Oil and Fat Hydrolysis with Lipase from *Aspergillus* sp.

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ABSTRACT: Hydrolysis of olive oil, soybean oil, mink fat, lard, palm oil, coconut oil, and a hydrogenated, hardened oil with lipase from an *Aspergillus* sp. has been studied. The lipase had high specific activity (60,000 U/g) and did not show any positional specificity. The lipase proved to be a more effective catalyst than Lipolase from *A. oryzae*, with an optimal activity at 37°C and pH 6.5–7.0. It was activated by Ca^{2+} but inactivated by organic solvents such as isopropanol and propanone. All substrates examined could be hydrolyzed to corresponding fatty acids with this enzyme at concentrations of 5–30 U/meq with yields of 90–99% in 2–24 h. The degree of hydrolysis was almost logarithmically linear with reaction time and occurred in two stages. The lipase was stable and could be repeatedly recycled for hydrolysis.
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KEY WORDS: Enzyme concentration, enzyme recovery, fatty acids, lipase, lipase activity, lipolysis, oil and fat, percent hydrolysis, reaction time, specificity.

When compared to the Colgate–Emery process of steam hydrolysis of oils and fats, a lipase-catalyzed approach provides the advantages of much lower energy costs and higher efficiency. It is especially suitable for preparing highly unsaturated fatty acids, which could be easily polymerized and decomposed at high temperatures (1). Several reports on this topic have been published in the last decade (2–5).

Lipase (E.C. 3.1.1.3) production and its application in hydrolysis of triglycerides are practiced commercially. A Japanese firm uses lipase from *Candida cylindracea* for hydrolyzing beef tallow and linseed oil to produce 4,000 tons of fatty acids per year (6,7). Rising costs, however, due in part to the low recovery of lipase, hamper the growth of production. New enzyme technology is expected to play an important role in the fatty acid industry in the future.

A new enzyme, Lipase 8901 from *Aspergillus* sp., has been shown to hydrolyze oils and fats. This paper describes the characterization and recovery of Lipase 8901. Various hydrolysis parameters, including type of oil, enzyme concentration, reaction time, and additives, were investigated.

EXPERIMENTAL PROCEDURES

Materials. Lipase 8901 powder (60,000 U/g) from *Aspergillus* sp. was supplied by Shenyang Institute of Applied Ecology, CAS (Shenyang, China). The microorganism was isolated from soil of an oleochemical plant. Lipolase (100 KLU/g ≈ 9,000 U/g) from *A. oryzae* was donated by Novo Nordisk A/S (Bagsveard, Denmark). Olive oil [free fatty acid (FFA) 2.0%] was purchased from Beijing Fangcao Medicine Manufacture Company (Beijing, China). Edible soybean oil (FFA 1.0%) was obtained from Hongyan Grain and Oil Shop (Dalian, China). Mink fat (FFA 1.0%) was from Dalian Surfactant and Detergent Chemical Factory (Dalian, China). Lard (FFA 2.6%), palm oil (FFA 2.0%), coconut oil (FFA 1.3%), and hydrogenated, hardened oil (FFA 0.8%) were supplied by Dalian Oleochemical Plant (Dalian, China). All other reagents were of reagent grade.

Assay of lipase activity. One unit of lipase activity was defined as the amount of lipase that liberated 1 μmole FFA from olive oil per minute at 37°C, pH 7.0. An olive oil emulsion was prepared as follows: 2 g polyvinyl alcohol (PVA) was dissolved in 98 mL boiled water and then filtered, and 75 mL of the filtered PVA solution and 25 mL olive oil were then blended in a homogenizer. In the present work, 4.0 mL olive oil emulsion, 5.0 mL 0.1M phosphate buffer at pH 7.0, and 1.0 mL 0.2% lipase solution were incubated in a water bath shaker for 30 min. The hydrolysis was stopped by adding 15 mL alcohol, and the solution was titrated with 0.05M NaOH and phenolphthalein as the indicator.

Lipolysis. Substrate (2.0 mL), 4.0 mL distilled water, 3.0 mL 0.1M phosphate buffer at pH 7.0, 0.5 mL 0.1M $CaCl_2$, and 0.5 mL lipase solution at specified concentrations were mixed in a 50-mL Erlenmeyer flask plugged with a rubber stopper. If the substrate was a hardened oil, the reaction mixture was composed of 2.0 mL oil, 5.0 mL isooctane, 2.0 mL buffer solution, 0.5 mL $CaCl_2$, and 0.5 mL enzyme solution. The flask was placed in a water-bath shaker at 120 rpm and at constant temperature for a prescribed period. At the end of reaction period, 10 mL of an isopropanol/propanone (1:1) solu-

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tion was added to inactivate the lipase, followed by titrating with 0.2M NaOH. The blank sample consisted of the same reactants, except that the lipase solution was replaced with distilled water.

Recovery of lipase. A 500-mL, three-necked flask, containing substrate, water, and an appropriate amount of lipase, was placed in a water bath at 35–40°C. After 8–10 h of mechanical stirring, the mixture was kept at the reaction temperature until three layers (consisting of glycerol–water, enzyme emulsion, and fatty acids) distinctly appeared. At that time, each layer was transferred to a separate beaker. The activities of the lipase in the emulsion and glycerol–water were assayed after centrifugal sedimentation. The lipase emulsion layer was reused in the next lipolysis procedure. The reaction mixture was composed of 150 g substrate, 90 g water, 15–30 g of lipase emulsion from the previous reaction and the recruited enzyme.

Thin-layer chromatography (TLC) analysis of hydrolysates. Hydrolysates were extracted with chloroform and deposited on TLC plates with Silica gel G as a stationary phase. Then the plate was developed in hexane/diethyl ether/acetic acid/pH 4.0 buffer solution (50:25:2:8) (8). The separated fatty acids were made visible with iodine vapor (9).

Gas chromatography/mass spectrometry (GC/MS) analysis of fatty acids. The fatty acids injected into the GC/MS analytical system were in the form of methyl esters. Oil (0.1 mL) and 2.0 mL 0.4M KOH–methanol solution were blended in a graduated test tube with a plug. Then, 2.0 mL of a benzene/petroleum ether (1:1) solution was added and vibrated. After 15 min, 12.0 mL H_2O was added to wash the upper layer, which contained the corresponding fatty acid methyl esters (10). The analysis was done on a Varian (Palo Alto, CA) 3700 gas chromatograph at a column temperature of 200–220°C and a detector temperature of 280°C, and was recorded on a C-R_{3A} Shimadzu recorder (Kyoto, Japan). Quantitation of each component was based on scan area normalization. In addition, an analysis on a MAT 312/SS_{200} chromatograph-mass spectrometer (Finning-MAT Co., Bremen, Germany) computer on-line instrument was performed under the following conditions: vacuum, 10^{-6} E; ion ventrical temperature, 200°C; accelerating voltage, 3 k V; and ionic potential, 70eV.

RESULTS AND DISCUSSION

Nature of Lipase 8901 from Aspergillus *sp.* Lipase 8901 was obtained from an as-yet-unidentified *Aspergillus* species. It had a specific activity of 60,000 U/g, which was higher than for any other lipase in the literature except for *Rhizopus arrhizus* (90,000 U/g) (11). Lipase 8901 activity remained unchanged in a refrigerator at 4°C for more than two years. TLC was utilized to follow the process of enzymatic lipolysis and to analyze the hydrolysates. The sequence of spots, starting from the origin, was as follows: monoglyceride, 1,2-diglyceride, 1,3-diglyceride, fatty acid, and triglyceride, as in Tahoun *et al.* (12). Oils and fats were rapidly hydrolyzed to fatty acids and glycerol at the initial stage of the reaction, with only small amounts of monoglycerides and diglycerides remaining at the end, and the amounts of 1,2-diglyceride and 1,3-diglyceride were apparently equal based on the relative spot areas on TLC. This indicates that Lipase 89801 lacks positional specificity.

Determination of optimal temperature and pH. Optimal temperature and pH for Lipase 8901-catalyzed hydrolysis were 37°C (Fig. 1) and 6.5–7.0, respectively. The hydrolysis temperature range examined was confined to the lipase's thermal stability and the substrate's meltability. Generally, lipolysis could be carried out at 30–40°C. As shown in Figure 2, only a narrow range of pH permitted enzyme activity. Lipase 8901 was not suitable for alkaline hydrolysis (13).

Effect of components of oils and fats. The fatty acid components released from oils and fats were analyzed by GC/MS to observe their effect on the hydrolysis. Olive oil and soybean oil, which are highly unsaturated, hydrolyzed most quickly (Table 1). Also, the more unsaturated fatty acids present, the faster the triglycerides hydrolyzed. These results agree well with previous publications (1,14). Because they are easily hydrolyzed substrates, highly unsaturated oils permit the use of reduced enzyme concentration and reaction time for complete hydrolysis.

The solidifying points of palm oil and the hardened oil were relatively higher (47 and 69°C, respectively) because they contain large amounts of saturated long-chain fatty acids. Only when initially dissolved in organic solvents could they be enzymatically hydrolyzed at 37°C.

Effect of enzyme concentration and reaction time. Table 2 summarizes the degree of hydrolysis at different lipase concentrations. Two interesting conclusions could be drawn.

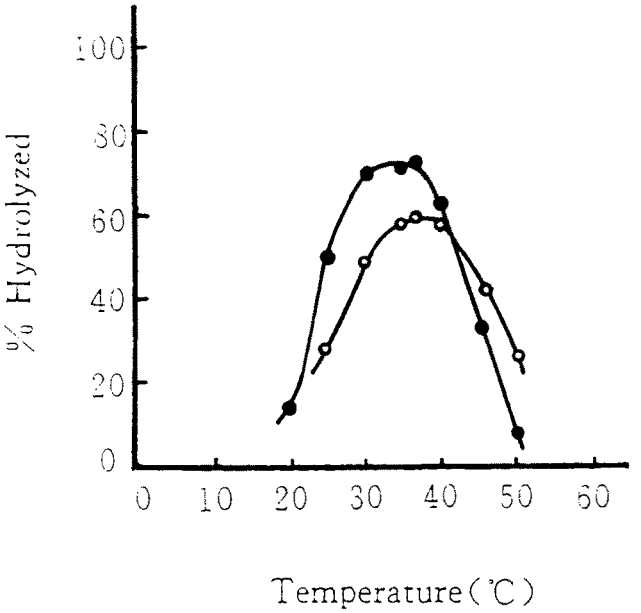

FIG. 1. Effect of temperature on lipolysis: ●, 40 U/meq mink fat, 2 h, pH 7.0; ○, 60 U/meq coconut oil, 1 h, pH 7.0. 1 meq corresponds to 1/3 millimole of lipid.

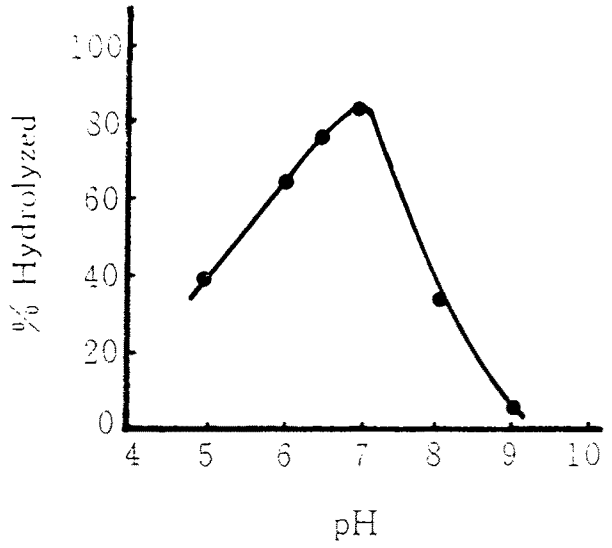

FIG. 2. Effect of pH on lipolysis: 40 U/meq mink fat, 2 h, 37°C.

Firstly, substrate hydrolysis increased noticeably with the increase of lipase concentration. If the concentration of enzyme was greater than 30 U/meq for olive oil and soybean oil, or greater than 60 U/meq for mink fat and lard, or greater than 120 U/meq for coconut oil, the amount of lipid hydrolyzed remained virtually constant. Secondly, the extent of hydrolysis greater than 95% for olive oil and soybean oil was reached in the first hour, whereas only 70% or less of the other three substrates could be hydrolyzed in 1 h, even at enzyme concentrations as high as 180 U/meq. Therefore, no matter how high the enzyme concentration, saturated fats were not rapidly hydrolyzed. These results also show that lipase concentration needs to be optimized for different oils.

Similar behavior was observed for the hydrolysis of palm oil and the hardened oil. Because Lipase 8901 has reduced activity at the solidifying temperatures of these triglycerides, the amount of enzyme required for the reaction is much greater. If the degree of hydrolysis vs. logarithm of enzyme concentration is plotted, an almost straight line can be obtained, provided that the reaction time was long enough to hydrolyze the oil.

As for the reaction time, two stages appear to be involved during the lipolysis process. In Figure 3A, the percent hydrolyzed is a function of reaction time. Olive oil was the most rapidly hydrolyzed with the least amount of lipase to obtain 95% hydrolysis, and mink fat required an additional 3 h, and palm oil was the most difficult substrate to hydrolyze due to its high solidifying point. Figure 3B shows the effect of the type of oil at a constant enzyme concentration. For soybean oil, the change in rate was around 1 h, 3 h for lard, and 5 h for coconut oil. The time required for this rate change varied with the substrates. It can be concluded that the initial rate of hydrolysis and time to the second stage, and levelling off of the percent hydrolyzed is related to the difference in fatty acid components in the substrate.

Because both enzyme concentration and reaction time were proportional to the percent hydrolyzed at a definite time, complete hydrolysis can be achieved by either lengthening the reaction time at low enzyme concentration or increasing the enzyme concentration for a shorter reaction time. The former is more preferable for industrial production when using an expensive enzyme. To achieve 90–98% hydrolysis for coconut oil and other oils, 5–30 U/meq of enzyme concentration were required if lipolysis was carried out as long as 24 h.

Effect of Ca^{2+} and organic solvents. Ca^{2+} served as an enzyme activator with all substrates. The data in Table 3 show percent of mink fat hydrolyzed with and without the presence of 0.1M $CaCl_2$. The addition of Ca^{2+} to Lipase 8901 improved the yields of fatty acids by about 30–40% in the beginning period of the reaction. However, varying the concentration of Ca^{2+} from 5 to 50% did not have an appreciable effect on lipolysis. This conclusion is somewhat in contradiction to a previous study (8).

The effect of organic solvents on lipolysis was also investigated. Isopropanol and propanone adversely affected the lipase activity, whereas hexane and isooctane had no such inhibitory action (90% of the hardened oil dissolved in isooctane was enzymatically hydrolyzed into fatty acids at 30 U/meq). This phenomenon is probably due to the interaction between polar groups of the enzyme active sites and polar organic solvents.

Comparison of catalytic action between Lipase 8901 and Lipolase from A. oryzae. The high catalytic activity of Lipase 8901 was verified by direct comparison with Lipolase from *A. oryzae*, offered by Novo Nordisk as a commercial detergent enzyme agent. The hydrolysis results with these two lipases under the same conditions are listed in Table 4. As shown in Table 4, even at the same enzyme activity concen-

TABLE 1
Effect of Components of Oils and Fats on Hydrolysis[a]

	Saturated fatty acid (%)					Unsaturated fatty acid (%)				
Substrate	C12:0	C14:0	C16:0	C18:0	Total	C16:1	C18:1	C18:2	Total	% Hydrolyzed
Olive oil			13.8	2.3	16.1		64.7	17.7	82.4	68.0
Soybean oil		6.5	11.2	3.4	21.1		20.9	54.3	75.2	47.1
Mink fat		6.2	24.5	2.7	33.4	22.7	26.4	8.5	57.6	35.7
Lard		1.5	26.3	14.6	42.4	2.6	48.5	4.3	55.4	29.9
Palm oil		1.7	63.2	5.5	70.4		24.0	2.2	26.2	
Coconut oil	43.8	16.2	18.3	5.7	84.0		13.5	2.3	15.8	26.8
Hardened oil		1.2	24.9	63.5	89.6		2.4		2.4	

TABLE 2
Effect of Enzyme Concentration on Percent Hydrolyzed[a]

	Enzyme concentration (U/mg)						
Substrate	3	6	30	60	120	180	250
Olive oil	30.8	57.9	89.4	95.0	98.3	99.2	99.0
Soybean oil	14.2	31.5	84.6	96.2	97.5	97.0	
Mink fat		26.5	60.4	72.5	68.2	62.7	
Lard		25.6	47.5	68.0	68.3	65.1	
Coconut oil	4.7	23.8	41.7	46.1	60.9	56.3	50.8

[a]Substrate was hydrolyzed for 1 h at 37°C, pH 7.0.

TABLE 3
Effect of Ca^{2+} on Percent Hydrolyzed[a]

	Reaction time (h)						
Additive	1	2	3	4	5	10	24
0.1M $CaCl_2$	65.0	76.8	83.6	84.2	89.9	96.4	98.0
—	28.5	40.0	51.9	59.6	70.0	83.4	94.9

[a]Mink fat was hydrolyzed at 37°C, pH 7.0, and 40 U/meq.

TABLE 4
Percent Hydrolyzed with Lipolase and Lipase 8901[a]

		Enzyme concentration (U/meq)			
	Reaction	Lipolase		Lipase 8901	
Substrate	time (h)	50	100	50	100
Mink fat	1	15.6	19.0	70.0	70.3
	2	21.9	25.3	77.1	74.5
Lard	1	19.6	24.2	66.2	68.6
	2	21.2	26.4	74.0	77.5

[a]Reaction conditions: 37°C, pH 7.0, and 0.05% (wt) $CaCl_2$.

tration (U/meq), the percent hydrolyzed by Lipolase was lower than that of Lipase 8901 by 40–50%. Two reasons are likely responsible for these results. Firstly, Lipolase has 1,3-positional specificity, while Lipase 8901 has no such selectivity, and the substrates can be rapidly and completely catalyzed to the corresponding fatty acids. Secondly, Ca^{2+} has a much stronger activation effect on Lipase 8901 than on Lipolase.

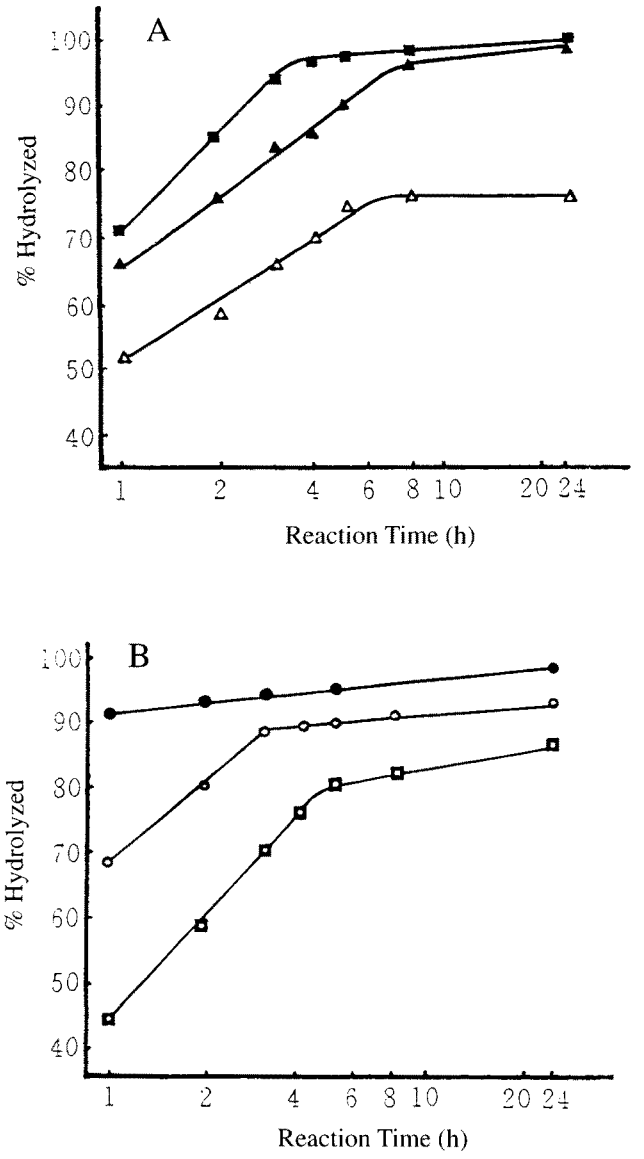

FIG. 3. Effect of reaction time on lipolysis. Substrate was hydrolyzed at 37°C (palm oil, 47°C), pH 7.0. A: ■, 12 U/meq olive oil; ▲, 40 U/meq mink fat; and △, 4000 U/meq palm oil. B: ●, 60 U/meq soybean oil; ○, 60 U/meq lard; and □, 60 U/meq coconut oil.

Results of lipase recovery. An experiment was designed to assess whether Lipase 8901 could be recovered from a reaction mixture and could be reused in a second hydrolysis reaction. The initial hydrolysis was performed with a mixture of oil, water, and lipase without a buffer and $CaCl_2$. At the end of the reaction period, most of the lipase accumulated in a emulsion layer between the fatty acids and the glycerol–water after the reaction due to the surface activity of the lipase. This emulsion layer could be separated from the lipolysis products. As shown in Table 5, the percent of lipase recovered in the emulsion was about 60% for coconut oil and 36% for lard because coconut oil could be hydrolyzed at the enzyme's optimal temperature of 37°C, whereas the lard required relatively higher temperature conditions of 40–50°C, which reduced the amount of enzyme recovered after hydrolysis.

A fraction of the total lipase remained in the glycerol–water, and about 30% of the lipase added could be recovered from this layer. This fraction was not reused because processing a large amount of glycerol–water by centrifugal sedimentation would be rather inappropriate in practice. The total lipase recovery was 94.3% for coconut oil and 65% for lard (Table 5).

Two comparison experiments were performed to verify the catalytic activity of the recovered lipase. First, both original and total 94.3% recovered lipase were used in hydrolyzing 150 g coconut oil for 12 h, which resulted in 95.8 and 94.7% hydrolysis, respectively. Second, all original and recovered lipase from emulsion and glycerol–water were used in hydrolyzing 150 g coconut oil for 4 h. The original lipase hydrolyzed 78.2% of the oil, the recovered emulsion lipase hy-

TABLE 5
Percent of Lipase Recovered After First Batch of Hydrolysis

Substrate	Lipase for hydrolysis	Lipase activity (U)	% of Lipase recovered
Coconut oil	Original lipase	36467	
	Recovered lipase from emulsion	22148	60.7
	Recovered lipase from glycerol–water	12239	33.6
Lard	Original lipase	36389	
	Recovered lipase from emulsion	13114	36.0
	Recovered lipase from glycerol–water	10578	29.0

drolyzed 72.5% of the oil, and the water-layer lipase was able to hydrolyze 50.8% of the oil.

To examine the effectiveness of enzyme recycling and reuse, a series of hydrolysis reactions was performed. The experiment consisted of six consecutive batches of hydrolysis, starting with the original enzyme being used in the first batch, and every subsequent batch was carried out with recovered emulsion lipase from the previous reaction and the recruited lipase. For coconut oil and lard, the recruited lipase were 35–40 and 65–70%, respectively. The recovered lipase could be cycled repeatedly to use within one week without an appreciable decrease in its activity. The average percentage of lipase recovered was 65% for coconut oil and 30% for lard. Moreover, the average percentage of substrate hydrolysis was over 92% for both coconut oil and lard.

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