Stereoselectivity of Lipases

II. STEREOSELECTIVE HYDROLYSIS OF TRIGLYCERIDES BY GASTRIC AND PANCREATIC LIPASES*

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In the present study, porcine pancreatic lipase, rabbit gastric lipase, and human gastric lipase stereospecificity toward chemically alike, but sterically nonequivalent ester groups within one single triglyceride molecule was investigated. Lipolysis reactions were carried out on synthetic trioctanoin or triolein, which are homogenous, prochiral triglycerides, chosen as models for physiological lipase substrates. Diglyceride mixtures resulting from lipolysis were derivatized with optically active R-(+)-1-phenylethylisocyanate, to give diastereomeric carbamate mixtures, which were further separated by high performance liquid chromatography. Resolution of diastereomeric carbamates gave enantiomeric excess values, which reflect the lipases stereobias and clearly demonstrate the existence of a stereopreference by both gastric lipases for the sn-3 position. The stereoselectivity of human and rabbit gastric lipases, expressed as the enantiomeric excess percentage, was 54% and 70% for trioctanoin and 74% and 47% for triolein, respectively. The corresponding values with porcine pancreatic lipase were 3% in the case of trioctanoin and 8% in that of triolein. It is worth noting that rabbit gastric lipase, unlike human gastric lipase, became more stereoselective for the triglyceride with shorter acyl chains (trioctanoin). This is one of the most striking catalytic differences observed between these two gastric lipases.

Lipases (triacylglycerol ester hydrolase; EC 3.1.1.3) are ubiquitous enzymes and occur widely in nature (1, 2). Their biological function, at least in plants and higher animals, is to catalyze the hydrolysis of triacylglycerols to yield free fatty acids, diacylglycerol, monoacylglycerol, and glycerol. Lipases are active at the oil/water interface in heterogeneous reaction systems (3). They are often difficult to assay and the interpretation of their kinetics is not straightforward (4).

During the last few decades, lipases have been used in the dairy and food industry, in detergents manufacture, and for tanning, sewage treatment, and cosmetics. One of the fields in which recent research in organic chemistry has been focusing is the development of methods for chiral synthesis, and lipases are becoming increasingly recognized as valuable chiral catalysts (5-14).

Another field of intense biochemical research, in which the stereospecificity of lipases might play a fundamental role, is dietary fat digestion in humans. It was thought until recently that the hydrolysis of alimentary lipids began in the intestinal lumen and was catalyzed exclusively by pancreatic lipase. The stomach was thought of as a transient storage organ, the role of which is limited to mixing and dispersing lipids with the other nutrients. The digestion of triacylglycerols by an enzymatic process in the stomach was assumed to be negligible and nonsignificant in comparison with the intestinal step. At our laboratory, screening assays were carried out on preduodenal lipases from several mammals, and the results indicated that in each mammal tested, the preduodenal lipase activity was associated mainly with a single tissue, which was located either in the lingual, the pharyngeal, or the gastric area (15). Results from several laboratories (16-18) indicate that gastric lipolysis is of prime importance for the digestion of dietary fat. The products released by gastric lipolysis trigger a further rapid digestion in the duodenum by pancreatic lipase. Thus, the complementarity between gastric and pancreatic lipases appears to be a general phenomenon and a detailed study of the stereoselectivity of the two major lipases of the digestive tract during glyceride hydrolysis would certainly be of fundamental physiological interest.

Milk fat is the dietary fat which has been generally used for studying gastric lipase specificity (16, 17). Lok (19) has shown, by 'H NMR spectroscopy with a shift reagent, that the predominant enantiomers of the diacyl glycerols present in fresh milk have the 1,2-sn configuration. It should be recalled that the 4:0, 6:0, and 8:0 fatty acids are mostly esterified at the sn-3 position of milk triglycerides (20-22). Thus, the reported (23, 24) preferential release of short chain and medium chain fatty acids by gastric lipase may in fact be partly attributable to its stereoselectivity and to a lesser extent to a short chain fatty acid specificity. The latter point of view was recently reinforced by Gargouri et al. (25), who reported that purified human gastric lipase was able to hydrolyze both short and long chain triglycerides at comparable rates under acidic conditions.

Several methods have been proposed for measuring lipase stereoselectivity, which will be critically discussed later. After taking stock of the previously published experimental procedures and considering their limitations as well as their practical features, we decided to set up a new method, based upon chromatographic separation of diastereomeric derivatives of diglycerides. Diglycerides, generated in a partial enzymatic hydrolysis of synthetic triacylglycerols with three identical acyl chains, were reacted with R-(+)-1-phenylethylisocyanate, to give diastereomeric carbamate mixtures, resolved then by...
HPLC. To our knowledge, no conclusive reports have been published on the stereospecificity of biochemically purified lipases. This prompted us to use purified gastric and pancreatic lipases, which are the main lipases of the mammalian digestive tract, as biocatalysts.

**EXPERIMENTAL PROCEDURES**

**Lipases**—PPL was purified, as previously described by Verger et al. (26), and has the specific activity given in Tables I and II. HGL was purified, as described by Tiruppathi and Balasubramanian (27) and has a specific activity of 1000 units/mg. RGL was purified as described by Moreau et al. (28) and has a specific activity of 1000 units/mg. HRL (25) and PPL (29) activities were determined titrimetrically at 37 °C under standard conditions, using tributyryl-glycerol as substrate.

**Lipolysis**—The progress of lipolysis of trioctanoin and triolein with PPL, HGL, and RGL was controlled by a pH-stat under the following conditions: 10 ml of 0.9% NaCl, 0.5 ml of trioctanoin or triolein, 6.5 ml of CHCl₃, and dried over MgSO₄, and solvent was evaporated.

**Carbamate Synthesis and Analysis**—The two-phase reaction mixtures (10.5 ml) resulting after lipolysis were extracted four times with 20 ml of CHCl₃ and dried over MgSO₄, and solvent was evaporated. The crude lipid extracts were separated on HPLC, using 5% ethyl acetate, with 5% ethyl alcohol/heptane as an eluent at 3.5 ml/min flow rate and corrected on a reference curve, established with weighed amounts of triglyceride and diglyceride.

**RESULTS**

The stereoselectivity of PPL, RGL, and HGL was tested in trioctanoin and triolein lipolysis reactions by derivatizing the resulting diglycerides, according to the principle depicted in Fig. 1. The lipolysis reactions were stopped by enzyme inactivation with CHCl₃, before any monoglyceride formation could be observed, and their progress was evaluated directly by HPLC (see Fig. 2, Table I and Table II). The lipid extracts were treated, as indicated under “Experimental Procedures,” and the mixtures of enantiomeric diglycerides thus obtained were reacted with R-(+)-1-phenylethylisocyanate, to produce diastereomeric carbamates with a 100% yield. The diastereomeric carbamates were separated by HPLC, as shown in Fig. 3, and then characterized by IR, H NMR (see Fig. 4), and mass spectrometry. The enantiomeric excess percentages of diglycerides were determined by HPLC separation of corresponding diastereomeric carbamates and are given in Tables I and II.

In all cases, 1,2-diglycerides (absolute configuration on chiral center S) were formed in excess and the excess isocyanate was removed from the reaction mixtures by eluting it with heptane on small silica gel columns (0.5 × 20 cm). The carbamates were then eluted with ethyl acetate, evaporated, and injected on HPLC to separate the pairs of diastereomeric carbamates with 0.5 or 0.7% ethyl alcohol/heptane as an eluent (in the case of diolein and dioctanoin, respectively) at a flow rate of 3.5 ml/min. The excess isocyanate was removed from the reaction mixtures by eluting it with heptane on small silica gel columns (0.5 × 20 cm).

**DISCUSSION**

As stated in the reviews by Jensen et al. (31), Wells and Di Renzo (32), Macrae (33), and Wang (34), the substrate specificity of the lipases involved in triglyceride hydrolysis can be subdivided into positional specificity (primary or secondary ester bond), fatty acid specificity, and stereospecificity (sn-1 versus sn-3 ester bonds).

Enzymatic stereospecificity in turn has two aspects: 1) discrimination by the enzyme between separate enantiomeric substrate molecules, which depends upon their reflective symmetry, and 2) discrimination between stereo-nondegenerate pairs of a particular prochiral substrate molecule, which depends on the rotational symmetry of the substrate with respect to these groups (35).

Most of the authors quoted in these reviews (31–34) have tested the “reflective symmetry” type (first aspect) of stereo-
Stereoselective Hydrolysis of Triglycerides by Digestive Lipases

**FIG. 1.** Principle of the method used to study the stereoselective hydrolysis of triglycerides by lipases. DG, diglyceride; TG, triglyceride.

**FIG. 2.** Typical examples of chromatograms of lipid mixtures after lipolysis. A, trioctanoin after 3-min hydrolysis with HGL (225 units): trioctanoin, \( R^f 4.76 \) min and 1,2(2,3)-dioctanoyl-sn-glycerol, \( R^f 8.54 \) min. B, triolein after 2.3-min hydrolysis with PPL (127 units): triolein, \( R^f 4.40 \) min, 1,2(2,3)-dioleoyl-sn-glycerol, \( R^f 6.50 \) min, and oleic acid (OA), \( R^f 8.45 \) min.

specificity of lipases, using as substrates synthetic enantiomeric acylglycerols with different acyl chains. The lipolytic stereospecificity has been determined by product analysis.

For example, Tetrug et al. (36), using synthetic 1,2-dipalmitoyl-3-oleoyl-sn-glycerol and 1-oleoyl-2,3-dipalmitoyl-sn-glycerol as substrates for pancreatic lipase, observed that the fatty acids in the two primary ester groups were released in equimolar proportions. In addition, the hydrolysis of trioleoylglycerol did not lead to a dioleoylglycerol with optical activity. It has also been suggested by Morley et al. (37), that pancreatic lipase may lack stereospecificity toward synthetic 1-palmitoyl-2-oleoyl-3-linoleoyl-sn-glycerol and 1,2-dipalmitoyl-3-oleoyl-sn-glycerol and their analogs as substrates. Although unequal yields of \( sn \)-1,2- and \( sn \)-2,3-diacylglycerols were recorded, the authors concluded that this was due to a preferential release of unsaturated fatty acid rather than to any pancreatic lipase stereospecificity. A study using the alkylacylglycerols as substrates was also consistent with the lack of stereospecificity of crude pancreatic extract (38).

Using premature human infant gastric aspirate as the source of lingual lipase and synthetic enantiomeric triacylglycerols containing oleic and palmitic acids, Jensen et al. (39) have demonstrated that the \( sn \)-3 ester was hydrolyzed about 4 times faster than the ester at the \( sn \)-1 position with no difference in the rates between 18:1 and 16:0. The \( sn \)-2 was also hydrolyzed to some extent. Szafran et al. (40) studied the sequential hydrolysis of the three acyl ester bonds in triolein by human gastric juice and found that the rates of all the steps were similar. Tiruppathi and Dalasubramanian (27) studied the positional specificity of an acid lipase purified...
from human gastric juice. These authors showed that the preferred site of hydrolysis in 1-palmitate-2-oleate-3-stearate of triacylglycerol that occurs in the lipase active site."

fatty acids by lingual lipase, "suggesting an acyl chain sorting from the lipase stereospecificity with any of the above methods, which may explain these apparently contradictory results. Furthermore, possible acyl migration can complicate the interpretation of the experimental data. To overcome these difficulties, enantiomeric alkylacylglycerols or alkylmonooacylglycerols have been synthesized and used as lipase substrates. The cleaved acyl moiety was the same in both enantiomers, which ruled out the possibility of any effects being due to fatty acid specificity. Furthermore, the presence of a nonester group at the 2-position prevented acyl migration. Such compounds, spread as lipid monolayers at the air/water interface, were used as digestive lipase substrates in the accompanying paper by Ransac et al. (42). Their chemical synthesis is difficult, however, and extrapolation of the results obtained on pseudoglycerides to triacylglycerides is not possible.

It should be recalled that the two primary ester positions of synthetic triacylglycerols with identical acyl chains are potentially nonequivalent as far as lipase action is concerned (because their symmetry is of the "rotational" type, as mentioned above). Two methods using prochiral triacylglycerol to potentially test lipase stereoselectivity were developed in the past. The first involved the use of a synthetic triolein and the specific optical rotation of the nonracemic 1,2(2,3)-diolein formed served as index to the lipase stereobias (36, 39). This method lacks sensitivity, however, and its use is limited to products having a known specific rotation. Second, Brockherhoff (43) proposed a new classical method for stereoselective triacylglycerol analysis, in which diacylglycerols generated from the partial hydrolysis of triacylglycerols are derivatized to give enantiomeric glycerophospholipids. These latter lipids are then subjected to a strict stereoselective hydrolysis by phospholipase A1, giving rise to 1-acyl-2-hydroxy-sn-glycero-3-phospholipids, a free fatty acid from the sn-2 position and unhydrolyzed 2,3-diacyl-sn-glycero-1-phospholipid. The stereoselectivity of the lipase can then theoretically be deduced from the fatty acid analysis after phospholipase A1 treatment (21, 43–45). Lands et al. (46) have described a different enzymatic procedure for stereospecific determination of triacylglyceride structure. The method developed uses the diacylglyceride kinase of Escherichia coli to selectively phosphorylate the 1,2-diacylglycerides from the mixtures of diglycerides formed by pancreatic lipase which has been shown to proceed independently of the long chain fatty acid composition of the glycerides. However, this generalization may not hold true for glycerides containing short chain acids (47). Uzawa et al. (48) recently described a method based on circular dichroism for determining the stereoselectivity of lipases. However, they used crude commercial pancreatic and microbial preparations in their experiments. The recently published methods for separating chiral diglyceride derivatives (49, 50) have not been applied to lipase stereoselectivity evaluation until now.

In our study, PPL, RGL, and HGL stereoselectivity toward chemically alike, but stereically nonequivalent, ester groups within one single triglyceride molecule was investigated. Lipolysis reactions were carried out on synthetic trioctanoin or triolein, which are homogenous, prochiral triglycerides, chosen as models for physiological lipase substrates. Diglyceride mixtures resulting from lipolysis were derivatized with optically active R-(-)-1-phenylethylisocyanate, to obtain diastereomeric carbamate mixtures, which were further separated by HPLC. Resolution of diastereomeric carbamates gave enantiomeric excess mixtures, which were further separated by HPLC. Resolution of diastereomeric carbamates gave enantiomeric excess mixtures, which were further separated by HPLC.

In our experiments, care was taken while preparing the carbamates to avoid the occurrence of any random diastereomer separation. Reactions were carried out until whole diglycerides were reacted, as systematically checked by TLC and HPLC. To ensure that the measured enantiomeric excess actually described only the stereoselectivity for the sn-1 or sn-3 position in the triglyceride substrate and was not influenced by the selective disappearance of the enantiomeric

Table I

**Stereoselective Hydrolysis of Triglycerides by Digestive Lipases**

<table>
<thead>
<tr>
<th>Lipase</th>
<th>t (min)</th>
<th>pH</th>
<th>y1,2</th>
<th>y1,3</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPL</td>
<td>1</td>
<td>8</td>
<td>1.2</td>
<td>0</td>
<td>2.0</td>
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<tr>
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<td>0</td>
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</tr>
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<td>11</td>
<td>6</td>
<td>14.6</td>
<td>0.4</td>
<td>56.2</td>
</tr>
<tr>
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<td>6</td>
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<td>69.7</td>
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<td>6</td>
<td>5.2</td>
<td>1.0</td>
<td>69.6</td>
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<td>8</td>
<td>6</td>
<td>7.5</td>
<td>1.8</td>
<td>70.2</td>
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<tr>
<td></td>
<td>11</td>
<td>6</td>
<td>11.2</td>
<td>1.8</td>
<td>70.3</td>
</tr>
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</table>

* Specific activity of PPLa was 8700 units/mg with added colipase and 1740 units/mg in absence of added colipase; 127 units of PPLb were used.
* Specific activity of PPLa was 1700 units/mg with added colipase and 800 units/mg in absence of added colipase. PPLa and PPLb are the two porcine pancreatic isolipases separated as described previously (26). 120 units of PPLa were used.
* HGL and RGL both had a specific activity of 1000 units/mg.
* 120 units of HGL were used.
* 594 units of RGL were used.
* 2375 units of RGL were used.

Yields based on the theoretical yield of total diglycerides (no monoglyceride formation, 1 mol of triglyceride gives 1 mol of diglyceride, taken as 100%). y1,2, 1,2- and 2,3-diglyceride yield; y1,3, 1,3-diglyceride yield; ee, enantiomeric excess.

Table II

**Lipolysis of triolein**

<table>
<thead>
<tr>
<th>Lipase</th>
<th>t (min)</th>
<th>pH</th>
<th>y1,2</th>
<th>y1,3</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPL</td>
<td>2</td>
<td>8</td>
<td>9.6</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>10.0</td>
<td>0</td>
<td>7.4</td>
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<tr>
<td>HGL</td>
<td>16</td>
<td>6</td>
<td>1.8</td>
<td>0</td>
<td>73.3</td>
</tr>
<tr>
<td>RGL</td>
<td>20</td>
<td>6</td>
<td>2.8</td>
<td>0</td>
<td>75.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>6</td>
<td>2.3</td>
<td>0</td>
<td>47.5</td>
</tr>
</tbody>
</table>

a Specific activity of PPLa was 8700 units/mg with added colipase and 1740 units/mg in absence of added colipase; 127 units of PPLb were used.

b Specific activity of PPLa was 1700 units/mg with added colipase and 800 units/mg in absence of added colipase. PPLa and PPLb are the two porcine pancreatic isolipases separated as described previously (26). 120 units of PPLa were used.
diglycerides formed, lipolysis was always stopped before any formation of monoglyceride could be observed. Thanks to this precaution, it was possible to assume that the measured enantiomeric excess reflects an intrinsic lipase stereopreference for the sn-1 or sn-3 position of the triglyceride molecule and did not result from a more complex kinetic pathway (e.g. simultaneous tri- and diglyceride hydrolysis). The enantiomeric excess percentages can be expected to remain constant during prochiral triacylglycerol lipolysis if no enantioselective and significant diacylglycerol disappearance occurs. This was
demonstrate the existence of a stereopreference by both gas-
in that of triolein. Lack of stereoselectivity of PPL in chiral
glycerides. Furthermore, any acyl migration within the diglyc-
eride molecule from the secondary to primary ester position
This is one of the most striking catalytic differences observed
from a physiological point of view, since gastric and pancreatic
agents, such as bile salts, to simplify the system. However,
steric preference by both gas-chromatography (36, 37). Our results
values with PPL were 3% in the case of trioctanoin and 8% and 74 and 47% for triolein, respectively. The corresponding
stereoselectivity of HGL and RGL, expressed as the enan-
tomeric excess percentage, was 54 and 70% for trioctanoin
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