The Positional and Fatty Acid Selectivity of Oat Seed Lipase in Aqueous Emulsions

5770

The positional and fatty acid selectivities of oat (*Avena sativa* L.) seed lipase (triacylglycerol hydrolase EC 3.1.1.3) were examined. Pure triacylglycerols were used as substrates. The products of lipolysis were examined by thin-layer chromatography and gas-liquid chromatography. Only symmetrical triacylglycerols were used as substrates; thus potential complications arising from stereobias were avoided. Controls were carried out with a lipase specific for primary positions. The lipase from oat seeds catalyzed the hydrolysis of both primary and secondary esters. When the lipase was tested upon mixtures of homoacid triacylglycerols (triacylglycerols composed of the same three fatty acids), the lipase acted most rapidly upon those containing oleate, elaidate, linoleate and linolenate. Strong intermolecular selectivity against homoacid triacylglycerols containing palmitate, petroselinate and stearate was observed. Comparison of assays performed at 26°C with those performed at 45°C showed that selectivity was temperature-independent. When mixed-acid triacylglycerols containing both oleate and stearate were treated with lipase, intramolecular selectivity was observed, with oleate hydrolysis predominating. From this work and earlier work, it can be concluded that the selectivity exhibited by the oat seed lipase is similar to that of the lipase from *Geotrichum candidum*, except that the oat seed lipase attacks elaidate, a fatty acyl group with a *trans* double bond, whereas the *G. candidum* lipase strongly discriminates against elaidate.

KEY WORDS: ***Avena sativa*, diacylglycerol, lipolysis, *Rhizopus delemar*, triacylglycerol, unsaturated fatty acid.**

In the lipase (triacylglycerol [TAG] hydrolase EC 3.1.1.3)-catalyzed hydrolysis of TAG, positional and fatty acid selectivity is often observed (1). However, prior studies of oat seed lipase have yielded conflicting observations: Analysis after partial hydrolysis of tributyrin showed that only secondary ester bonds were cleaved (2). When natural oils and fats were used as substrates, the oat seed lipase hydrolyzed primary and secondary esters (3). Selectivity for oleate over stearate was observed, but no discrimination against palmitate was noted. When tallow was utilized as a substrate, the liberated fatty acids were enriched in oleic acid and depleted in both stearic and palmitic acids (4). Little fatty acyl selectivity was observed when lipolysis was conducted in organic solvents (5,6). Here, the positional and fatty acid specificity or selectivity of the oat seed lipase is examined with synthetic TAGs of high purity, emulsified in an aqueous medium. Such studies are important prerequisites to the design of systems employing lipase-promoted fat splitting and restructuring.

MATERIALS AND METHODS

Materials. The following are the materials used and their company sources: Benzene, J. T. Baker Chemical Co. (Phillipsburg, NJ); *n*-hexane and methanol, Burdick and Jackson (Muskegon, MI); ethyl acetate and formic acid, Eastman Kodak Co. (Rochester, NY); ethyl ether and sulfuric acid, Mallinckrodt (Paris, KY); acetic acid, Aldrich Chemical Co. (Milwaukee, WI); lipids and other biochemicals, Sigma Chemical Co. (St. Louis, MO); glycerol tri-[1-^{14}C] oleate, Amersham (Arlington Heights, IL); silica-gel G thin-layer chromatography (TLC) plates, Analtech (Newark, DE).

Oat seed lipase preparation. Oat seed lipase was prepared as previously described (4). Briefly, 5 g oat seeds (U.S. Grain Co., Timonium, MD) were homogenized in 35 mL cold 10 mM N-2-hydroxyethyl piperazine-N′-ethanesulfonic acid (Hepes) buffer (pH 7.4) for 2 min in a Waring commercial blender (Model 31BL91) fitted with a 110-mL stainless-steel mini jar. The homogenate was filtered through two layers of Miracloth (Calbiochem, San Diego, CA) and centrifuged at 120 *g* for 15 min. The supernatant was used as the source of lipolytic activity.

Rhizopus delemar *lipase preparation. Rhizopus delemar*, ATCC 34612, was obtained from the American Type Culture Collection (Rockville, MD). Progenies of individual spores were propagated for use as inocula. Spore suspensions were prepared by inoculating malt extract agar (per liter: 20 g malt extract [Difco Laboratories, Detroit, MI], 20 g glucose, 1 g Bacto Peptone, 20 g agar) with a small number of spores. The plate was incubated at 30°C until the culture had sporulated. A sterile glass rod was then used to tease the spores off the mycelial pad into M9 salts solution (7). Spore densities obtained in this manner were approximately 10^6 per mL.

Basal growth medium, adapted from that of Westergaard and Mitchell (8), contained 3.7 mM monobasic potassium phosphate, 0.136 mM calcium chloride, 0.86 mM sodium chloride, 1 mM magnesium sulfate, 25 mM potassium nitrate, 37 mM ammonium chloride, 0.5% (wt/vol) Casamino Acids (Difco Laboratories), 5 μg biotin per liter, trace elements (9), and 30 mM glycerol. Cultures were inoculated with 0.1% vol of spore suspension and shaken at 150 rpm on a New Brunswick Model G10 rotary shaker at 30°C. One-liter cultures were grown in 2-L Erlenmeyer flasks.

At the peak of enzyme activity, approximately 36 h after inoculation, the mycelia were removed by filtration over cheesecloth. After passage over Whatman #1 paper, the filtrate was concentrated approximately tenfold by lyophilization. The concentrated solution was dialyzed against 14 L of cold water twice for 24 h and lyophilized to dryness. The residue was dissolved in an amount of water equal to 130th of the original media volume and was stored at −80°C until used.

Protein assay. Protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). The assay was calibrated with Sigma protein standard.

Lipolysis of mixtures of homoacid triacylglycerols. Partial lipolysis was conducted in a disposable borosilicate glass culture tube (12 × 75 mm). The tube contained eight different monoacid TAGs (25 mg each) along with 0.88 mL 100 mM Hepes buffer, 0.96 mL 10% gum arabic and 0.56 mL 235 mM $CaCl_2 \cdot 2H_2O$ (final pH 7.6). The contents of the tube were warmed in a water bath until all of the TAGs were melted, and the mixture was immediately sonicated, allowed to cool, and the oat seed lipase preparation was added (0.33 mL for those experiments performed at 45°C and 1.0 mL for those performed at 26°C). The reaction was quenched with 0.68 mL 1.5% H_2SO_4 after 15 min, and the reaction mixture was transferred to a screw-capped 20-mL vial containing 5 mL-hexane/isopropanol (60:40, vol/vol) and 5 mL 6.7% aqueous Na_2SO_4. The mixture was shaken, allowed to separate, and the upper (organic) layer was removed. The aqueous phase was extracted with hexane/isopropanol at least nine more times, or until TLC showed no lipid remaining in the aqueous phase. The individual extractions were combined and taken to dryness under a steam of nitrogen.

Cyclohexane (6 mL) was added to the dried lipids, and a 20-μL aliquot was spotted on a washed (10) 250-micron silica gel G TLC plate. The plate was developed as described previously (11), and the lipid classes were quantified with a TLC scanner (CAMAG Scanner II, Muttenz, Switzerland) at 440 nm. To convert peak area to a mol value, calibration curves were prepared by means of acylglycerol and fatty acid standards. The fraction of lipolysis was calculated by dividing the mol of released fatty acid by the sum of the mol residing in all lipid classes.

For the analysis of the composition of the liberated fatty acids, one-quarter of the lipids in cyclohexane were applied to a washed 500-micron silica gel G plate, and the plate was developed as described previously (11). Positions of the lipids on the TLC plate were determined by spraying the TLC plate with a 0.1% ethanolic solution of 2′,7′-dichlorofluorescein followed by visualization under ultraviolet (UV) radiation. The liberated fatty acids were extracted from the silica gel with ethylacetate/methanol (80:20, vol/vol). The dichlorofluorescein was removed by partitioning between cyclohexane and ethanol/water (50:50, vol/vol). The free fatty acids were methylated with diazomethane. The fatty methyl esters were baseline-resolved by gas-liquid chromatography (GLC) (Hewlett-Packard 5880A, Palo Alto, CA; Supelco fused-silica capillary column, SP-2340, Bellefonte, PA; the column oven program was 150°C to 160°C at 0.5°C/min, 160°C to 170°C at 1.0°C/min, and 170°C to 190°C at 1.5°C/min). Peak heights were corrected by subtracting the peak areas obtained from experiments in which heat-inactivated oat lipase was used. The corrections amounted to, at most, a 2% decrease in the peak area. The validity of the free fatty acid data was checked with the method of Schwartz and Gadjeua (12), which demonstrated that the fatty acids were not fractionated by incomplete extraction from the silica gel.

Reactions of mixed acid TAGs and analysis of lipolysis products. Each reaction mixture contained 0.12 mL 10% gum arabic, 0.11 mL 100 mM Hepes buffer, 0.07 mL 235 mM $CaCl_2 \cdot 2H_2O$, 25 mg TAG, and 0.06 mL oat seed lipase. The final pH of the mixture was 7.6. The mixture was subjected to sonication immediately prior to the lipase addition. Reactions were conducted for 15 min at 45°C. The liberated fatty acids and acylglycerols were analyzed by the procedures described above.

Hydrolysis rate assay with radiolabel. Assays with radiolabelled TAGs were performed as follows. The reaction contained 1.26 mL 10% gum arabic, 1.7 mL 100 mM Hepes, 32.1 mg (36.2 μmol) triolein, 3 μCi (0.5 μmol) glycerol tri[1-^{14}C]oleate and 0.54 mL oat lipase. The final pH of the mixture was 7.6. The mixture was sonicated prior to the addition of lipase. At each of the indicated times, a 0.5-mL aliquot of the reaction mixture was withdrawn. After workup, TLC was performed on TLC plates that contained standards spotted at the far left and right of the plate. The radiolabelled material was streaked across the central portion of the plate. After development, the lanes containing the standards were snapped off and charred (11) to reveal the positions of the acylglycerols and fatty acid. Silica-gel bands corresponding to the standards were scraped off the plate into vials that were filled with 16 mL Ecoscint O (National Diagnostics, Manville, NJ). Radioactivity was determined with a Beckman LS 8100 liquid scintillation (Fullerton, CA). It was corrected for background by using a silica-gel band containing no acylglycerol or fatty acid.

RESULTS AND DISCUSSION

A typical time-course experiment, in which the fate of radiolabelled triolein was followed during its lipolysis by oat seed lipase, is shown in Figure 1. In the reaction mixture, the levels of 1,2(2,3)- and 1,3-diolein rose until a plateau (steady-state) was reached. Control experiments were performed with the lipase from *Rhizopus delemar,*

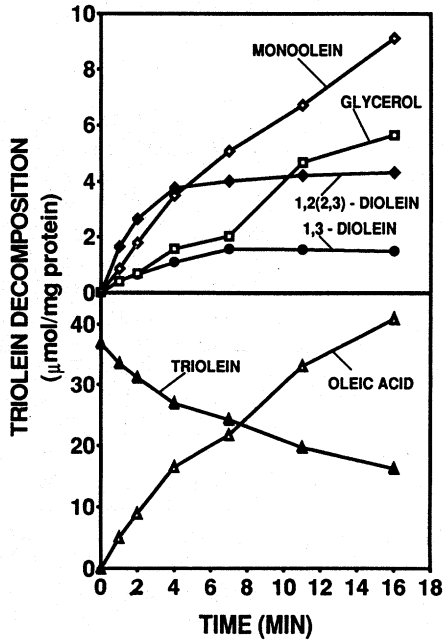

FIG. 1. The kinetics of triolein decomposition by oat lipase. Assays for radiolabelled acylglycerols were conducted as described in Materials and Methods. Free glycerol concentrations were calculated from the measured concentrations of free fatty acids, monoacylglycerols and diacylglycerols.

an enzyme that is known from prior work to be specific for the primary esters of glycerol (13). In four experiments the amounts of 1,3-diolein formed by each lipase, as measured by the level of radioactivity associated with these fractions, were compared. The amount of 1,3-diolein generated by the oat seed lipase was 7 to 15 times higher than that generated by the *R. delemar* lipase. Because identical reaction conditions and extraction procedures were used with both lipases, the 1,3-diolein generated by the oat seed lipase must arise mostly from an enzyme-catalyzed pathway, rather than through nonenzymatic interesterification. Thus, oat seed lipase catalyzes the hydrolysis of both primary and secondary esters. This conclusion was also reached in an earlier study of oat seed lipase (3). There, oleic acid was preferentially released from the secondary position of cocoa butter.

The data of Figure 2 are from analyses of free fatty acid fractions obtained from partial lipolyses of equimolar mixtures of homoacid TAGs at 26°C and 45°C (a homoacid TAG is a TAG composed of the same three fatty acids). The data show that there are at least three features of fatty acyl selectivity that the oat seed lipase and the lipase from *Geotrichum candidum* share (14–16). Triolein (C18:Δ9*c*) and trilinolein (C18:2Δ9,12*c*) are hydrolyzed most rapidly (14). As is seen with many isozymes of *G. candidum*, discrimination against stearate (C18:0) is greater than discrimination against palmitate (C16:0) (15). Both lipases show strong discrimination against petroselinate (C18:1Δ6*c*) (14). One difference between the oat seed lipase and the lipase from *G. candidum* is that the former shows only modest discrimination against the fatty acyl group with a *trans* double bond, elaidate (C18:1Δ9*t*), while the latter shows strong discrimination against elaidate (16). Changing the temperature from 26°C to 45°C caused almost no change in selectivity. The lack of temperature sensitivity is particularly notable for trielaidin, which is a solid at 26°C and a liquid at 45°C, indicating that physical state has little or no influence on the results.

The fatty acid analyses data found in Table 1 support the contention that the oat lipase can distinguish against stearate intramolecularly. The fatty acid fraction derived from 1,3-dioleoyl-2-steroyl-glycerol (OSO) is composed of only 10.2% stearic acid compared to a level of 33.5% in the TAG substrate. The fatty acid fraction derived from 1,3-distearoyl-2-oleoyl-glycerol (SOS) contains 54.0% stearic acid, rather than the 66.3% found in the TAG substrate. As expected, the DAG and monoglycerol fractions were enriched in stearate (data not shown).

Lipolyses of triolein, OSO and SOS were conducted with the primary ester-specific lipase from *R. delemar*. When the fatty acid fractions were examined by GLC, only oleate was detected from the lipolyses of OSO and only stearate from SOS (Table 1). These observations demonstrate that the structures of OSO and SOS are correctly represented and also show that nonenzymatic acyl migration did not occur during workup.

As shown in Table 2, in a typical experiment with oat seed lipase, the level of 1,3-DAG was reduced when OSO was the substrate compared to that when triolein was the substrate. When SOS was the substrate, the level of 1,3-DAG was also reduced somewhat, probably as a result of an overall reduction in the hydrolysis rate. However,

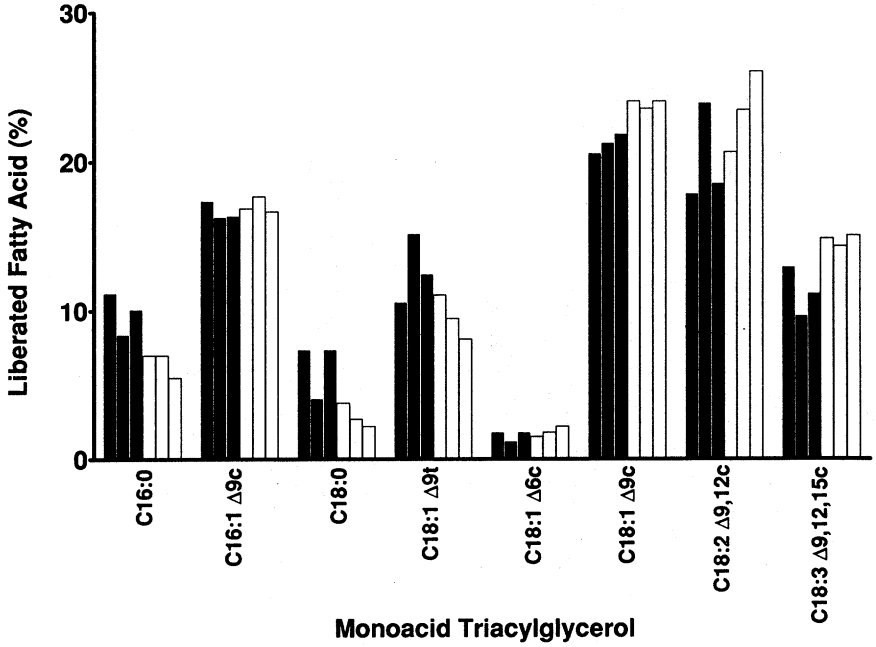

FIG. 2. Fatty acids released from equal molar mixtures of homoacid triacylglycerols. The reactions were conducted for 15 min, and the liberated fatty acids were isolated and analyzed as described in Materials and Methods. Lipolysis was conducted at 26°C (□) and 45°C (■). The bars over the indicated fatty acid represent results from separate experiments. The mean rate of lipolysis at 26°C was 3.4 μmol/min, the amount of added protein was 5.2 mg, and the mean degree of lipolysis was 8.6%. The mean rate of lipolysis at 45°C was 4.9 μmol/min, the amount of added protein was 1.7 mg, and the mean degree of lipolysis was 10.1%.

TABLE 1

Analysis of the Composition of the Fatty Acids Liberated from the TAGs, OSO and SOS[a]

	OSO (%)[b]	SOS (%)[b]
Starting material[c]		
oleate	66.5	33.7
stearate	33.5	66.3
Fatty acid fraction liberated by oat seed lipase[d]		
oleate	89.8	46.0
stearate	10.2	54.0
Fatty acid fraction liberated by *R. delemar* lipase		
oleate	100	0
stearate	0[e]	100

[a]TAGs, triacylglycerols; OSO, 1,3-dioleoyl-2-stearoyl-glycerol; SOS, 1,3-disteroyl-2-oleoyl-glycerol.

[b]Each analysis was conducted 3–6 times. The standard error of the mean was 3.1% or less.

[c]Determined after hydrolysis with KOH/methanol.

[d]Reactions were conducted as described in Materials and Methods. The enzymatic activity on triolein was 1.5 μmol/min, and the amount of added protein was 0.46 mg.

[e]Limit of detection: 0.3% hydrolysis.

TABLE 2

Weight of Some of the Products of the Partial Lipolysis of OSO and SOS by Oat Lipase in a Typical Experiment[a]

Fatty derivative[b]	Triolein (mg)	OSO (mg)	SOS (mg)
1,3-DAG	0.9	0.5	0.6
1,2(2,3)-DAG	2.5	3.0	0.7

[a]Abbreviations: See Table 1, footnote a; DAG, diacylglycerol.

[b]Reactions were conducted as described in Materials and Methods. The enzymatic activity on triolein was 1.5 μmol/min, and the amount of added protein was 0.46 mg.

most of the impact caused by the presence of stearate in the primary positions of the TAG was upon the level of 1,2(2,3)-DAG, which was reduced more than threefold compared to that observed in the lipolysis of triolein. At the temperature at which the experiments were conducted (45°C), all of the TAGs were in the liquid state, and therefore, changes in the character of the substrate emulsion due to changes in state cannot be influencing the DAG level; thus, the observations also demonstrate intramolecular selectivity. The rate of hydrolysis of stearate was slow relative to that of oleate, causing shifts in the pathway of flow into the DAG pool: with OSO, primary ester cleavage was favored, and with SOS, secondary ester cleavage was favored.

In conclusion, the lipase from oat seeds exhibits almost no positional specificity, but significant selectivity for long-chain Δ9 unsaturated fatty acyl groups when hydrolysis is conducted in an aqueous medium free of organic solvent. This conclusion is consistent with our analyses of fatty acid fractions from partially hydrolyzed tallow (4). Oleic acid was enriched in these fractions, and palmitic and stearic acids were depleted. Berner and Hammond (3) found no discrimination against palmitate in their study of the lipase from oat seeds. Most likely, the degree of discrimination against palmitate depends upon the cultivar of oat used. Although there has not been a systematic study of this in oats, a survey of the selectivity of lipase isozyme preparations from *G. candidum* has been made by comparing the initial rates of hydrolyses of several fatty esters of 4-methylumbelliferone (15). Some of the isozymes could not discriminate against palmitate but showed selectivity against stearate. That the isozymes of *G. candidum* show variable selectivity upon palmitate has also been noted in another study (17). Thus, the oat seed lipase and the lipase from *G. candium* are similar with regard to positional and fatty acyl selectivity, except in one respect: the oat seed lipase will hydrolyze elaidate at a relatively rapid rate.

REFERENCES

1. Jensen, R.G., F.A. Dejong and R.M. Clark, *Lipids 18*:239 (1983).
2. Peers, F.G., *Nature 171*:981 (1953).
3. Berner, D.L., and E.G. Hammond, *Lipids 5*:572 (1970).
4. Piazza, G., A. Bilyk, D. Schwartz and M. Haas, *Biotechnol. Lett. 11*:487 (1989).
5. Piazza, G.J., *Ibid. 13*:173 (1991).
6. Piazza, G.J., and H.M. Farrell, Jr., *Ibid. 13*:179 (1991).
7. Maniatis, T., E. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, p. 440, 1982.
8. Westergaard, M., and H.K. Mitchell, *Am. J. Bot. 34*:573 (1947).
9. King, R. (ed.), *Handbook of Genetics*, Vol. 1, Plenum Press, New York City, 1974, p. 432.
10. Maxwell, R.J., S.W. Yiesley and J. Unruh, *J. Liq. Chromatogr. 13*:2001 (1990).
11. Bilyk, A., G.J. Piazza, R.G. Bistline, Jr. and M.J. Haas, *Lipids 26*:405 (1991).
12. Schwartz, D.P., and D.G. Gadjea, *J. Am. Oil Chem. Soc. 65*:378 (1988).
13. Okumura, S., M. Iwai and Y. Tsujisaka, *Agric. Biol. Chem. 40*:655 (1976).
14. Jensen, R.G., D.T. Gordon, W.H. Heimermann and R.T. Holman, *Lipids 7*:738 (1972).
15. Baillargeon, M.W., *Ibid. 25*:841 (1990).
16. Jensen, R.G., *Ibid. 9*:149 (1974).
17. Jocobsen, T., and O.M. Poulsen, *Can. J. Microbiol. 38*:75 (1992).