Enzymic Hydrolysis of Animal Fats in Organic Solvents at Temperatures Below Their Melting Points

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Lipase from *Candida rugosa* catalyzed the hydrolysis of inedible beef tallow and pork lard (edible and inedible) in the presence of organic solvents at temperatures below the melting point of the fat. Reactions were carried out at 50% substrate with 180 lipase units per gram of fat in a two-liter reactor. In the presence of isooctane (5–10%) beef tallow yielded 94% hydrolysis in 24 hr both at 37° and 31°C. Edible pork lard yielded 97% hydrolysis under these conditions and at temperatures as low as 25°C, while inedible lard gave hydrolysis intermediate between the other two fats.

KEY WORDS: Animal fats, hydrolysis, lipase, organic solvents.

Enzymatic hydrolysis of fats is an attractive alternative to the currently used, high-pressure and high-temperature process for the industrial production of fatty acids and glycerol. For this reason, it is being actively investigated (1–4). Because of their high melting points, hydrolysis of animal fats is, in general, more difficult than that of liquid oils (such as most vegetable oils), and there are fewer studies with the former substrates. Yet, beef tallow and pork lard are important industrial raw materials. Highly active thermostable lipases suitable for hydrolysis of solid fats are still at the research level (5,6) or under development (7). Of all the lipases currently available in large quantities, that of the yeast *Candida rugosa* is most suitable for complete fat hydrolysis on account of its kinetic characteristics (8–10), and because high activity preparations have recently become available. However, commercial preparations of this lipase are not stable at (or above) the melting points of these animal fats (5) for the time necessary to achieve a high degree of hydrolysis.

Nevertheless, as we report in this paper, this lipase can be employed successfully, provided these solid fats are liquified at temperatures below their melting points by the addition of small amounts of organic solvents. For this reaction to be of practical application, it must be carried out at high substrate concentrations and it must yield a high percentage of hydrolysis. In the present contribution we report the results of our investigations realized under these conditions in a two-liter reactor.

EXPERIMENTAL PROCEDURES

Materials. Inedible beef tallow and pork lard were provided by Lascaray S.A. (Vitoria, Spain). Edible, bleached pork lard was purchased from Grasas Guijuelo, S.A. (Salamanca, Spain). Olive oil was purchased from a local market. All three substrates were used without further purification. Lipase from *Candida rugosa* (Lipase OF, formerly *C. cylindracea*) was purchased from Meito Sangyo, Ltd. (Tokyo, Japan). The activity of the powder was 360 units/mg of product (as stated by the manufacturer). All other reagents were analytical reagent grade or better.

Methods. The animal fats used in this study did not exhibit a well-defined melting point. For our purposes, the melting point was considered to be the temperature at which a given fat became liquid, although the liquid was not yet transparent. These melting temperatures were: inedible beef tallow, 42°C; inedible pork lard, 40°C; edible pork lard, 39°C.

Enzymatic hydrolyses were carried out in a two-liter glass reactor (Biolafitte, St. Germain, France), provided with a water jacket, temperature and agitation control. Reaction mixtures contained 500 g animal fat or 500 mL olive oil, organic solvent and lipase dissolved in various amounts of water to a final volume of 1000 mL. The amounts of water and organic solvent used in each case are indicated in the corresponding tables. Lipase powder was dissolved in tap water because preliminary experiments indicated no loss of activity in unbuffered deionized or tap water. These preliminary determinations of enzymic activity were carried out in two ways. Initial velocities for the hydrolysis of olive oil (as model substrate) were determined as described (11) in 0.1 M potassium phosphate buffer pH 7.0, unbuffered deionized or tap water. The enzymic activity of lipase dissolved in buffer, unbuffered deionized or tap water was also determined by calculating the degree of hydrolysis obtained after 24 hr under the same conditions as described in Table 2 with no isooctane added. Therefore, lipase solutions for the experiments reported herein were prepared by dissolving the required amount of enzyme in tap water.

Fat (or oil) was mixed with the specified amount of organic solvent in the reactor and equilibrated at the desired temperature with an agitation of 700 rpm until a homogeneous solution was obtained. Reactions were started by the addition of lipase solution previously equilibrated at the reaction temperature. The degree of hydrolysis obtained in 24 hr varied slightly with increasing agitation speed (96.0% at 300 rpm, 97.5% at 500 rpm, 96.5% at 700 rpm, and 96.0% at 1000 rpm). A constant speed of 700 rpm was used throughout this study to provide good mixing with beef tallow at lower temperature.

Samples were taken at intervals and placed in a 90°C water bath to inactivate the enzyme and separate the aqueous and organic phases. Separation of the phases was achieved in 10 min but the interphase was not well defined, and samples were routinely left at 90°C for one hour. After this period of time the organic layer was withdrawn. The remaining solvent and small amount of water contained in the organic layer were removed by heating at 115°C for a few minutes. To calculate the degree of hydrolysis (or percent hydrolysis) of the samples, acid values (AV) and saponification values (SV) were determined by titration as described (12). Blanks

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without oil or fat were also prepared. Saponification values (SV) for the beef tallow, pork lard and olive oil used were 207, 205 and 202, respectively. The degree of hydrolysis (or percent hydrolysis) was calculated by the following equation: percent hydrolysis = 100 × AV/SV.

The precision of this titration method was determined as follows. A series of standards were prepared by mixing known amounts of free stearic acid with unmodified triglyceride. These standards contained 85%, 90%, 92%, 94%, 96%, 98% and 99.5% free fatty acid. They were treated and their AV determined in triplicate as described above. The differences in the AVs obtained were significant ($P<0.001$).

To determine the fatty acid composition of the substrates, samples were saponified and free fatty acids were extracted with hexane after acidification. Samples were dissolved in tetrahydrofuran (THF) and separated without further modification on two 25 cm × 1/4″ columns (Spherisorb ODS-2.5 μm) connected in series, essentially as described by Hammond and Irwin (13). The mobile phase used was THF: acetonitrile: water (5:70:25 v/v/v) containing 0.1% acetic acid. The underivatized fatty acids were detected with a Konik UVIS-200 detector at 214 nm.

RESULTS

Amount of enzyme. In a preliminary experiment, the minimum amount of lipase needed to achieve a degree of hydrolysis above 90% was determined in the presence of isooctane (Table 1). This solvent was selected to liquify beef tallow at 37°C based on previous studies (10,14,15), which reported it to be the least disrupting for this enzyme activity. Over 94% hydrolysis was obtained in 24 hr with as low as 180 units/g of fat. From the point of view of practical applications, amounts of enzyme higher than 360 units/g of fat may increase excessively the cost of the overall process. Conversely, the use of less enzyme which yielded degrees of hydrolysis below 90% would result in increased downstream processing costs. Lower amounts of enzyme than those used in the present study can also yield 90% hydrolysis provided the reaction time is extended to 72 hr, in agreement with the results of Linfield and co-workers (9). Because long reaction times are inadequate for practical applications, it was decided to limit it to 24 hr with 180 lipase units per gram of fat, which represents 0.5 g of commercial enzyme powder per kilogram of fat.

Nature of the solvent. Several organic solvents of different hydrophobicities were used to liquify beef tallow at 37°C and the extent of hydrolysis in the presence of 180 units/g fat was determined at various time intervals up to 24 hr (Fig. 1). Degrees of hydrolysis above 90% were obtained only in the presence of isooctane and n-heptane. More polar solvents, such as acetonitrile, greatly inhibited the hydrolysis reaction. These results are consistent with those of Mukataka and co-workers (16) who showed that lipase had higher activity in branched hydrocarbons than in straight-chain hydrocarbons.

The reaction in isooctane proceeded at a slightly higher overall rate than with no solvent, although essentially the same extent of hydrolysis was obtained in 24 hr. The optimum amount of isooctane was between 50 and 100 mL, which corresponds to 5% and 10%, respectively (Table 2). Lower degrees of hydrolysis were obtained with higher and lower concentrations of solvent.

Effect of temperature. We have observed in parallel investigations that at high substrate concentrations (between 40% and 60%) the optimum temperature for

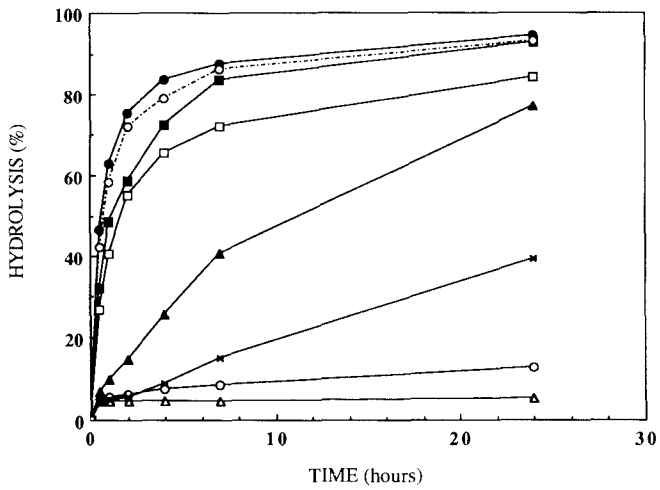

FIG. 1. Time course for the hydrolysis of beef tallow in the presence of 100 mL organic solvent and 400 mL water. Reactions were carried out at 37°C as described in Experimental Procedures with 180 lipase units/g fat. Isooctane: ●; n-heptane: ■; ethyl ether: ▲; n-pentane: □; chloroform: ○; acetonitrile: △; tetrahydrofuran: ×; without organic solvent: ····○····.

TABLE 1

Effect of the Amount of Lipase on Beef Tallow Hydrolysis[a]

Lipase units/g beef tallow	% Hydrolysis[b]
504	99.1
360	95.1
180	94.3
36	81.5

[a] Reactions were carried out as described in Experimental Procedures at 37°C in the presence of 100 mL isooctane and 400 mL water.
[b] Hydrolysis obtained in 24 hr.

TABLE 2

Hydrolysis of Beef Tallow in the Presence of Different Amounts of Isooctane[a]

Isooctane (mL)	Water (mL)	% Hydrolysis[b]
0	500	93.1
10	490	91.3
30	470	91.4
50	450	94.3
100	400	94.3
150	350	92.0
200	300	92.1
300	200	89.6

[a] Reactions were carried out as described in Experimental Procedures with 180 lipase units/g fat at 37°C.
[b] Hydrolysis in 24 hr.

hydrolysis with the Candida lipase is around 30°C (Mertxe de Renobales and co-workers, unpublished observations). Thus, we wished to see if this would also be the case in the presence of isooctane. To liquify the substrate at temperatures below 37°C, higher amounts of solvent had to be used. For example, at 25°C beef tallow could not be liquified in less than 30% isooctane. Essentially the same maximum degree of hydrolysis (94%) was obtained at 37°C and 31°C, although the reaction was carried out in higher concentration of isooctane at 31°C (Table 3). Isooctane appeared to improve the hydrolysis reaction at lower temperatures. In the presence of 30% isooctane, the percentage hydrolysis at 25°C (Table 3) was considerably higher than that at 37°C under the same conditions (Table 2).

Other substrates. Pork lard is an industrial raw material used for the preparation of fatty acids and glycerol. Its characteristics are similar to those of beef tallow except that it has a higher content of unsaturated fatty acids (Table 4). Of the three types of animal fats used in the present study, edible pork lard had the highest content of unsaturated fatty acids (oleic and linoleic), while that of beef tallow was the lowest. The degree of hydrolysis obtained with edible lard was the highest for the three animal fats tested (Table 5). As the temperature of the reaction decreased, and the amount of isooctane present had to be increased, the degree of hydrolysis did not vary significantly. At 37°C the results obtained with inedible lard were intermediate between those of tallow and edible lard. Olive oil was included as a comparison because it is a frequently used substrate. The degree of hydrolysis obtained in the absence of isooctane with olive oil was comparable to that of edible lard, both at 31° and 37°C. Contrary to the results obtained with the three animal fats, olive oil yielded slightly lower degrees of hydrolysis in the presence of isooctane.

The time course of the reaction with olive oil, edible lard and inedible tallow showed large differences in the hydrolysis obtained during the first two hours in the presence of 10% isooctane (Fig. 2), with olive oil clearly being the best substrate. At the end of 24 hr these differences were much smaller. Essentially the same results were obtained with these three substrates both at 37°C and 31°C. While 24 hr were needed with beef tallow to reach 95% hydrolysis, the same result could be obtained with pork lard in only 7 hr, permitting a considerable economy of time.

TABLE 3

Hydrolysis of Beef Tallow at Different Temperatures[a]

Temperatures (°C)	Isooctane (mL)	Water (mL)	% Hydrolysis[b]
31 ± 1	100	400	94.2
	150	350	94.1
	200	300	93.9
	300	200	91.5
25 ± 1	300	200	92.6

[a] Reactions were carried out as described in Experimental Procedures with 180 lipase units/g fats.

[b] Hydrolysis in 24 hr.

TABLE 4

Fatty Acid Composition of the Substrates

Fat	Percent distribution				
	Palmitic	Stearic	Oleic	Linoleic	Others
Inedible beef tallow	18.8	14.3	48.4	5.6	12.4
Inedible pork lard	23.1	13.9	51.4	6.1	5.5
Edible pork lard	24.3	8.2	58.0	5.4	4.2
Olive oil	7.5	2.8	75.5	6.6	7.6

TABLE 5

The Hydrolysis of Different Substrates in the Presence of Varying Amounts of Isooctane[a]

Substrate	Temperature (°C)	Isooctane (mL)	Water (mL)	% Hydrolysis[b]
Edible pork lard	37 ± 1	0	500	96.4
		50	450	97.0
		100	400	97.1
	31 ± 1	50	450	97.1
		100	400	96.6
	25 ± 1	200	300	96.9
Inedible pork lard	37 ± 1	0	500	94.9
		100	400	95.5
Olive oil	37 ± 1	0	500	96.7
		100	400	95.7
	31 ± 1	0	500	97.1
		50	450	96.2

[a] Reactions were carried out as described in Experimental Procedures with 180 lipase units/g fat.

[b] Hydrolysis in 24 hr.

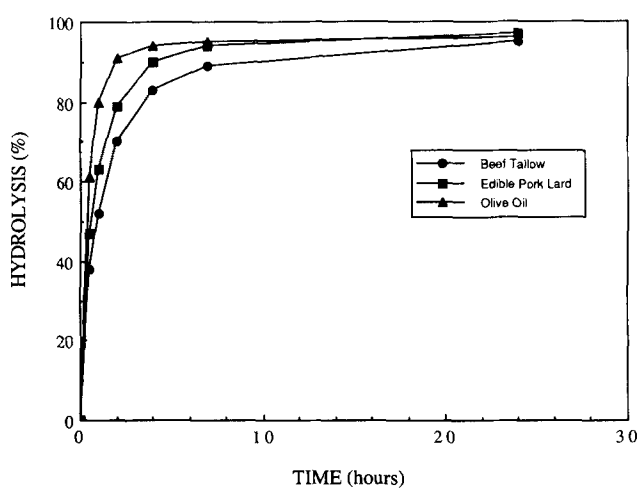

FIG. 2. Time course for the hydrolysis of beef tallow (■), edible pork lard (▲) and olive oil (●). Reactions were carried out as described in Experimental Procedures, at 37°C and in the presence of 100 mL isooctane and 400 mL water.

DISCUSSION

The hydrolysis of high-melting animal fats has been studied for practical applications at high substrate concentrations, and at temperatures below their melting points to minimize thermal denaturation of the lipase employed. The results presented in this paper show that the temperature range for the hydrolysis of these fats can be significantly extended by including in the reaction mixture small amounts of isooctane to liquify the substrates at temperatures as low as 25°C. Of all the solvents tested, hydrocarbons and particularly isooctane were the only ones in which high degrees of hydrolysis were obtained. These results suggested that the polarity of the solvent could play an important role in lipase activity (17), although at present the underlying mechanism is not clearly understood.

Over 90% hydrolysis was consistently obtained in 24 hr under the conditions presented, reaching 97% with edible pork lard. This substrate appeared to be better than beef tallow and inedible lard for several reasons in addition to the significantly higher hydrolyses obtained: i) lower amounts of isooctane were necessary; ii) 95% hydrolysis could be obtained in slightly over one-third the time needed with beef tallow; iii) the temperature and amount of isooctane did not seem to influence the final degree of hydrolysis.

Although *Candida* lipase is non-specific with respect to the fatty acid positions in the triacylglycerol, it does exhibit some selectivity toward fatty acids with a *cis* double bond in position 9 (8). The degree of hydrolysis obtained during the first few hours of the reaction at 37°C with the three animal fats tested, both in the absence and in the presence of isooctane (10%), correlates well with their content of unsaturated fatty acids. The same correlation is observed with olive oil during the first few hours of the reaction. The presence of 10% isooctane improved the degree of hydrolysis obtained with the three animal fats, but it appeared to have a slightly deleterious effect with olive oil. The positive effect of isooctane could in part be due to the fact that it liquified solid fats, resulting in better mixing of the two phases. Linfield and co-workers (9) observed almost a 70% inhibition of beef tallow hydrolysis in the presence of 50% hexane, both at 40°C and 25°C, with respect to the same reaction at 40°C without solvent. It is not clear why their results are so different from the data presented in this paper. Our results demonstrate that a high degree of hydrolysis can be obtained with beef tallow at 25°C in the presence of isooctane.

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