Characterization of New Yeast Lipases

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ABSTRACT: Previously, we used a simple, sensitive agar plate method to screen lipase activity from 1229 selected cultures, including 508 bacteria, 479 yeasts, 230 actinomycetes and 12 fungi, that covered many genera and species. About 25% of the cultures tested were lipase-positive. We also expanded our screening method to focus specifically on the pH dependence and thermostability of these lipase activities. In this report, we have characterized 25 yeast lipases, obtained from our screening program, on the basis of their positional specificity against triglycerides. Lipase was produced by growing cultures on nutrient medium in the presence of vegetable oil at 25°C for 4 d. Of the 25 new yeast lipases analyzed, 19 showed 1,3-positional specificity and 6 showed random specificity. No 2-positional specific lipases were found. Among those cultures with highest lipase activity are: *Candida silvicola* NRRL YB-2846 (random); *Candida* sp. 55 (random); *Candida* sp. 125 (random); *Pichia americana* NRRL Y-2156 (1,3-specific); *P. muscicola* NRRL Y-7005 (random); *P. petersanii* NRRL YB-3808 (1,3-specific); and *Yarrowia lipolytica* NRRL YB-423 (random). Characterization of *Candida* sp. strain 55 lipase on its substrate preference showed that this enzyme hydrolyzed soybean oil triglyceride species LLLn, LLL, LLO, and LLP more readily than LOO, LOP, OOO, LOS, and POO, where L = linoleic, Ln = linolenic, O = oleic, P = palmitic, and S = stearin.

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Lipases (3.1.1.3) are among the most important enzymes in the oils and fats industry. Lipases catalyze the hydrolysis of triglycerides or acyl and aryl esters. Many of them are also capable of catalyzing organic reactions in nonaqueous media (1). Large quantities of microbial lipases are being produced on an industrial scale for medical and industrial uses. Lipases hydrolyze or synthesize triglycerides with positional and fatty acid specificities. For example, lipases from *Aspergillus niger* and *Rhizopus delemar* hydrolyze/form ester bonds only at positions 1 and 3 of glycerol (2). In contrast, lipases from *Geotrichum candidum* and *Penicillium cyclopium* hydrolyze or form ester bonds at all three positions (2). Lipase-catalyzed esterification processes have been commercialized by Unichema International (Gouda, The Netherlands) (3) for the production of high-quality fatty esters, such as isopropyl myristate, isopropyl palmitate and 2-ethylhexyl palmitate, which are used as ingredients in skin creams and other personal-care products.

In recent years, interest in the use of enzymes as hydrolytic or synthetic chiral catalysts has risen rapidly. In particular, the search for selective enzyme inhibitors and receptors, agonists, or antagonists is one of the keys for target-oriented research in the pharmaceutical industry. Extracellular microbial lipases are particularly suited for this application. Because of their availability, more than one dozen commercial lipases have been studied extensively by researchers in both industry and academia. For example, Sih *et al.* (4) studied several commercial lipases for the enantiospecific hydrolysis of 2-arylpropionic acid esters, an important class of nonsteroidal antiinflammatory drugs, and the aryl-thio-2*S*-methyl-propionic acid esters, key chiral intermediates for the synthesis of the antihypertensive agent captopril. However, under most conditions, enantioselectivity has not been high enough to permit economically useful production of the desired enantiomer. More recently, Patel *et al.* (5) used lipases to synthesize chiral intermediates for the chemical synthesis of three drugs: (i) taxol, an anticancer compound; (ii) thromboxane A_2 antagonist; and (iii) antihypertensive drug captopril.

Industry continues to look for economical sources of lipases with high activity. New lipases from microbial sources have been reported sporadically (6–8). The need for novel lipases is obvious, but little effort has been made for conducting a large-scale systematic screening for new lipases. At the National Center for Agricultural Utilization Research (NCAUR, Peoria, IL), we had the opportunity to conduct a large-scale screening for lipase activity with selected cultures from the Agricultural Research Service (ARS) Culture Collection (Peoria, IL). About 25% of the cultures screened were lipase-positive (9). Some of these lipases also function as esterases, stereospecifically hydrolyzing 2-ethylhexyl butyrate to produce *S*(+)2-ethylhexanol (10). We also expanded our screening method to focus specifically on pH dependence and thermostability of these lipases (11). We have now characterized 25 highly active yeast lipases with respect to their positional specificity against triglyceride. Nineteen of them showed 1,3-positional specificity, and six showed random specificity. This paper presents the results of our lipase characterization.

MATERIALS AND METHODS

Microorganisms. Microbial cultures were obtained from the ARS Culture Collection. Yeasts were grown on potato dex-

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trose (PDA) medium that contained (per liter): 26 g PDA (Difco Lab, Detroit, MI), pH 5.5 at 25°C. For preparing agar plates, 10 g agar was added into the medium. For the production of lipase, yeast was grown on PDA medium containing 1% soybean oil at 25°C for 4 d. The yeast cells were removed by centrifugation, and the extracellular lipase in the supernatant was dried by lyophilization or was precipitated by adding 3 vol of acetone at –20°C.

Chemicals. Commercial lipases, Rhodamine 6G, triolein, soybean oil, and olive oil were purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer precoated Kieselgel 60F_{254} plates were obtained from EM Science (Cherry Hill, NJ). Simple triglycerides (tripalmitin, tristearin, triolein, and trilinolein) were obtained from Nu-Chek-Prep (Elysian, MN). All other chemicals were reagent-grade and were used without further purification. Solid-phase extraction cartridges, Bond Elut, were purchased from Varian (Harbor City, CA).

Lipase assay. The reaction mixture, composed of 500 µg tributyrin and a given amount of enzyme (in 0.5 mL 0.05 M sodium phosphate buffer, pH 7.0), was incubated with shaking (200 rpm) for 30 min at 40°C. The reaction was terminated by adding 0.25 mL 0.1 M HCl in 95% ethanol. Liberated free fatty acid was extracted with 5 mL ether. The ether layer was evaporated to dryness and was assayed colorimetrically with Rhodamine 6G (12). One unit of lipase activity is defined as 1 µmol fatty acid liberated/min at 40°C.

Identification of lipase hydrolysis products. Filter-sterilized triolein (0.5 g) was added to a 1-d-old culture of yeast grown on 50 mL PDA medium at 28°C, and the incubation continued for an additional 3 d. For commercially available lipase, 100 lipase units of Sigma lipase II (from porcine pancreas) were added to 10 mL 0.05 M sodium phosphate buffer solution (pH 7.5) containing 0.3 g triolein and incubated at 40°C for 24 h. At the end of the incubation, the reaction mixture was acidified with dilute HCl to pH 2 and extracted twice with an equal volume of diethyl ether. The ether extract was concentrated in a rotary evaporator and analyzed by thin-layer chromatography (TLC). TLC plates were developed with a two-stage development procedure: (i) benzene/ether/ethyl acetate/acetic acid (80:10:10:1, by vol), development finished when solvent was 10 cm above the origin; (ii) hexane/ether/formic acid (80:20:2, vol/vol/vol), development in the same direction to the top of the TLC plate. After development, products on the plate were identified first by exposure to iodine vapor and then by spraying with 60% aqueous sulfuric acid and charring.

Separation of triglycerides. The reaction mixture was acidified and extracted with solvent as described above. The solvent fraction was evaporated to dryness, and the residue was mixed with 50 mg activated carbon and 0.25 mL *n*-hexane. After 10 min, the mixture was transferred to the top of a 0.5-g SE-cartridge, which had been previously activated with 1.5 mL *n*-hexane. The cartridge was eluted with 0.4 mL *n*-hexane to give fraction 1 (nonpolar materials); 3.5 mL of a mixture of diethyl ether/*n*-hexane (10:9, vol/vol) to give fraction 2 (triglycerides); and 3.5 mL methanol to give fraction 3 (polar materials). The triglyceride fraction was evaporated to dryness and redissolved in a small amount of methylene chloride.

High-performance liquid chromatography. Triglyceride species were separated with a Spectra-Physics analytical high-pressure liquid chromatograph (HPLC) (San Jose, CA). A Zorbax ODS 0.5 µm, 4.6 mm i.d. × 50 cm column (Rainin, Woburn, MA), a solvent gradient of methylene chloride/acetonitrile (40:60 to 80:20, vol/vol) for 120 min at 0.8 mL/min, and an evaporative light-scattering detector (Varex, Burtonsville, MD) were used. The nebulizer gas flow was 45 mL/min, the drift tube temperature was 116°C, and the exhaust gas temperature was 70°C. Triglyceride species were identified by comparing their HPLC retention times with authentic triglycerides and also with those reported in papers from our Center (13,14).

RESULTS AND DISCUSSION

Lipase hydrolysis products. The lipase hydrolysis products, 1,3- or 1,2-diglycerides, monoglycerides, and free fatty acids were analyzed by TLC with a two-solvent system. Figure 1 is a typical TLC plate with lipase hydrolysis products. Lipases that produced 1,2-diglycerides, monoglycerides, and free fatty acids but not 1,3-diglycerides were classified as 1,3-positional-specific enzymes. On the other hand, lipases that produced all of the abovementioned products were classified as random specific enzymes. Among the 25 yeast lipases ana-

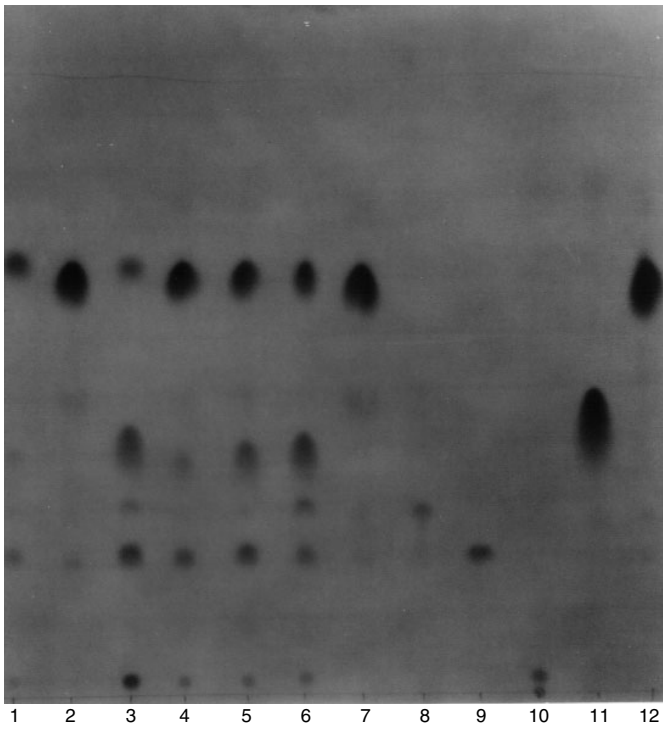

FIG. 1. A typical thin-layer chromatogram plate to show lipase hydrolysis products. Track 1, *Candida silvicola*, YB-2846; 2, *C. silvanorum*, Y-7782; 3, *Pichia muscicola*, Y-7005; 4, *P. Canadensis*; 5, Novo IM-20 (Sigma, St. Louis, MO); 6, *Geotrichum candidum*, Y-552; 7, blank (no microbes); 8, 1,3-diolein; 9, 1,2-diolein; 10, mono-olein; 11, oleic acid; 12, triolein

TABLE 1
Characterization of New Yeast Lipases: Positional Specificity[a]

Microbes	Growth (650 nm)	Products identified by TLC				
		1,2-	1,3-	mono-	FFA	Specificity
Known lipases:						
C. cylindracea Y-17506	24.5	+++	+/–	–	++++++	Random
G. candidum Y-552	21.6	++	++	+/–	+++++	Random
Novo IM-20	–	+++	–	++	++++	1,3-
New strains:						
C. apicola Y-2481	4.5	+	–	–	+/–	1,3-
C. azyma Y-17067	4.8	++	–	+/–	+/–	1,3-
C. cacaoi Y-7302	13.6	++	–	–	+/–	1,3-
C. magnoliae Y-2333	12.4	+	–	+/–	+	1,3-
C. magnoliae YB-4226	13.8	+/–	–	–	+/–	1,3-
C. melinii Y-1514	5.9	+++	–	+	+++	1,3-
C. quercuum Y-12942	17.3	+++	–	+/–	++	1,3-
C. silvicola YB-2846	10.7	++	++	+	+++++	Random
C. silvanorum Y-7782	7.6	++	–	–	+/–	1,3-
C. sp. YB-2064	8.5	++	–	–	+	1,3-
P. americana Y-2156	20.9	+++	–	+/–	++++++	1,3-
P. bimundalis Y-5343	14.2	+	–	–	+/–	1,3-
P. candensis Y-2340	8.5	+++	–	++	+++	1,3-
P. holstii Y-7914	15.4	++	+	+/–	+/–	Random
P. lynferdii Y-7723	4.0	++	–	+/–	++	1,3-
P. muscicola Y-7005	5.0	+++	+	+++	+++++	Random
P. muscicola Y-7006	10.2	++	–	–	+	1,3-
P. petersanii YB-3808	13.0	+++	–	++	+++++	1,3-
P. sydowiorum Y-7130	13.2	++	–	++	++	1,3-
Saccharomycopsis crataegensis YB-192	3.3	+++	–	–	++	1,3-
Yarrowia lipolytica YB-423	12.9	+++	+	+	+++	Random
Y. lipolytica Y-2178	8.6	+/–	–	–	+/–	1,3-
Zygosaccharomyces fermentati Y-17056	11.9	+	–	+/–	+/–	1,3-
Strain #55[b]	14.1	++	++	–	+++++	Random
Strain #125[b]	13.9	++	++	+	+++++	Random

[a]Abbreviations: *C.*, *G.* and *P.* represent *Candida, Geotrichum,* and *Pichia,* respectively; 1,2- and 1,3- are diglycerides; FFA is free fatty acid; +/–, +, ++, +++, etc. indicate visual intensity of the thin-layer chromatography (TLC) spot.
[b]Note: These numbers are not NRRL official numbers.

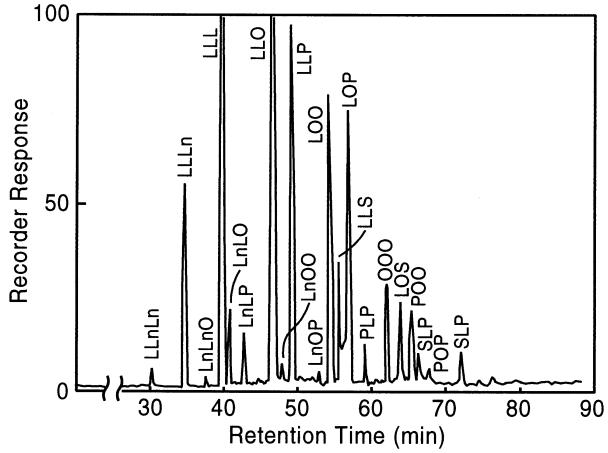

FIG. 2. A typical high-performance liquid chromatographic (HPLC) pattern of soybean oil triglyceride species. HPLC conditions: C-18 Zorbax ODS column (5 µm, 50 × 0.46 cm) (Rainin, Woburn, MA). Solvents: CH_2Cl_2/CH_3CN = 40:60 to 80:20 gradient, 120 min. Flow rate = 0.8 mL/min. Detection: evaporative light-scattering detector. L, linoleic; Ln, linolenic; O, oleic; S, stearic; P, palmitic.

lyzed (Table 1), 19 of them showed 1,3-positional specificity, and 6 showed random specificity. No 2-positional specific lipases were found. Cultures with the best lipase activity are: *Candida silvicola* NRRL YB-2846 (random); *Candida* sp. 55 (random); *Candida* sp. 125 (random); *Pichia americana* NRRL Y-2156 (1,3-specific); *P. muscicola* NRRL Y-7005 (random); *P. petersanii* NRRL YB-3808 (1,3-specific); and *Yarrowia lipolytica* NRRL YB-423 (random).

Cellular location of lipase activity in strain 55. A time-course study on the production of lipase activity in strain 55 showed that lipase activity reached its maximum after 5 d of incubation (data not shown). Therefore, after 6 d of

TABLE 2
Cellular Location of Lipase in Strain 55

Location	Lipase activity (U)	Total activity (%)
Supernatant	279.5	94.2
Rinse	4.06	1.3
Cell pellet	13.14	4.5

TABLE 3
High-Performance Liquid Chromatographic Analysis of Triglyceride Species Before and After Treatment with Lipase-Active Strain #55

Retention time (min)	Triglyceride species[a]	Original (%)	Remaining (%)
33.1	LLLn	5.9	0
38.0	LLL	17.9	2.0
42.0	LnLP	1.5	0
45.2	LLO	21.0	2.1
47.9	LLP	13.8	1.8
53.6	LOO	9.4	16.6
55.3	LLS	3.3	5.8
56.3	LOP	8.9	25.8
59.6	PLP	0.9	4.5
62.5	OOO	2.6	13.3
64.0	LOS	2.3	11
65.4	POO	1.7	9.2
67.1	SLP	0.6	2.8
68.7	POP	0.1	0
73.0	SOO	0.4	2.3
76.1	SOP	0.1	0.6

[a]Abbreviations: L = linoleic, Ln = linolenic, O = oleic, S = stearic, and P = palmitic.

growth, the culture medium was centrifuged to separate cells and the supernatant. The cell pellet was rinsed with a small amount of 0.05 M phosphate buffer pH 7.5 and centrifuged again to obtain the rinse fraction. About 94% of the lipase activity was located in the cell-free supernatant fraction (Table 2). The cell pellet contained only 4.5% of the total lipase activity. Therefore, the majority of the lipase produced is secreted extracellularly rather than being cell-associated.

Determination of the substrate preference of strain 55 lipase. To examine which triglyceride species are preferred by strain 55 lipase, 0.5 g soybean oil was incubated with a strain 55 lipase preparation (an acetone powder with about 200 lipase units) in 10 mL 0.05 M phosphate buffer pH 7.5 for 24 h at 40°C; a control run without lipase was also conducted. After 24 h, the triglyceride species remaining in both reaction mixtures were determined by HPLC. This method is capable of separating the numerous triglyceride species existing in soybean oil; a typical HPLC profile is shown in Figure 2. The triglyceride species of the original soybean oil and those obtained from the control run (without lipase) are identical. However, the composition of triglyceride species in the lipase-treated sample was different. These data are compared in Table 3. It is clear that strain 55 lipase hydrolyzes soybean oil triglyceride species LLLn, LLL, LLO, and LLP more readily than LOO, LOP, OOO, LOS and POO, where L = linoleic, Ln = linolenic, O = oleic, S = stearic, and P = palmitic. Differences in preference toward the various triglyceride species likely exist among different lipases found in nature.

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