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Kinetic Properties of Soluble and Immobilized *Candida rugosa* Lipase

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ABSTRACT

Immobilized lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) from *Candida rugosa* has been immobilized on commercially available microporous polypropylene and used for the batch hydrolysis of different animal fats. The effect of the reaction products at concentrations similar to those obtained at 90% hydrolysis, both on soluble and immobilized lipase, was studied. Glycerol showed low inhibitory effect but oleic acid caused 50% inhibition. A mixture of free fatty acids present in the complete hydrolysis of beef tallow inhibited lipase activity more than 70%. The stability of the enzyme (both soluble and immobilized) was highest in the presence of 20% isooctane. The apparent Michaelis constant for each substrate for the soluble enzyme did not change on immobilization.

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INTRODUCTION

Enzymatic hydrolysis of fat and oils has been studied intensively as an alternative method for the conventional chemical method (Colgate-Emery) used for the industrial production of fatty acids and glycerol. This chemical hydrolysis is carried out for 2 h at 30–50 atm and at 250°C and provides poor quality free fatty acids (FFA) and a diluted solution (12% v/v) of glycerol. The resulting FFA must be redistilled to remove the colored substances and other contaminating products. In addition to the problems derived from the undesired side reactions occurring at high temperatures between unsaturated FFA, the process is highly energy consuming and requires expensive reactors.

Enzymatic hydrolysis with lipases offers several advantages over chemical hydrolysis, since it can occur at atmospheric pressure and at 30–50°C. Moreover, because of the specificity of lipases, negligible amounts of undesired products are obtained and no purification of products is required for many applications.

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of triacylglycerols. The most widely studied lipases for this application are microbial extracellular enzymes produced by fermentation of some yeasts, fungi and bacteria. Among these, the lipase from *C. rugosa* is *a priori* one of the most attractive commercially available lipases for the complete hydrolysis of triacylglycerols because of its ability to liberate all types of acyl chains, regardless of their position in the glycerol (*1*).

As it is well known, hydrolysis of fats by lipase occurs at the interphase between the organic and aqueous phases of the reaction. For this reason, since the early work of Benzonana and Desnuelle (*2*), the substrate concentration and the value of K_m have been expressed as interfacial area per volume. Recently, Kierkels et al. (*3*) demonstrated that the total amount of interfacial area changes as the reaction progresses employing R,S-glycidyl butyrate in a lipase-esterase type reaction. The particle size of various hydrolysis reactions with different animal fats at high substrate concentrations (prepared as described in 4) vary with the size of the reactor (1, 2, and 30 L) (de Renobales et al., unpublished results). In addition, model substrates are frequently used (*3,5,6*) in this type of study. For these reasons, we report in this paper a kinetic study of soluble and immobilized *C. rugosa* lipase in terms of the fat concentration in the reaction mixture, a parameter that might be easier to use in an applied situation for reactor design and scaleup.

MATERIALS AND METHODS

Materials

Solid lipase OF (360 U/mg of solid) from *Candida rugosa* (formerly *Candida cylindracea*) was obtained from Meito Sangyo Co. (Tokyo, Japan) and used without further purification. Microporous polypropylene powders Accurel® EP-100 (particle size 200–400 μm) were purchased from Enka AG (Obernburg, Germany). Edible pork lard (mp 38–40°C) was obtained from Grasas Guijuelo, S.A. (Salamanca, Spain). Inedible pork lard (mp 39–41°C) ad beef tallow (mp 41–43°C) were provided by Lascaray, S.A. (Vitoria, Spain). Olive oil (maximum acidity 0.3°) was purchased in a local market. Fat-free bovine serum albumin (BSA) was from Sigma Chemical Co. (St. Louis, MO). Oleic acid and glycerol were from Merck (Darmstadt, Germany). All other organic solvents and chemicals used were of analytical reagent grade or better.

Enzyme Immobilization

Lipase was immobilized as previously described (*7*). One gram of polypropylene powder wetted with 2.5 mL ethanol was incubated with 50 mL of a lipase preparation (0.25 mg solid lipase OF powder/mL) adjusted to pH 7.0 with 0.1*M* potassium phosphate buffer. The mixture was stirred for 90 min at room temperature, and the immobilized lipase was collected by filtration under reduced pressure and washed with 3 vol of distilled water to remove completely the residual unadsorbed soluble enzyme. From now on the term immobilized lipase will mean lipase-loaded support.

Enzyme Assays

Lipase activity was assayed essentially as described (*7*) at 37°C in 0.1*M* potassium phosphate buffer, pH 7.0 (standard buffer) by measuring the release of free fatty acids (FFA) from edible pork lard (unless otherwise stated). The substrate emulsion was prepared by mixing the melted fat, BSA (25 mg/mL) and standard buffer in the ratio 1 g: 1 mL: 2.7 mL, respectively. The mixture was emulsified by sonication for 4 min (at 12 microns of amplitude) at room temperature in a Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT). The resulting preparation contained 208 mg fat/mL.

Soluble lipase was assayed for 10 min in continuously shaken vessels containing 1 mL of standard buffer and 1 mL of the substrate emulsion. The reaction was initiated by the addition of 50 μL of a soluble enzyme preparation (0.4 mg solid lipase OF/mL standard buffer) and stopped by the addition of 5 mL of the mixture composed of isopropanol, *n*-hexane, and 2*N* H_2SO_4 (40:10:1, v/v/v) followed by 3 mL of water and 3 mL of

n-hexane. After vigorous stirring, aliquots of 2 mL of the upper organic phase were withdrawn and titrated with a 10 m*M* ethanolic KOH solution, using phenolphthaleine as a pH indicator.

Immobilized lipase activity was determined using the same conditions and reaction mixture as those used with the soluble enzyme except that the reaction was started by the addition of 1 mL of substrate emulsion to the vessel containing 1 mL of standard buffer and 10 mg of immobilized lipase. The reaction time was 30 min. The reaction was stopped and FFA evaluated as above.

One unit of enzyme activity (*U*) was defined as the amount of enzyme that catalyzed the production of 1 μmol of FFA/min under the indicated experimental conditions.

Effect of Reaction Products and Solvents

The effect of glycerol and free fatty acids (a mixture obtained from a beef tallow hydrolysate) as well as the presence of different solvents on the lipase activity were tested using the assay conditions described above, but the corresponding buffer volume was replaced by the product or solvent in a final volume of 2 mL.

Soluble Lipase Stability

The stability of the soluble enzyme was studied at 37°C in the presence of solvents as follows. The enzyme preparation (500 mL) containing 0.5 mg/mL of solid lipase OF in distilled water was stirred for 24 h at 800–1000 rpm and 37°C in the presence of 20% (v/v) of different solvents. At timed intervals aliquot samples were withdrawn and the residual activity assayed by the standard procedure.

RESULTS AND DISCUSSION

Effect of Reaction Products on Lipase Activity

The effect of glycerol and a mixture of free fatty acids, obtained from a beef tallow hydrolysate, on the soluble and immobilized lipase activity is summarized in Table 1. Glycerol moderately inhibited the lipase activity. At the maximal concentration present in the fat hydrolysates (i.e., 12–15% w/v, which corresponds to 1.30–1.63*M*) only inhibition lower than 15% was observed. At concentrations three times higher, more than 60% of the initial activity was routinely measured.

However, oleic acid, the main fatty acid in olive oil, which represents nearly 50% of the total fatty acids of beef tallow (*8*), significantly, inhibited in a similar way, both the soluble and the immobilized enzyme activity.

Table 1
Inhibition of Soluble and Immobilized
C. rugosa Lipase by the Products of Animal Fat Hydrolysis[a]

Reaction product	Concentration, *M*	Inhibition, %	
		Soluble enzyme	Immobilized enzyme
None, control	—	0.0	0.0
Glycerol	0.43	0.0	5.2
	1.30	13.9	8.3
	3.91	34.3	36.0
Oleic acid	0.07	22.1	36.7
	0.22	43.1	40.1
	0.33	53.5	56.1
Mixture of FFA from a beef tallow hydrolysate[b]	0.07	51.4	64.8
	0.22	53.3	59.4
	0.33	70.7	75.2

[a]Assays were carried out as detailed in Materials and Methods.
[b]The FFA composition of the mixture was: 48.4% oleic, 18.8% palmitic, 14.3% stearic, 5.6% linoleic, and 12.4% others (*8*). An average molecular weight of 282 was assumed for this mixture of FFA.

Thus, more than 50% of inhibition was observed at about 0.33*M* oleic acid, which corresponds to the fatty acid concentration obtained when 90% of hydrolysis of fats was attained. A mixture of FFA (at a concentration equivalent to 0.33*M*) obtained by distillation from a hydrolysate of beef tallow, inhibited the lipase activity by more than 70%. The highest inhibitory capacity of this mixture compared with that of pure oleic acid can be explained by the inhibitory effect of other fatty acids (probably palmitic acid) by themselves or by some synergistic and/or cumulative effect owing to the simultaneous presence of several FFA in the reaction mixture.

Effect of Solvents on the Lipase Activity

In order to carry out hydrolysis reactions at temperatures below their melting points a number of organic solvents were used to liquefy animal fats. The presence of solvents in the reaction mixture was studied under three different points of view:

1. The effect of the initial rate;
2. The effect on the kinetic constants; and
3. The effect on the enzyme stability.

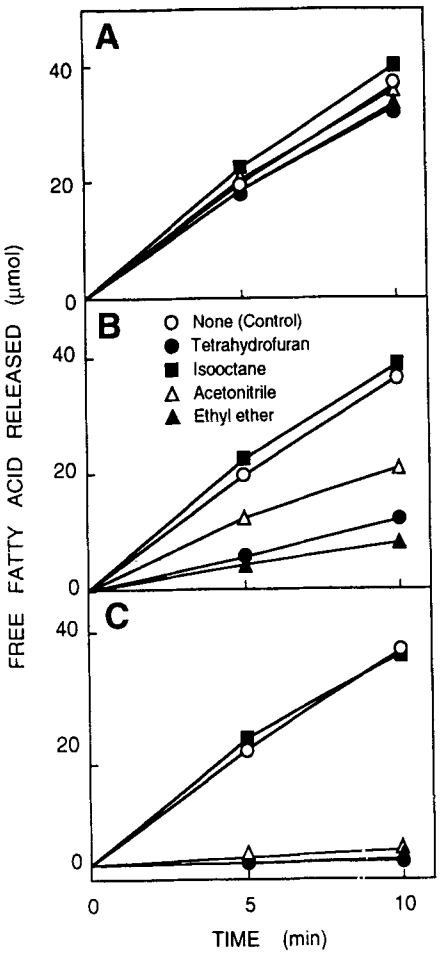

Fig. 1. Hydrolysis of edible pork lard by the soluble C. *rugosa* lipase in the presence of organic solvents. The final concentrations of solvent in the reaction mixture were: **A**, 0.5% (v/v); **B**, 2.0% (v/v); and **C**, 10% (v/v). Reaction conditions were as detailed in the Materials and Methods section.

Initial hydrolysis rates showed by the soluble (Fig. 1) and immobilized (Table 2) lipase depended on the nature and concentration of the solvent used. At lower solvent concentrations (0.5% v/v), similar rates were observed with the soluble enzyme (Fig. 1A), but at higher concentrations (10% v/v), only isooctane produced no inhibition of the activity (Fig. 1C). Similiar results were obtained for the immobilized lipase (Table 2). Only isooctane appeared to be suitable at such concentrations. The rest of the solvents at concentrations higher than 2% (v/v), led to a progressive loss of activity of both the soluble and the immobilized enzyme that depended on the polarity of the solvent. So, the higher the polarity of the solvent used, the greater the loss of enzyme activity. These results are in agreement with those obtained in hydrolysis reactions (*8,12*), where only in the presence of branched hydrocarbons (like isooctane) high degrees of hydrol-

Table 2
Effect of Some Organic Solvents
on the Activity of the Immobilized *C. rugosa* Lipase

Organic solvent	Concentration, % v/v	Relative lipase activity, %[a]
None, control	—	100.0
Isooctane	2	108.4 ± 1.4
	10	86.9 ± 11.6
Tetrahydrofuran	2	46.8 ± 0.6
	10	0.4 ± 0.5
Acetonitrile	2	44.9 ± 0.9
	10	4.6 ± 0.3
Ethyl ether	2	31.8 ± 8.2
	10	2.9 ± 0.4

[a]One hundred percent activity corresponded to 78 mU/mg protein. The activity was determined as described in Materials and Methods using edible pork lard as substrate.

Table 3
Effect of Some Organic Solvents
on the Kinetic Parameters of Soluble *C. rugosa* Lipase[a]

Organic solvent	Concentration, %, v/v	K_m apparent, mg fat/mL	V_{max} apparent, U/mg lipase
None, control	—	15.4	216
Isooctane	2	21.0	189
	4	27.0	290
Acetonitrile	2	20.0	160
	4	10.0	66
Tetrahydrofuran	2	4.0	75
	4	5.0	36
Ethyl ether	2	4.0	53
	4	8.0	35

[a]Determinations were done as described in Materials and Methods with edible pork lard as substrate.

ysis were obtained. We did not observe the significant increase in activity reported by Kim et al. (*10*) as the concentration of isooctane increased from 3 to 30% (v/v).

The effect of different organic solvents, at two concentrations, on the kinetic parameters K_m and V_{max} of soluble *C. rugosa* lipase was studied. K_m and V_{max} were calculated from linear double-reciprocal plots. As it can be inferred from the results presented in Table 3, the effect of solvent was complex. It could be explained if one assumes a direct effect on the lipase

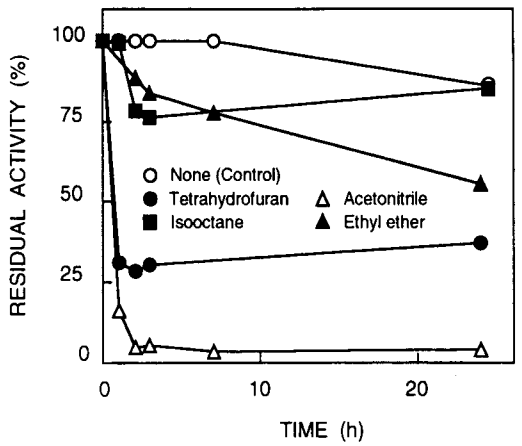

Fig. 2. Stability of the soluble *C. rugosa* lipase in the presence of organic solvents. The enzyme was incubated at 37°C for different time periods with stirring as described in the Materials and Methods section in the presence of 20% (v/v) organic solvent. The residual activity was assayed with edible pork lard as substrate. Activity was expressed as a percentage of the initial activity.

itself accompanied by some indirect effects on the enzyme microenvironment, in addition to the physicochemical characteristics of the substrate emulsion, which in turn can interfere with the substrate accessibility to the lipase active site. The values reported in Table 3 for edible pork lard, in the presence of isooctane, are of the same order of magnitude, although slightly lower, as those reported for other natural fats in the presence of isooctane (*9,13*).

The stability of the soluble enzyme at 37°C in the presence of 20% (v/v) solvents in the reaction mixture is shown in Fig. 2. In the absence of solvents *C. rugosa* lipase maintained more than 80% of its initial activity after 24 h of incubation with stirring. The addition of some polar solvents significantly decreased the stability of the enzyme. Thus, after 1 h in the presence of acetonitrile, the enzyme completely lost its activity. Residual enzyme activity after 24 h of incubation in the presence of isooctane was similar to that observed in the absence of solvents, although the inactivation profiles were different in each case. The residual activity, measured in the presence and absence of isooctane, at temperatures up to 50°C were also similar (only 4.5% of its initial activity was retained at 50°C with and without isooctane) (data not shown).

Kinetics of Lipase with Other Animal Fats

The hydrolysis time-course of several animal fats catalyzed by the soluble enzyme was assessed by following the release of FFA. As shown in Fig. 3, the hydrolysis rate was linear with time in all cases. Edible pork lard appeared to be the best animal fat substrate. The rates of hydrolysis measured with this animal fat were similar to those obtained with olive

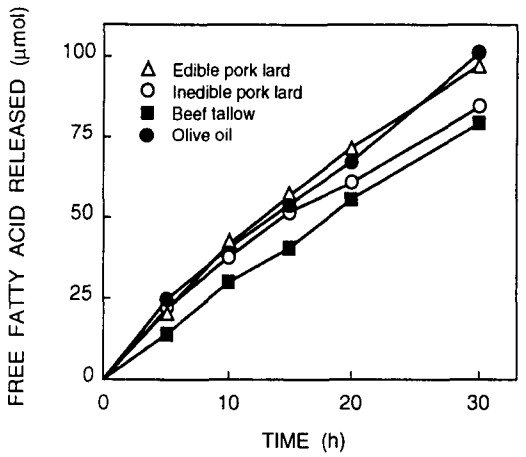

Fig. 3. Hydrolysis of different fats by the soluble *C. rugosa* lipase. Experimental details are described in Materials and Methods.

Table 4
Kinetic Parameters for the Hydrolysis of Several Animal Fats and Olive Oil Catalyzed by the Soluble or Immobilized *C. rugosa* Lipase

	$K_{m_{apparent}}$, mg fat/mL		$V_{max_{apparent}}$, U/mg[a]	
Animal fat substrate	Soluble	Immobilized	Soluble	Immobilized
Edible pork lard	15.4	20.8	216	0.11
Inedible pork lard	7.7	10.2	174	0.10
Beef tallow	6.9	5.3	146	0.09
Olive oil	11.9	9.6	24	0.14

[a]Rates are referred to mg of lipase powder (soluble enzyme) or to mg (wet wt) of enzyme loaded support (immobilized enzyme).

oil, which was included as a control substrate. The lowest hydrolysis rate was measured when beef tallow was used as a substrate.

The kinetic properties of both soluble and immobilized *C. rugosa* lipase were analyzed using different animal fats as substrates. Table 4 summarizes the apparant kinetic parameters, K_m and V_{max}. There is no significant difference between the apparent K_m values for the soluble and immobilized enzymes for each substrate. This suggests that diffusion caused by matrix support was negligible under the conditions studied.

Although *C. rugosa* lipase has been reported as an unspecific enzyme with respect to fatty acid position into the glycerol molecule, it has been demonstrated (*14*) that the enzyme shows a slight preference for fatty acids with a cis double bond in position 9 as in oleic acid. This fact is in agreement with the calculated V_{max} values, which increased with the percentage of oleic acid present in the animal fat used as a substrate (*8*). Edible pork lard showed the highest degree of hydrolysis for the three animal fats studied (*4,8*).

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