linearly increased 4-fold in this concentration range. For the compounds containing a primary hydrolyzable ester bond (R_1, S_1, R_2, and S_2) the apparent K_m was not evidently affected by a change in the interfacial substrate concentration. In Table III apparent affinity constants and apparent catalytic turnover rates at 0.04 mole fraction of substrate in Triton X-100 are compiled. For reason of comparison the relative velocities of the enzyme acting on the monomolecular surface layers of the pure substrate at a surface pressure of 25 mN/m are inserted. It can be seen that at the maximal interfacial substrate concentration a 9-fold difference in K_m^{app} is reached between the two optical antipodes of the triglyceride containing a secondary ester bond. Similar results were found if Triton X-100 was replaced by a zwitterionic detergent (SB-16). The kinetic parameters obtained with this detergent are inserted in Table III between brackets. Again a 10-fold difference in apparent K_m is observed at a mole fraction of 0.025 substrate. In nonionic and zwitterionic detergents the same kinetic phenomena, in particular the remarkable direct correlation between k_{cat}^{app} and K_m^{app}, are observed.

Table III: Enzymatic Velocities of *P. glumae* Lipase Measured with the Surface Barostat Technique and the pH-stat Technique for All Six Pseudotriglycerides[a]

	surface barostat[b]	pH-stat[c]	
	V at 25 mN/m in 10^{13} molecules/(cm^2·min)	K_m^{app} (mM)	k_{cat}^{app} (s^{-1})
R_1	3.4	0.3	40
S_1	2.2	0.4	16
R_1/S_1	1.5	0.8	2.5
R_2	10	0.3	44
S_2	4.4	0.3	90
R_2/S_2	2.3	1	0.5
R_3	21	0.9 (0.7)[d]	405 (190)[d]
S_3	0.41	0.1 (0.07)[d]	11 (4)[d]
R_3/S_3	51	9 (10)	38 (48)

[a] Full names and stereoconfigurations are depicted in Scheme I. For standard monolayer conditions see Materials and Methods. [b] Activities were measured under standard conditions with an experimental error within 10%. [c] The apparent V_{max} and K_m were obtained by variation of the micelle concentrations (for further experimental detail see Material and Methods). [d] Activities were measured in mixed micelles with SB-16 at a molar fraction of 0.025 substrate.

To what extent can we compare the results obtained by the two techniques presented in Table III? By comparing, we directly observe the puzzling different behavior of the enzyme acting on the R_2/S_2 couple: in pure substrate films the R_2 isomer is preferred whereas in mixed micelles the reverse is found. Although NMR, $[\alpha]_D^{20}$, and chromatographic behavior of R_2 and S_2 gave no indications of a possible contaminant or error in the chemical synthesis, we decided to resynthesize S_2 by a different route (see Materials and Methods). The hydrolysis kinetics, measured by surface barostat and pH-stat technique, of this newly synthesized S_2 compound fully confirmed the results given in Table III. For the enantiomeric couple R_1/S_1 the pH-state results are in perfect agreement with those obtained by the surface barostat technique: the enzyme possesses a slight preference for the R-isomer. The same accounts for the R_3/S_3 couple, containing a single hydrolyzable secondary ester function. The stereopreference of the enzyme for the R_3-isomer is observed again in mixed micelles of substrate and Triton X-100. Taking all isomers into consideration, the highest activity rates in the surface barostat and pH-stat were detected on R_3.

Discussion

The determination of the positional and stereospecific preference of lipases acting on triglycerides is subject to several problems. Enantiomeric triglycerides containing three different acyl groups are in principle ideal substrates to investigate the properties of lipases. However, it remains a problem that the hydrolysis products of these compounds are substrates as well. In addition to this, lipases might exert a certain degree of fatty acid specificity. In this study we wanted to investigate whether synthetic triglyceride analogues containing only one single hydrolyzable acyl ester bond might be useful to rapidly screen positional specificity of various lipases without problems of acyl migration. Chirally pure enantiomeric substrates were prepared to analyze simultaneously the stereospecific properties of these enzymes.

The enantiomeric pseudoglycerides were used to investigate the kinetic properties of porcine pancreatic and *P. glumae*

lipase. From Table II it can be seen that similar breakdown rates are found for these positional and stereoisomeric pseudotriglycerides containing a primary ester function. This indicates that PPL lacks stereoselectivity in the hydrolysis of the enantiomeric R_1/S_1 and R_2/S_2 couples. In contrast, the substrate analogues R_3 and S_3 having a single secondary ester group are not substrates for the pancreatic enzyme. The lack of activity of PPL on secondary ester bonds has been reported for a variety of substrates (Brockerhoff & Jensen, 1974; Leger & Charles, 1980).

The microbial lipase PGL differs from PPL in several aspects. The activity–surface pressure profiles are clearly distinct, and in general *P. glumae* lipase is at least 20 times more active in the monolayer (higher activity rates are detected upon addition of the same amount of enzyme). As shown in Table III, the stereoselectivity of PGL acting on pseudoglycerides with a primary ester function (R_1/S_1 and R_2/S_2) was modest, as was also demonstrated for PPL. The position of the acyl chains (being adjacent in the case of R_1/S_1 or separated in R_2/S_2) does not seem to have a large effect on the hydrolysis rates. Striking, however, is the behavior of PGL acting on R_3 and S_3. PPL is unable to cleave either one of these stereoisomers. The microbial enzyme, however, strongly discriminates between these two stereoisomers in both assays. With the hydrolysis of a secondary ester PGL favors the R-configuration, which is 40–50 times more rapidly hydrolyzed than its optical antipode. The much higher stereoselectivity of PGL acting on the secondary ester containing enantiomers R_3/S_3, as compared to the couples R_1/S_1 and R_2/S_2, can be explained by the direct attachment of the secondary ester group to the chiral center. A similar explanation has been reported (Horrevoets et al., 1989) for the substrate specificity of *Escherichia coli* outer membrane phospholipase A (=OM PLA). This enzyme is a rather aspecific acyl-hydrolase, which has a preference for the primary ester bond of phospholipids. OM PLA is aspecific in its phospholipase A_1 activity, but is absolutely stereoselective in its phospholipase A_2 activity.

From Figure 5 it is clear that the stereoselectivity of *glumae* lipase is dependent on the surface pressure, i.e., the packing density of the substrate molecules in the monolayer. Such an observation has been reported recently for other lipases as well. Ransac et al. (1990b), using enantiomeric diglyceride analogues as substrate, showed that the stereoselectivity of human gastric lipase increased by *decreasing* the packing density of the substrate film. Such modulations of stereoselectivity with surface pressure are hard to explain when the amount of enzyme acting in the film is unknown. In theory this difficulty can be overcome by the pH-stat technique: upon addition of increasing amounts of mixed micelles of substrate and Triton X-100 (at a fixed ratio = 1:25), all enzyme can be directed to the micellar interface ($E \rightarrow E^*$). However, even when all enzyme is bound to the interface as E*, the measured enzymatic velocity is not V_{max} because the interfacial binding constant is not known. If maximal velocities are determined as a function of the two-dimensional substrate concentration [S], it is possible to get at least an idea of the magnitude of K_m^*. For mixed micellar systems composed of substrate with Triton X-100 a perfectly linear relationships over an 8-fold increasing substrate concentration was found. From this we can conclude that $K_m^* \gg [S]$.

An interesting observation is the fact that an increase in the interfacial substrate concentration of R_3 resulted in a simultaneous increase of the apparent binding constant. This behavior cannot be explained by the model of Verger and de Haas (1976). Therefore, to interpret the kinetic behavior of

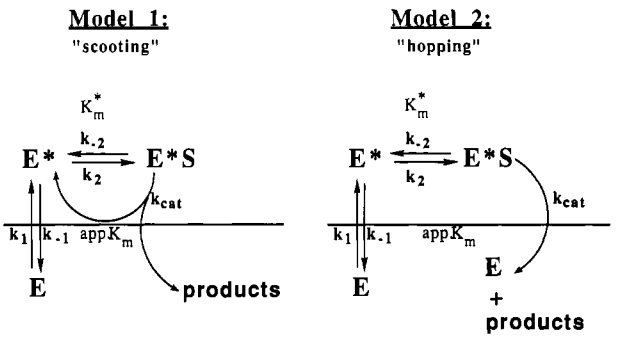


FIGURE 6: Two different models for the action of lipolytic enzymes at interfaces. Model 1 is the scooting mode of catalysis, and model 2 describes the pure hopping mode of catalysis. The following symbols have been used: E_0, total enzyme concentration in (molecules/volume); E, bulk enzyme concentration (molecules/volume); E^*, penetrated enzyme concentration (molecules/surface); E^*S, penetrated enzyme–substrate complex concentration (molecules/surface); k_{-1}, desorption rate constant for E* to E (time)$^{-1}$; k_1, penetration rate constant for E to E* (surface/volume)$^{-1}$·(time)$^{-1}$; k_{-2}, rate constant for the dissociation of E*S into E* + S (time)$^{-1}$; k_2, rate constant for the complex formation E*S (molecules/surface)$^{-1}$·(time)$^{-1}$; k_{cat}^{app}, experimentally determined catalytic rate constant (time)$^{-1}$; k_{cat}, catalytic rate constant when $K_m^* \ll [S]$ (time)$^{-1}$; K_m^{app}, experimentally determined K_m (surface/volume); K_m^*, interfacial Michaelis–Menten constant (molecules/surface); K_m, dissociation constant for the enzyme-mixed micellar complex (surface/volume); I, total interfacial area (surface); S, two-dimensional surface concentration of substrate (molecules/surface); v, enzymatic activity in (molecules·volume^{-1}·time^{-1}); v_{max}^{app}, experimentally determined activity at which nearly all enzyme is absorbed at the interface (molecules·volume^{-1}·time^{-1}); V, total volume (volume).

P. glumae lipase, two different models [i.e, "hopping and scooting", as initially proposed by Upreti and Jain (1980)] for the interfacial catalysis were taken into consideration. Both support the generally accepted postulation that there are two important steps in lipolysis: binding of the enzyme to the interface ($E \rightarrow E^*$) followed by binding of a single substrate molecule (E*S) and decomposition into its products. This overall mechanism offers two extreme modes for interfacial catalysis: scooting and hopping, which are represented as separate models in Figure 6. The difference between the two modes lies in the pathway through which E* is recycled. We will first shortly discuss both models.

Model 1: Scooting. In this model the first step is binding to the interface. Many catalytic turnover cycles takes place with the liberation of reaction products before the enzyme leaves the interface. In the extreme situation it remains bound to the interface until all substrate has been depleted (the enzyme remains in the so-called scooting mode). A more detailed description and quantitative interpretation (see Appendix) of this previously proposed model can be found in Verger et al. (1973), Verger and de Haas, (1976) and Jain and Berg (1989).

Model 2: Hopping. Obviously, again binding to the interface is the first step in lipolysis. In this case, however, surface binding is weak. Only a small amount of the substrate (if any) will be hydrolyzed in each binding event. In the extreme situation desorption of bound enzyme is supposed to take place after each catalytic turnover cycle. Quantitative predictions and underlying boundary conditions of this model are also presented in the Appendix.

The experimental hydrolysis rates of both kinetic models under the conditions $K_m^* \gg [S]$ can be described by the simplified equation where

$$v = \frac{k_{cat}^{app} E_0}{1 + K_m^{app}/(I/V)}$$

$$k_{cat}^{app} = k_{cat}\frac{k_2 S}{k_{-2} + k_{cat}}$$

From the mathematical treatment (see Appendix) it follows that discrimination between the two models is possible on the basis of analysis of the K_m^{app} values. In the scooting model the binding step is not a part of the catalytic cycle and hence K_m^{app} is only dependent on the on rate (k_1) and the off rate (k_{-1}):

$$K_m^{app}(\text{scooting}) = \frac{k_{-1}}{k_1}$$

An important theoretical aspect of kinetic model 2 is the prediction that not only the rate of equilibration of the enzyme over bulk and interface but also catalytic turnover rates influence the value of K_m^{app}:

$$K_m^{app}(\text{hopping}) = \frac{k_{-1} + k_{cat}^{app}}{k_1}$$

Only when all equilibria are rapid relative to k_{cat}, enzyme distribution between bulk and interface will not be affected by the rate of hydrolysis. However, when k_{cat}^{app} is large compared to the off rate k_{-1}, K_m^{app} will be dependent on k_{cat}^{app}. Indeed, we observed, by increasing the mole fraction from 0.005 to 0.04, a linear increase for the k_{cat}^{app} as well as for the K_m^{app}. The 10-fold difference in K_m^{app} (Table III) for the R_3 and S_3 enantiomers in fact also illustrates this effect of k_{cat}^{app} on K_m. If the enzyme were acting on the scooting mode only, no such effect would have been expected. The fact that for the R_3 and S_3 enantiomers an effect of k_{cat}^{app} on K_m is observed means that with these substrates the enzyme leaves the interface after a limited number of catalytic cycles. Although kinetics can never prove a mechanism, it can disprove one. The observed contribution of k_{cat}^{app} to K_m^{app} cannot be explained in the light of the scooting model, but the quantitative interpretation of the hopping model agrees very well with the kinetics obtained with *P. glumae* lipase. A similar conclusion was reached by Bengtsson and Olivecrona (1983) to explain the kinetic behavior of lipoprotein lipase. The kinetic behavior of another lipolytic enzyme, porcine pancreatic phospholipase A_2, is probably best explained by the scooting model. Pattus et al. (1979) showed that activity initially detected in a monomolecular film of substrate was almost completely recovered after the film transfer. Jain et al. (1989) demonstrated the extreme high apparent affinity of porcine phospholipase A_2 for anionic interfaces. The authors calculated that phospholipase A_2 did not leave the interface before all substrate in the outer layer of their vesicle was hydrolyzed.

In conclusion, the synthetic pseudotriglycerides used in this study provide a convenient way to rapidly characterize lipases as has been demonstrated with PPL and PGL. The lipase from *P. glumae* is a potent enzyme capable of hydrolyzing both primary and secondary ester bonds. Our kinetic data suggest that lipolytic enzymes can efficiently hydrolyze lipids via hopping model. The highest activity and stereopreference was found for the R-enantiomer of the pseudotriglyceride containing a secondary ester. This may be of potential interest for enzymatic synthetic purposes. With the hydrolysis of this latter isomer it was shown that the microbial lipase leaves the interface after a limited number of catalytic events.

Acknowledgments

We thank Dr. N. Dekker for the NMR analyses of our products. We are grateful to Dr. R. Verger for this fruitful discussions.

Appendix

Kinetic Treatment of Model 1: Scooting. When binding of the protein to the interface is energetically favored, the enzyme remains in the interface during many catalytic turnover cycles. A detailed description and quantitative interpretation of this model are previously described (Verger et al., 1973; Verger & de Haas, 1976; Jain & Berg, 1989). The steady-state resolution is obtained from the following enzyme fluxes:

$$\frac{dE}{dt} = -k_1\frac{I}{V}E + k_{-1}\frac{I}{V}E^* \quad (1)$$

$$\frac{dE^*}{dt} = k_1\frac{I}{V}E\frac{V}{I} - (k_{-1} + k_2 S)E^* + (k_{-2} + k_{cat})E^*S \quad (2)$$

$$\frac{dE^*S}{dt} = (k_2 S)E^* - (k_{-2} + k_{cat})E^*S \quad (3)$$

and the conversion of enzyme mass, given by

$$E_0 = E + (E^* + E^*S)\frac{I}{V} \quad (4)$$

By combining them, we thus obtain

$$v_1 = \frac{k_{cat}E_0}{1 + \frac{k_{-2} + k_{cat}}{k_2 S}\left(\frac{k_{-1}}{k_1(I/V)} + 1\right)} \quad (5)$$

Equation 5 can be rearranged into

$$v_1 = \frac{k_{cat}E_0\frac{k_2 S}{k_{-2} + k_{cat}}}{1 + \frac{k_{-1}}{k_1(I/V)} + \frac{k_2 S}{k_{-2} + k_{cat}}}$$

If we define k_{cat}^{app} as

$$k_{cat}^{app} = k_{cat}\frac{k_s S}{k_{-2} + k_{cat}} = k_{cat}\frac{S}{K_m^*}$$

and simplify the equation for the extreme situation where the interfacial substrate concentration is far below the interfacial Michaelis–Menten constant (K_m^*), we obtain

$$v_1 = \frac{k_{cat}^{app}E_0}{1 + \frac{k_{-1}}{k_1(I/V)}} = \frac{v_{max}^{app}}{1 + \frac{K_m^{app}}{(I/V)}} \quad (6)$$

where

$$K_m^{app}(\text{scooting}) = \frac{k_{-1}}{k_1} \quad (6)$$

From eq 7 it is obvious that in scooting model 1 the experimentally determined K_m^{app} is not affected by k_{cat} if $[S] \ll K_m^*$.

Kinetic Treatment of Model 2: Hopping. If binding and desorption of bound enzyme take place in *every* catalytic turnover cycle, a quantitative interpretation can be obtained (in analogy to model 1) from the following fluxes of all enzyme species:

$$\frac{dE}{dt} = -k_1\frac{I}{V}E + k_{-1}E^*\frac{I}{V} + (k_{cat}E^*)S\frac{I}{V} \quad (8)$$

$$\frac{dE^*}{dt} = k_1\frac{I}{V}E\frac{V}{I} - (k_{-1} + k_2 S)E^* + (k_{-2}E^*)S \quad (9)$$

$$\frac{dE^*S}{dt} = (k_2 S)E^* - (k_{-2} + k_{cat})E^*S \quad (10)$$

The conservation of enzyme mass can be expressed by

$$E_0 = E + (E^* + E^*S)\frac{I}{V} \tag{11}$$

Under steady-state conditions the following general formula can be derived (from the enzyme fluxes and enzyme mass conservation):

$$v_2 = \frac{k_{cat}E_0\dfrac{k_2S}{k_{-2}+k_{cat}}}{1 + \dfrac{k_{-1}}{k_1(I/V)} + \dfrac{k_2S}{k_{-2}+k_{cat}}\left(1 + \dfrac{k_{cat}}{k_1(I/V)}\right)} \tag{12}$$

This can be simplified again by use of

$$k_{cat}^{app} = k_{cat}\frac{k_2S}{k_{-2}+k_{cat}}$$

which gives

$$v_2 = \frac{k_{cat}^{app}E_0}{1 + \dfrac{k_{-1}}{k_1(I/V)} + \dfrac{k_{cat}^{app}}{k_1(I/V)} + \dfrac{k_2S}{k_{-2}+k_{cat}}} \tag{13}$$

In all experiments the enzyme activity at saturation is directly proportional to the molar fraction of substrate in the mixed micelles (vide infra). With use of the assumption that [S] $\ll$ K_m^*, eq 13 can be rewritten as

$$v_2 = \frac{k_{cat}^{app}E_0}{\left(1 + \dfrac{k_{-1}+k_{cat}^{app}}{k_1(I/V)}\right) + \dfrac{k_2S}{k_{-2}+k_{cat}}} \tag{14}$$

Since

$$\frac{k_2S}{k_{-2}+k_{cat}} \ll 1$$

we can deduce the simplified expression

$$v_2 = \frac{k_{cat}^{app}E_0}{1 + \dfrac{K_m^{app}}{I/V}} = \frac{v_{max}^{app}}{1 + \dfrac{K_m^{app}}{I/V}} \tag{15}$$

where

$$K_m^{app}(\text{hopping}) = \frac{k_{-1}+k_{cat}^{app}}{k_1} \tag{16}$$

It can be seen from eqs 12 and 16 that only if $k_{cat}S/K_m^*$ is negligible compared to k_{-1} then the K_m^{app}(hopping) will approach K_m^{app}(scooting).

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