Evaluation of Lipase Selectivity for Hydrolysis[1]

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The positional selectivity of several commercial lipases was reevaluated from the point of view of synthetic utility. A 1,2-diglyceride was synthesized, and exposed to typical conditions of lipase-catalyzed hydrolysis (without the enzyme). Little, or no, acyl migration was observed. The recovery of oleic acid from several lipase-catalyzed hydrolyses of 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl glycerol (POS) reported here, therefore, must be regarded as due to the presence of the lipases themselves. This could limit the use of such catalysts in schemes requiring high selectivity for the primary positions of triglycerides. Fatty acid selectivity data of the enzymes studied also are presented.

KEY WORDS: Acyl migration; 1,2-diglycerides; lipase; positional selectivity.

Prospective applications of lipases (triacylglycerol hydrolases, 3.1.1.3) in the fats and oils industries include transformations that depend critically on the positional reactivity of the lipase. For example, a recent review points out that pure 2-monoglycerides may be synthesized by employing a lipase that is specific for the hydrolysis of the primary ester groups of triglycerides (1). In addition, 1,3-regioselective interesterifications allow the formation of fats and oils that have altered triglyceride compositions, but in which the fatty acid substituent in the 2-position has remained unchanged. Benefits are foreseen for improved nutritional value by retaining unsaturated fatty acids in that position, or by improvement in other physical properties of the material if the acids introduced into the primary positions are saturated, thereby producing a more hardened fat. Several patents exist that are based on 1,3-specificity of certain lipases. For example, cocoa butter substitutes can be prepared from palm oil fractions by incorporating palmitic (2) or stearic acid (3) into the 1- and 3-positions of the natural triglycerides by using selected lipases. Also, diglycerides can be obtained by the glycerolysis of oils in the presence of 1,3-positionally specific lipases (4), and a hardened butter has been prepared from corn salad oil by 1,3-interesterification with stearic acid and lipase (5).

A related phenomenon is that of fatty acid selectivity. A notable example is the preference for the hydrolysis of fatty acid chains containing a *cis*-9 double bond. Lipases with such properties occur in the genus *Geotrichum,* and the most thoroughly documented of these is that of *G. candidum* (6). An application of this selectivity has been patented in which tallow was lipolytically cleaved with this enzyme to produce an acid fraction slightly enriched in oleic acid and lower in polyenoic acid content than tallow itself (7).

Excellent reviews are available that discuss the selectivities of lipases and describe methods for their assessment (8–10). In general, the techniques for determining positional selectivity consist of partial hydrolysis of synthetic triglycerides, separation by preparative thin-layer chromatography, extraction and weighing and/or conversion to methyl esters for quantitation by gas-liquid chromatography. The structures of synthetic triglycerides are often confirmed by lipase-catalyzed hydrolysis by means of the "known" selectivity of the lipase (11). Such enzymatic digestion has to be brief (≤5 min) or acyl residues from the 2-position migrate to a free primary hydroxyl group and are then hydrolyzed. The situation for determining lipase positional selectivity, at least, was labor-intensive and not without frustrating ambiguities. As long as a brief enzymatic exposure can be maintained, lipases that are relatively selective for the primary position can serve as tools for analysis (12), but greater selectivity may be required for a synthetic procedure. In reviewing the potential of enzyme technology for lipid industries, Yamane (13) expressed the view that lipases probably run the gamut of selectivity from positionally nonspecific to highly 1,3-specific. He also noted the complexity created by nonenzymatic acyl migration. Apparently improved methods for determining both positional and fatty acid preferences of lipases would be beneficial, and that augmenting those selectivities would make patentable processes more efficient. We report here information that bears on the topic of nonenzymatic acyl migration, and the positional and fatty acid selectivities of certain commercially available lipases when the degree of conversion in hydrolysis is permitted to exceed the low values normally practiced in analytical procedures.

EXPERIMENTAL

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Jeol JNM-GX 400 FTNMR spectrometer, deuteriochloroform as the solvent, and tetramethylsilane as the internal standard. Infrared spectra were obtained on a Perkin-Elmer Model 1310 spectrophotometer by using 3% solutions in CCl_4. Gas-liquid chromatography (GLC) was performed with a Shimadzu GC-Mini 2 instrument with flame ionization detector and a (nonpolar) SPB-1 capillary column (0.25 mm × 30 m) with a 50:1 split ratio and He carrier gas at temperatures indicated below. A Hewlett-Packard 3390A integrating recorder was used for area determinations. High-performance liquid chromatography (HPLC) was accomplished with a SpectraPhysics Model 8800 ternary pump, a S.-P8480XR ultraviolet (UV) detector, a Supelco 5μ silica gel column (4.6 mm i.d. × 25 cm), and a corresponding 10 mm i.d. preparative column. Thin-layer chromatography was done with 0.25 mm silica gel 60 plates supplied by Merck (Montreal, Canada) and bromothymol blue spray was used for visualization. Titrations were conducted with a Radiometer AGU 80 Autoburette. A Heat Systems Ultrasonic Inc. W-220 unit was employed to sonicate mixtures for 5–10 sec at 25 W.

Solvents were reagent grade or better, and all reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used directly. Octanoate esters of 1- and 2-octanol (14), and butyl palmitate and oleate (15) were

[1] Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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prepared by standard procedures. The specific triglyceride was obtained from Dr. R. G. Jensen, University of Connecticut, and its synthesis has been reported (11). Enzymes employed were (supplier, trade name, identity, specific activity on olive oil in μmol free fatty acids (FFA), min^{-1}, mg^{-1}): Enzeco (New York, NY), R, *Candida cylindracea*, 9.45; Amano Co. (Troy, VA), GC-20, *Geotrichum candidum*, 6.2; Sigma Chemical Co. (St. Louis, MO), Type II, porcine pancreatic lipase, 2.0; Tanabe (Tokyo, Japan), Food Grade, *Rhizopus delemar*, 3.85; Miles (Naperville, IL), Fine Grade, *R. delemar*, 140; Gist-Brocades (Charlotte, NC), Lipase S, *Rhizomucor miehei*, 21.3; Amano Co., AP-12, *Aspergillus niger*, 120 (label activity).

Synthetic reactions. Synthesis of 1-stearoyl-3-t-butyldimethylsilyl-glycerol, **2**. This compound was prepared as described (15). *Synthesis of 1-stearoyl-2-(4'-phenyl)butyroyl-3-t-butyldimethylsilylglycerol*, **3**. The silyl ether **2** (2.0 g, 4.35 mmol) was dissolved in 20 mL of dry pyridine. The solution was cooled in an ice bath, and 4-phenylbutyroyl chloride (1.7 g, 9.2 mmol) was added directly thereto. The resulting mixture was stirred overnight at 25°C. Methanol (8 mL) was added and, after stirring another hour, the mixture was diluted with 100 mL of hexane and extracted with 3 × 100 mL of H_2O. The organic phase was dried (Na_2SO_4) and concentrated. The product was purified by passage through 30 g of silica gel (60–200 mesh) eluting with 10% ethyl acetate-hexane to give 2.17 g of **3**: thin-layer chromatography (TLC) (25% ethyl acetate-hexane) R_f 0.67; infrared (IR): 3080,3060,3010,1740 cm^{-1}; ^1H NMR δ 7.11, 7.21 (*m*, 5H, C_6H_5), 5.1 (*m*, 1H, COOCH), 4.38 (*m*, 2H, $COOCH_2$), 3.75 (*m*, 2H, $SiOCH_2$), 2.69 (*m*, 2H, $C_6H_5CH_2$), 2.37 (*m*, 4H, $COCH_2$), 2.00 (*m*, 2H, CH_2), 1.62 (*m*, 2H, CH_2), 1.3 (*m*, ~28H), 0.91 (*m*, 12H, CH_3, $SiC(CH_3)_3$), 0.08 (*s*, 6H, $SiCH_3$); mass spectrum m/e (relative abundance), 562 (27, M-*t*-butyl), 342 (36), 221 (100), 171 (26), 147 (92), 131 (33), 129 (30), 117 (26).

Desilylation of **3** *to prepare 1-stearoyl-2-(4'-phenyl)-butyroyl glycerol*, **1**. The desilylation procedure employed is analogous to that reported (16,17). Compound **3** (2.08 g, 3.45 mmol) was dissolved in tetrahydrofuran (THF) (5 mL), dimethylsulfoxide (DMSO) (45 mL), and H_2O (5 mL) to which was added a few crystals of hydroquinone. The flask was protected from light with aluminum foil, and then *N*-bromosuccinimide (2.88 g, 16.2 mmol) was added. The mixture was stirred overnight at room temperature. Aqueous $Na_2S_2O_3$ (100 mL of 1%) was added, and the resulting mixture was extracted with 3 × 100 mL of hexane. The organic phase was washed with H_2O twice, dried (Na_2SO_4) and concentrated. TLC indicated incomplete reaction, and the procedure was therefore repeated. Although still incomplete, the reaction product seemed free of the 1,3-isomer of **1** (TLC: 4% acetone-$CHCl_3$). The product was chromatographed on 40 g of silica gel with ether-hexane; the 1,2-diglyceride, **1**, eluted with 100% ether and contained ~15% of the 1,3-isomer. Preparative HPLC (2% i-propanol-hexane at 6.0 mL/min) gave pure **1**: R_t 17.6 min; IR 3,600b, 3030,1740 cm^{-1}; ^1H NMR δ 7.11, 7.21 (*m*, 5H, C_6H_5), 5.01 (*q*, 1H, COOCH), 4.20 (*d* of *d*, 2H, $COOCH_2$), 3.64 (*d*, 2H, CH_2OH), 2.59 (*t*, 2H, $C_6H_5CH_2$), 2.27 (*m*, 4H, $COCH_2$), 1.90 (*m*, 2H), 1.54 (*m*, 2H), 1.18 (*m*, *ca.* 28H), 0.81 (*t*, 3H, CH_3); ^{13}C NMR key signals: δ 173.77, 173.02, 141.2, 128.44, 128.38, 126.03, 72.19, 61.97, 61.43; mass spectrum: 505.6 (M^+, 0.6), 487 (M−18, 21), 396 (21), 383 (27), 343 (20), 342 (87), 313 (11), 267 (38), 262 (13), 239 (12), 222 (15), 221 (100), 220 (80), 207 (31).

The corresponding 1,3-diglyceride, **4**, was prepared: mp 45–58°C; IR: the same as **3**; ^1H NMR: δ 7.11, 7.21 (*m*, 5H, C_6H_5), 4.1 (*m*, 5H, CHO, CH_2O), 2.59 (*t*, 2H, $C_6H_5CH_2$), 2.29 (*m*, 4H, $COCH_2$) 1.90 (*m*, 2H), 1.55 (*m*, 2H), 1.18 (*m*, ~28H), 0.81 (*t*, 3H, CH_3); ^{13}C NMR key signals: 173.89, 141.16, 128.44, 128.38, 126.03, 68.32, 65.05, 64.98; mass spectrum: essentially the same as **3**.

Lipase-catalyzed hydrolyses of octanoate esters. Equimolar mixtures of the octanoate esters of 1- and 2-octanol (**14**) (0.64 g, 2.5 mmol) were sonicated briefly in 5 mL of 0.05 M phosphate buffer at pH 7.0 containing a few drops of 10% gum arabic and 0.10 g of commercial enzyme powder. Reaction mixtures were stirred magnetically at 30°C for the indicated periods of time (Table 1). They were then diluted with 50 mL of ethanol and titrated to pH 9.65 with 0.100 N NaOH. Titrations were corrected for blanks to obtain the fraction of esters converted to free acid. The mixtures were transferred to separatory funnels with 100 mL of H_2O, and the organics were extracted with 3 × 50 mL of hexane. The hexane extract was washed twice with H_2O, dried ($MgSO_4$), and concentrated for analysis by GLC: SPB-1 column at 220°C; the retention time of 2-octanol octanoate was 8.0 min, and for the primary ester, 10.0 min.

Lipase-catalyzed hydrolyses of 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl-glycerol, POS. Each sample was prepared from POS (11) (287 mg, 1 meq of potential FFA), 5 mL of 0.05 M phosphate buffer at pH 7.0, 5 droplets of 10% gum arabic, and 0.10 g of commercial lipase powder. After brief sonication, the mixture was stirred magnetically at 30°C for the indicated time period (Table 2). Periodically the vials were swirled by hand to resuspend materials deposited on the walls. The mixture was then titrated for FFA to determine the degree of conversion as described above.

Extraction and separation of acids from neutrals was as follows: the titrated mixture was transferred to a separatory funnel with 50 mL of 2N HCl and extracted with 3 × 25 mL of hexane. Emulsions occurred occasionally that could be broken with sodium sulfate. The combined organic phase was washed with 25 mL of H_2O, dried ($MgSO_4$), and concentrated. The oily residue was transesterified by means of NaOMe (0.3 g, excess) in 6 mL of methanol at 25°C for 1 hr. The fatty acids are thereby converted to sodium salts. Water (15 mL) was added, and the methyl esters were extracted with 3 × 20 mL of ether

TABLE 1

Lipase-Catalyzed Hydrolysis of 1- and 2-Octanol Octanoates at pH 7

Lipase	t(hr)	C[a]	Ratio (2:1)[b]
C. cylindracea	1.0	0.174	0.511:0.489
G. candidum	2.0	0.206	0.520:0.480
	2.0	0.215	0.500:0.500
R. delemar	1.0	0.192	0.517:0.483
	1.0	0.194	0.528:0.472

[a]Mole fraction of total esters converted to FFA.

[b]Ratio of recovered esters with 2-octanol ester listed first.

TABLE 2

Lipase-Catalyzed Hydrolysis of POS[a] at pH 7.0

Lipase	C[b]	Unreacted[c]			Reacted[d]		
		P	O	S	P	O	S
A. niger	0.502	0.250	0.481	0.269	0.424	0.152	0.424
	0.614	0.220	0.567	0.213	0.419	0.141	0.429
C. cylindracea	0.862	0.153	0.774	0.073	0.406	0.270	0.325
G. candidum	0.500	0.324	0.285	0.373	0.391	0.337	0.273
	0.779	0.187	0.369	0.444	0.367	0.320	0.313
Porcine pancreas	0.290	0.269	0.482	0.249	0.446	0.115	0.439
	0.386	0.272	0.484	0.244	0.433	0.124	0.443
R. delemar[e]	0.511	0.214	0.604	0.182	0.447	0.035	0.518
	0.643	0.118	0.804	0.905	0.436	0.073	0.491
R. delemar[f]	0.919	0.130	0.774	0.096	0.337	0.299	0.364
R. miehei	0.782	0.191	0.647	0.162	0.355	0.245	0.399
	0.876	0.196	0.628	0.176	0.330	0.290	0.377

[a]POS = 1(3)-palmitoyl-2-oleoyl-3(1)-stearoylglycerol; reaction times were 1–4 hr at 30°C; conversions are ± 0.01; methyl ester fractions were calculated from GLC areas averaging 2–3 injections and are ± 0.01.

[b]Fraction of esters (POS) converted to FFA; i.e., fraction of reaction.

[c]Proportions of unreacted acid residues expressed as a fraction.

[d]Proportions of free acids expressed as a fraction.

[e]Tanabe food grade.

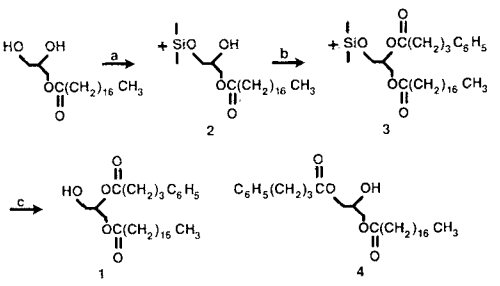
[f]Miles fine grade.

(emulsion formation was not a problem, and the major concern is fractionation of acid residues). The ether extract was then washed with H_2O (2 × 10 mL). The aqueous phase and associated solids were then acidified with 2N HCl (5 mL) and extracted with 3 × 10 mL of hexane to obtain the acids liberated by the lipase. The acids were recovered and converted to methyl esters with methanol·BF_2 (5 mL) at 25°C for 4 hr. The esters were then recovered by diluting the mixture with H_2O and extracting with hexane. The unreacted and reacted materials were analyzed as methyl esters by GLC (SPB-1 column at 220°C; methyl ester, retention time, k′): palmitate, 7.4 min, 2.2; oleate, 11.5 min, 4.0; stearate, 12.4 min, 4.4.

Control experiments. A sample of POS was transesterified as described above to provide the data required for peak area normalization. A gravimetric mixture of methyl esters was prepared for comparison. The POS showed a slightly higher value for oleic acid (~4%), and therefore the data from the synthetic sample was used for area normalization. The extractive method described was checked by using dummy product mixtures of triolein + palmitic acid, and tripalmitin + oleic acid. The methyl esters representing these products ideally should contain only the ester of a single fatty acid. There was some cross contamination (1–2% at most), and the method was therefore deemed useful even if not absolutely quantitative.

RESULTS AND DISCUSSION

Preparation and stability of a 1,2-diglyceride. Methods for preparing 1,2- and 1,3-diglycerides have been reviewed (18) as has some of the chemistry of acyl migration in partial glycerides (19). We have found little in the literature dealing with acyl migration under conditions employed for enzymatic hydrolysis, and we therefore synthesized 1-stearoyl 2-(4′-phenyl)butyroyl glycerol, 1 (Scheme 1). 1-Monostearin was silylated at the 3-position with *t*-butyldimethylsilyl chloride (15), acylated at the 2-position by means of 4-phenylbutyroyl chloride, and then desilylated with *N*-bromosuccinimide. The desilylation occurs without acyl migration (17). Final purification was achieved by preparative HPLC.



SCHEME 1. Synthesis of a 1,2-diglyceride, 1-stearoyl-2-(4′-phenyl)-butyroyl glycerol. a, *t*-butyldimethylsilyl chloride, imidazole, THF; b, 4-phenylbutyroyl chloride, pyridine; c, *N*-bromosuccinimide, DMSO, H_2O, THF.

The 1,2-diglyceride, containing a convenient UV chromophore, was sonicated in water buffered at pHs 6,7, and 8 (0.05 M phosphate buffer) with a little gum arabic to create an emulsion. The 1,3-isomer was not observed even after 4 hr at 30°C at pHs 6 and 7 (see below for results of lipase-catalyzed hydrolysis of triglycerides), and was only present to the extent of about 4% at pH 8. In contrast, an ethereal solution of 1 containing either silica gel or a trace of *p*-toluene-sulfonic acid developed 9–11% of the 1,3-diglyceride in less than 2 hr. The equilibrium ratio of 60:40 favoring the 1,3-isomer (19) was achieved

in those latter solutions by allowing them to stand overnight at room temperature. It is useful to know that heating such a mixture briefly in benzene with catalytic *p*-toluenesulfonic acid produces the 1,3-diglyceride only. The equilibrium position is affected by the reaction medium, and this observation may have some synthetic application.

Positional selectivity experiments. An equimolar mixture 1- and 2-octanol octanoates was exposed to each of several commercial lipases in aqueous medium buffered at pH 7. The sonicated mixtures were stirred magnetically at 30°C, titrated to determine the fraction of starting mixture converted to free acid and, after suitable extractive workup, analyzed by GLC for the ratio of the unreacted esters (Table 1). None of the three enzymes examined showed significant preference for the primary ester. If a 1:1 reaction mixture is allowed to proceed to 20% conversion and only one substrate reacts, the ratio of unreacted substrates would be 0.625:0.375. The data of Table 1 indicate that none of these enzymes reacts exclusively with the primary ester. The lipase of *C. cylindracea* has been characterized as a positionally nonspecific (random) lipase, and reaction of the *G. candidum* lipase with oleic acid residues also is not affected by position. However, the apparent ease of reaction for the sterically encumbered secondary ester by the *R. delemar* lipase, characterized originally as 1,3-specific (20), is noteworthy; and although these esters bear little resemblance to triglycerides, the observations suggested that the characterizations of lipases for selectivity ought to be reevaluated.

The synthetic triglyceride POS (11) was treated with seven lipases under identical reaction conditions as described above, and the fraction conversions were obtained again by titrimetry. The proportions of P, O, and S in the recovered partial/triglycerides and in the free fatty acids were determined by GLC of methyl esters and are presented in Table 2. The *C. cylindracea* lipase, known to lack position preference under these conditions, seems to show slight selectivity, cleaving the P and S residues that are at the 1- and 3-positions faster than the 2-oleoyl group. One should note that the figures for unreacted glycerides do not indicate more than a slight preference—the reaction has gone to 86% completion (the POS reacts considerably faster than the octanol octanoates). The *G. candidum* lipase is similar to the *C. cylindracea* in its reactivity and may actually be even less selective. The other lipases all indicate a preference for hydrolyzing the primary ester groups though the selectivity is not absolute. The most selective enzyme product is Tanabe's *R. delemar* preparation, which generates only 3-4% oleic acid at 51% conversion. As expected, as the conversion increases (64%), the proportion of oleic acid in the product also increases (7%). The law of mass action takes hold: the enzyme is being exposed to unreacted acid residues that have increasingly higher proportions of oleic acid. Nonenzymatic acyl migration of intermediate 1,2-diglycerides is unlikely in these reactions, and the reportedly faster rearrangement of 2-monoglycerides (8) also seems unlikely to account for the amount of oleic acid being generated. The (crude) commercial preparations have some propensity for cleaving a secondary alcohol ester in partial/triglycerides or, the enzyme has the ability to function as an acid-base catalyst to cause a "nonenzymatic" acyl migration followed by enzymatic cleavage from a primary position. The commercial *G. candidum* lipase shows some preference for P and O over S, but does not show the striking properties of the originally described strain (6). Appropriate checks of the methods used are given in the Experimental section.

Additional studies with G. candidum *lipase.* There is considerable interest in cloning the gene for *G. candidum* lipase to learn the basis for fatty acid selectivity and, perhaps, to improve it by altering the amino acid sequence (21). Our own work with several strains and sources of *G. candidum* (15) indicated that a very selective isoform of the lipase occurs in nature. A complementary approach to obtaining selectivity involves using the crude lipase and altering reaction conditions to change the fatty acid preference. Hydrolysis of equimolar mixtures of butyl palmitate and oleate was conducted at pH intervals of 0.5 from 5.5 to 7.9 for 1-2 hr at 37°C. At lower pH, the oleate ester reacted 1.5 times as fast as the palmitate, and that figure decreased to unity at pH 7.0. There was no significant preference at higher pH. The reaction was conducted at 50°C, and samples of enzyme incubated at 50°C for varying lengths of time before being used as catalysts were also employed. Neither the change in temperature (T), or the attempts to partially denature the commercial enzyme (mixture) altered selectivity. Various metal ions (calcium, cupric, ferrous, ferric, magnesium, manganese, zinc) were employed to no avail (ferric ion reduced reactivity significantly). Another strategy for improving selectivity involves reversible chemical transformation of the oleate double bond hoping to reduce the binding of the lipase and allow preferential hydrolysis of butyl palmitate. Thus butyl oleate "dibromide" and butyl "epoxy" oleate were prepared by standard methods. These reacted only slightly more slowly than did the palmitate. Finally, esterification of oleic-palmitic acid mixtures in several solvent systems containing *n*-butanol were evaluated. In dry hexane, oleic > palmitic (1.2:1), and in wet hexane, oleic > palmitic (3.4:1). It seems unlikely that these approaches will lead to a more selective catalyst.

In conclusion, acyl migration of 1,2-diglycerides during enzymatic reactions may be less of a problem than previously thought. Reactions that are being conducted to determine the positional selectivity of a lipase can, and should, be carried to greater degrees of conversion to assess more realistically positional preference. In the experiments reported here *R. delemar* lipase was quite selective, but porcine pancreatic lipase favors reaction at the primary positions in POS, only slightly. Thus, in its present form, the latter lipase could only be used to make token amounts of 2-monoglycerides, and its use in interesterifications would be limited according to the actual relative rate of exchange at the primary and secondary positions and the importance attached to retaining the composition at the secondary position. Similarly, the available *G. candidum* lipase is of no utility in chemical transformations that require high selectivity for oleic acid, and our efforts to alter that selectivity failed.

Several of the fungal lipases are mixtures of enzymatically active principles (21-23). An examination of these could prove useful, because the reactivities of individual purified isoforms can vary. For example, Lan and Jones (24) have shown that isozymes of pig liver esterase exhibit differing stereoselectivities in hydrolyses of

selected esters. Since high selectivity in lipase reactions can be the basis for types of industrial reactions not currently possible with conventional technology, identification of very selective isoforms seems critical to furthering such objectives.

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