Carica papaya Latex Lipase: *sn*-3 Stereoselectivity or Short-Chain Selectivity? Model Chiral Triglycerides Are Removing the Ambiguity

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ABSTRACT: Short-chain fatty acids are usually located at position *sn*-3 in natural triglycerides, particulary in dairy fats. As a result, it is extremely difficult to differentiate between *sn*-3 stereospecificity and short-chain typoselectivity in many lipases and acyltransferases that perform in this way. This ambiguity can be removed through successive use of a chiral triglyceride with a short fatty acid in position *sn*-1 and of its racemic in controlled hydrolysis reactions. After checking that the proposed method effectively confirmed the type of activity of control biocatalysts (*Candida cylindracea* nonspecific lipase and *Mucor miehei* 1–3 regiospecific lipase), we confirmed that *Carica papaya* latex has a strict *sn*-3 stereospecificity.
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KEY WORDS: *Carica papaya* latex, chiral triglyceride, enzymatic hydrolysis, racemic triglyceride, stereospecificity, typoselectivity.

Lipases (E.C 3.1.1.3) play a predominant role in the digestion mechanism of humans and animals (1). Therefore, there is a considerable amount of literature on the hydrolysis of acylglycerols by these enzymes (2–4). A distinction can be made between three kinds of lipases: nonspecific; stereoselective, preferentially hydrolyzing one of the triglyceride positions; and typoselective lipases, which preferentially hydrolyze given fatty acids, irrespective of their positions.

There is probably no enzyme that is specific to a single fatty acid; usually, a marked preference for a category of fatty acids, such as short-chain fatty acids from C_4 to C_{10}, is observed. In dairy fats (5) and in the great majority of natural fats and oils, short-chain fatty acids are distributed in position *sn*-3. Studying *sn*-3 specificity is therefore intricate because it becomes difficult to make a distinction between short-chain fatty acid typoselectivity and *sn*-3 stereoselectivity. We recently proposed a procedure (6) for synthesis of chiral triglycerides with a short-chain fatty acid in position *sn*-1 and their corresponding racemics.

Such molecules remove ambiguity. This article concerns the hydrolytic performance of *Carica papaya* latex, an acyltransferase obtained from a plant extract known for its preference for short chains in dairy fat bioconversion operations. But, is it really short-chain fatty acid typoselective or *sn*-3 stereospecific? The approach taken in this work provides the answer.

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EXPERIMENTAL PROCEDURES

Triglycerides. The synthesis and stereochemical analysis of triglycerides were described recently (6).

Enzymes. The *Carica papaya* latex was a purified extract (Sigma, Saint Priest, France), and the granular material was ground up before use. The lipozyme IM (Novo Nordisk, Copenhagen, Denmark) was the lipase from *Mucor miehei*, fixed on an ion exchange resin. The *Candida cylindracea* lipase (Sigma) was a preparation that contained lactose.

Hydrolysis. Seventy mg of triglycerides was added to 2.3 mL of 0.2M Tris HCl buffer (pH 8); then 10 mg of enzyme preparation was added and shaken at 40°C for a time, t, in an ultraturax (IKA-Werk, Paris, France) at 10,000 rpm to obtain a hydrolysis rate of about 10% (t = 2 min for *Carica papaya*, t = 30 s for *Candida cylindracea* and for Lipozyme IM).

The hydrolysis was stopped by the addition of 1.5 mL of saline acid solution (0.1M H_2SO_4, 2.5M NaCl). The fat was extracted three times with 3 mL of ether/heptane solution (75:25, vol/vol). The lipolysis products were then separated by thin-layer chromatography, and the sample was applied with an automatic applicator (Camag Linomat III; Camag, Ltd., Muttenz, Switzerland) to a preparative plate (Kieselgel 60, 0.25 Mesh; Merck, Darmstadt, Germany). Development was carried out in a hexane/ether/acetic acid mixture (50:50:1, vol/vol/vol).

The diglycerides and free fatty acids bands were located by transparency and transformed into isopropyl esters.

Measurement of the degree of lipolysis. The degree of lipolysis was obtained by KOH titration of the free acids in the reaction medium from a 1-mL sample taken from the 9 mL of ether/heptane extract (75:25, vol/vol).

Derivation into isopropyl esters (7). The total amount of diglycerides and free fatty acids recovered were dissolved in a hexane/isopropanol mixture (3:2, vol/vol). Then, 400 µL of 6M H_2SO_4 and 1 mL isopropanol were added. The tube was hermetically sealed, shaken, and placed in the oven for 1 h at 100°C. The tube was then cooled in an ice bath, and 5 mL dis-

tilled water were added to trigger phase separation. The hexane phase was recovered and injected directly into the gas chromatograph.

Gas chromatography (GC). Isopropyl esters were analyzed on a fused-silica capillary column (Carbowax 20M; DB Wax, J and W, Montpellier, France) with the following characteristics: length, 30 m; internal diameter, 0.28 mm; film thickness, 0.1 μ. GC was carried out under the following conditions: carrier gas, helium; pressure, 1 bar; column flowrate, 2 mL/min; splitting ratio, 1:30; injector, 250°C; and detector, 250°C. The temperature settings were as follows: 2 min at 38°C, 38–210°C at 10°C/min, 15 min at 210°C.

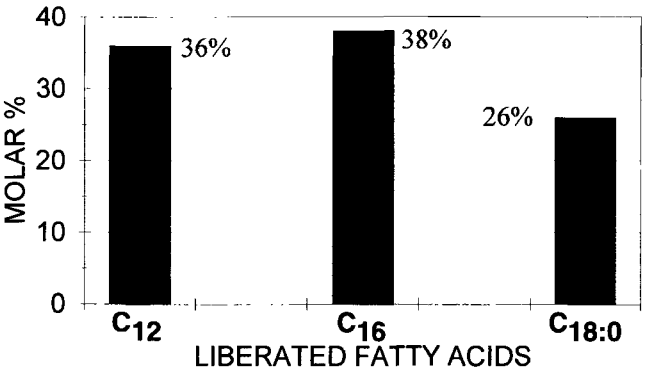
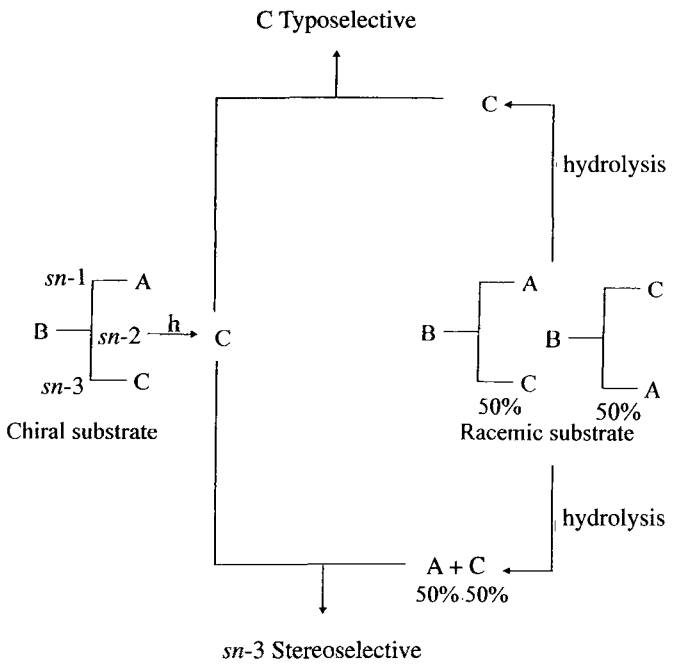


FIG. 1. Hydrolysis of 1(3)-lauroyl 2-stearoyl 3(1)-palmitoyl *rac* glycerol (LSP) with *Candida cylindracea* lipase.

RESULTS AND DISCUSSION

With the bioconversion of oils and fats, it is extremely useful to be able to distinguish clearly between the typoselectivity and stereoselectivity of a lipase. To do so, it is necessary to have a chiral triglyceride and the racemic one in each case. For example, if the enzymatic hydrolysis of a chiral triglyceride of the type 1-A 2-B 3-C *sn* glycerol (where A, B, and C are three different fatty acids) releases fatty acid C, it is impossible to know whether this lipase is C typoselective or *sn*-3 stereoselective. Therefore, we have to hydrolyze the racemic form of the triglyceride at the same time—this substrate corresponds to an equimolar mixture of 1-A 2-B 3-C *sn* glycerol and 1-C 2-B 3-A *sn* glycerol. If the hydrolysis of the racemic only releases fatty acid C, the lipase is typoselective for this acid; if it releases acids A and C in equivalent quantities, the enzyme is *sn*-3 stereoselective (Scheme 1).

It is only worth studying the specificity of a lipase if the triglyceride hydrolysis rate is limited (less than 15%) and also for a limited period (less than 15 min), to avoid isomerization and hydrolysis of the diglycerides formed, in which case the results obtained would be meaningless. Consequently, we had to adjust the reaction time for each enzyme in accordance with the activity of the lipase used (see Experimental Procedures section). Initially, we studied lipases of known specificity, to check that our method gave identical results to those already described in the literature. The two enzymes used as controls to validate our study were nonspecific *Candida cylindracea lipase* (CCL) (8) and 1,3-regioselective *Mucor miehei* lipase (MML). Hydrolysis of the racemic triglyceride, 1(3)-lauroyl 2-stearoyl 3(1)-palmitoyl *rac* glycerol by CCL released all the fatty acids, irrespective of their position on the triglyceride skeleton (Fig. 1). The nonspecificity of this lipase is thus confirmed.



SCHEME 1

For MML, hydrolysis was performed on 1-butyroyl 2-stearoyl 3-palmitoyl *sn* glycerol (chiral BSP) and 1(3)-butyroyl 2-stearoyl 3(1)-palmitoyl *rac* glycerol (*rac* BSP), both synthesized by a previously published method (6). No conclusion could be drawn by studying the results on the chiral triglyceride alone (Fig. 2A). Indeed, at this stage of the study, two hypotheses could be put forward—either the MML was 1,3 regioselective, or it presented a combination between short-chain fatty acid typoselectivity and *sn*-3 stereospecificity. An analysis of the results obtained on the racemic triglyceride enabled us to opt for 1,3 regioselectivity (Fig. 2B). As the results obtained tallied with those in the literature, we went on to study the specificity of *Carica papaya* latex, following a protocol that was identical to that used for our control enzymes. The chiral triglyceride, BSP, was then hydrolyzed (Fig. 3A). The results revealed preferential release of palmitic acid in a molar ratio of around 85:15. However, it was not yet possible to distinguish between palmitic acid typoselectivity and *sn*-3 stereospecificity. Hydrolysis of the racemic revealed that butyric and palmitic acid are released in virtually the same quantities (Fig. 3B). Under our hydrolysis conditions, *Carica papaya* latex acts like a strict position *sn*-3 stereoselective enzyme and not as a short-chain fatty acid selective enzyme, as might have been expected from its activity on dairy fat. This property could be exploited in bioconversion of oils and fats and suggests new applica-

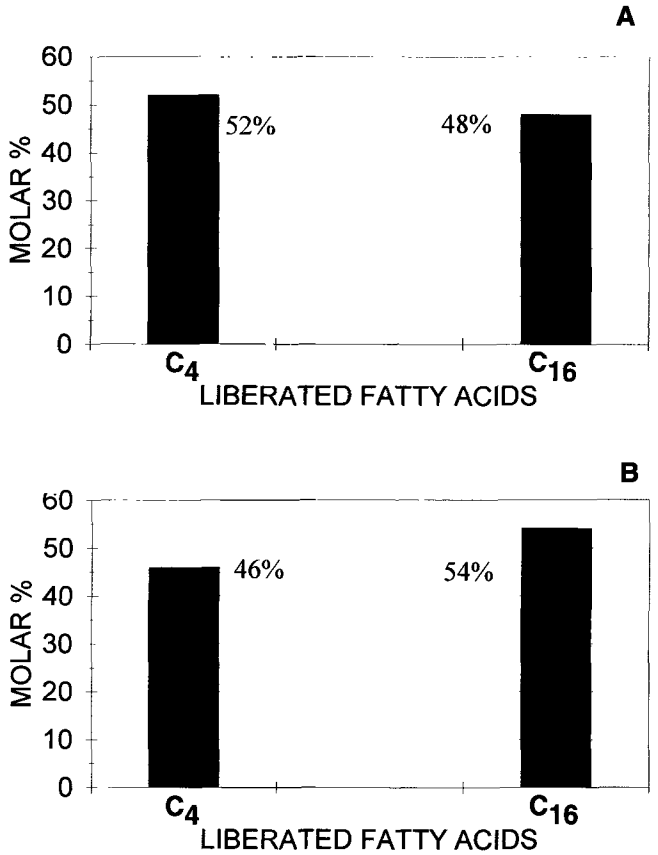


FIG. 2. A: Hydrolysis of 1-butyroyl 2-stearoyl 3-palmitoyl *sn* glycerol with *Mucor miehei* lipase. B: Hydrolysis of 1(3)-butyroyl 2-stearoyl 3(1)-palmitoyl *rac* glycerol with *M. miehei* lipase.

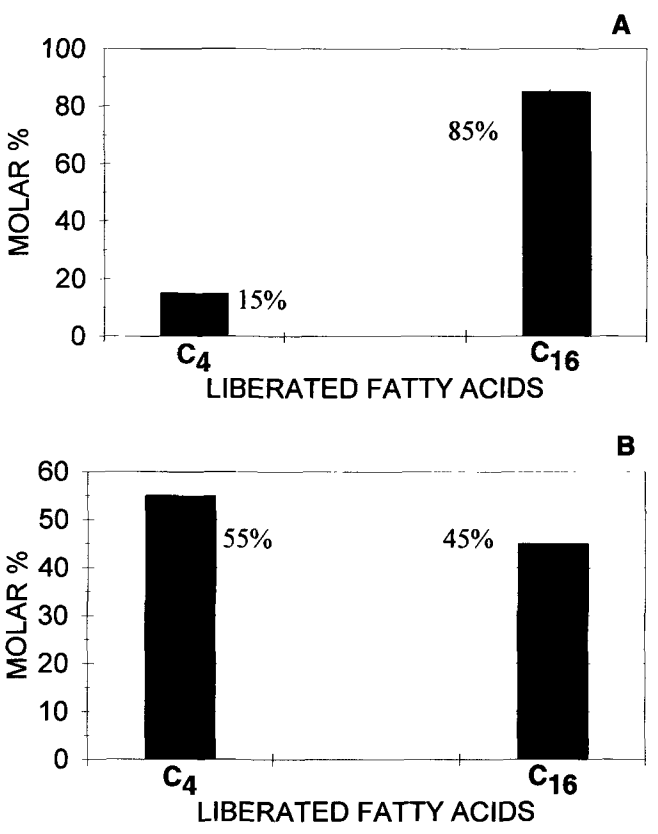


FIG. 3. A: Hydrolysis of 1-butyroyl 2-stearoyl 3-palmitoyl *sn* glycerol with *Carica papaya* lipase. B: Hydrolysis of 1(3)-butyroyl 2-stearoyl 3(1)-palmitoyl *rac* glycerol with *Carica papaya* lipase.

tions for this enzyme preparation, which is already widely used for its proteolytic activity (papain). It is also clear that this method can be applied to a wide range of lipases of various origins. In this article, we have shown *sn*-3 specificity, but it would be just as easy to define typoselectivities, non-specificities, or even *sn*-1 and *sn*-2 specificities for other enzymes.

This method, which is complementary to other studies already published (2), is easy to implement and merely requires model chiral and racemic substrates, whose simple and rapid synthesis have been described (6).

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