Pancreatic Lipase Assays with Triglycerides as Substrate: Contribution of Each Sequential Reaction to Product Formation

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Human pancreatic lipase assays are usually performed in the presence of either emulsified triglycerides or diglycerides within the limits of their solubility. Two reactions are catalyzed in the presence of triglycerides: hydrolysis of triglycerides to diglycerides, and diglycerides to monolycerides. The contribution of each reaction to the final result was determined after extensive kinetic studies on the appearance and/(or) accumulation of intermediates and/(or) products. Acylated glycerides were analyzed after extraction from the reaction mixture, separation of lipid classes by thin-layer chromatography, and quantification by capillary gas chromatography. The results show that after 10 min of reaction in the presence of high concentrations of triolein, 75% of the released fatty acids arise from the first reaction. Relative merits and disadvantages of each substrate (triglyceride or diglyceride) are discussed in terms of practicability.

Indexing Terms: *lipids/fatty acids/thin-layer chromatography/pancreatitis/capillary gas chromatography*

Pancreatic lipase (EC 3.1.1.3) assays are most often performed in the presence of triglycerides, probably because the use of such substrates reflects the in vivo conditions of intestinal lipolysis (see reviews *1–4*). Both indirect procedures (*5–8*) and optimized titrimetric procedures have performed in this way (*4, 9, 10*). Enzyme coupling procedures are usually carried out in the presence of diglycerides (*11–13*), with few exceptions (*14*). The main reason for using diglycerides seems to be their increased solubility in water: When based on the use of soluble substrates the assays are easier to automate. In recent reports Lessinger et al. (*15, 16*) pointed out considerable interassay discrepancies in the plasma lipase activities in samples from patients with acute pancreatitis. With five different routine assays, they found lipase activity to vary by a factor of up to 16. This problem may be reduced considerably with the use of an appropriate calibrator. Therefore, the main cause of discrepancies in lipase assays seems to be the use of different substrates and calibrators. Moreover, the reaction catalyzed by pancreatic lipase is complex in the sense that the initial substrate (triglyceride) yields a product (fatty acid) and another intermediate substrate (diglyceride), which is further hydrolyzed to another fatty acid and a monoglyceride. The existence of such complex

substrates and sequential reactions hinders the establishment of reference methods. The lack of a systematic study on the rates of hydrolysis of triglycerides, diglycerides, and monoglycerides in assays involving human lipolytic enzymes is obvious. For this reason, we studied the rates of hydrolysis of triolein, diolein, and monoolein by purified human pancreatic lipase by separating the products of lipolysis by thin-layer chromatography and quantifying them by gas chromatography.

Materials and Methods

Reagents

Colipase (~95% pure) was provided by Boehringer Mannheim (Mannheim, Germany). Internal standards for lipid analysis were from Larodan (Malmoe, Sweden). All other reagents and enzymes used were purchased from Sigma Chemical Co. (St Louis, MO); they were of the highest purity available. Thin-layer chromatography silica gel plates were also from Sigma (cat. no. T-6770).

Lipase Purification

Lipase was purified from human pancreatic juice as described previously (*17*). After dilution in a 18 mmol/L sodium taurodeoxycholate solution we obtained an enzyme preparation containing 910 U/L, defined as micromoles of fatty acids released per minute under the conditions described in the next section (triolein was present at 50 mmol/L).

Lipase Assays

The substrate was prepared according to a procedure already described (*4, 17*). The reaction mixture contained emulsified triolein (at concentrations indicated in *Results*) and, per liter, 12 g of hydroxypropylmethylcellulose, 0.2 mg of colipase, 70 mmol of sodium deoxycholate, and 0.5 mmol of calcium chloride. Titrimetric assays were carried out at 37°C, pH 9.0, with an automated titrator (Mettler DL-21, Greifensee, Switzerland). The volume of the sample was 100 μL in a final reaction mixture of 25 mL.

Analysis of Lipolysis Products

The reaction mixture (final volume 5 mL) used for these studies was as follows: 2.5 mmol/L triolein, 50 mmol/L Tris-HCl, pH 9.0, and other cofactors as in the titrimetric assay described above. To achieve complete hydrolysis of triolein within 90 min, we used in these assays 100 μL of undiluted purified human enzyme (29 000 U/L). To quantify triglycerides, diglycerides, and monoglycerides, we followed a procedure consisting of four steps: extraction of lipids from the reaction mix-

ture, separation of lipid classes by thin-layer chromatography, transesterification with methanol, and quantification of fatty acid methyl esters by capillary gas chromatography. As internal standards we used triheptadecanoin, 1,2-diheptadecanoin, 1,3-triheptadecanoin, and monoheptadecanoin. The procedure was as follows:

Extraction of lipids. Lipids were extracted from the reaction mixture by the method of Dole (*18*). Aliquots (0.25 mL) were removed at several time points, then added to 1.25 mL of isopropanol:heptane:0.5 mol/L sulfuric acid (40:10:1 by vol) already containing the internal standards. After 15 min at room temperature, 0.5 mL of heptane and 0.75 mL of water were added. After stirring for 1 min the upper organic phase containing the lipids was removed. Heptane was evaporated under nitrogen.

Separation of lipid classes. The residue obtained after evaporation was redissolved in 50 μL of chloroform: methanol (2:1 by vol). The separation of lipid classes was carried out by thin-layer chromatography with silica gel plates. Aliquots (7-μL) were spotted onto the plates. The mobile phase was petroleum ether:diethyl ether:acetic acid (80:20:1 by vol). The retention factors of lipid classes were 0.8 for triglycerides, 0.25 for 1,3-diglycerides, 0.2 for 1,2-diglycerides, and 0.07 for monoglycerides. Spots were made visible under ultraviolet light after spraying with dichlorofluorescein.

Transesterification. Spots of triacylglycerol, diacylglycerols, and monoacylglycerol were scraped off the silica gel plates. Transmethylation was achieved in methanol:sulfuric acid (96:4 by vol) by overnight incubation at 60°C.

Quantification by gas chromatography. The fatty acid methyl esters produced by transesterification of glycerides were extracted with 1 mL of petroleum ether and 1 mL of water. After evaporation of petroleum ether under nitrogen the residue was redissolved in carbon disulfide. Aliquots (1 μL) were injected into a Hewlett-Packard (Palo Alto, CA) 5890 Series II chromatograph equipped with a Carbowax capillary column from Alltech (Deerfield, IL). The column temperature was 210°C. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. A flame-ionization detector was used for analysis of effluents. The retention times for the internal standard (methyl heptadecanoate) and for methyl oleate were 3.3 and 4.1 min, respectively.

Results

Substrate Concentration and Enzyme Activity

Different substrate concentrations have already been used in lipase assays (*4–14*). Under our assay conditions, maximal activity was observed with 50 mmol/L triolein (Fig. 1). We calculated that a final concentration of 2.5 mmol/L triolein provides nearly 25% of the maximal activity.

Kinetic Studies

Hydrolysis of triglycerides by pancreatic lipases proceeds as a two-step reaction. First, a molecule of triglyceride is hydrolyzed, yielding a fatty acid and, moreover, a
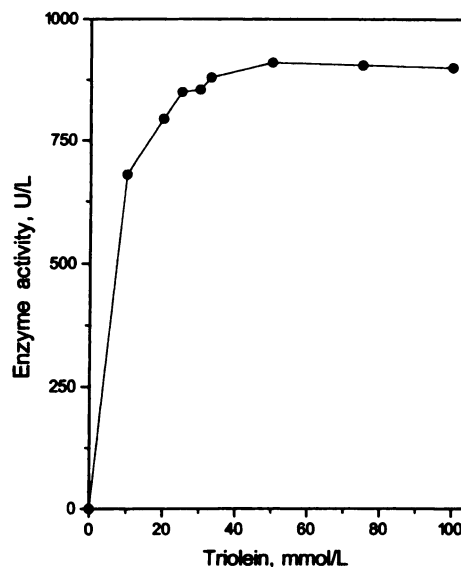

Fig. 1. Purified human lipase activity vs triolein concentration in th presence of 70 mmol/L deoxycholate and 0.2 mg/L colipase.

1,2-diglyceride that is then hydrolyzed to a 2-monoglycer ide and a fatty acid. Therefore, diglyceride constitutes botl the first product of lipolysis and the substrate for the sec ond reaction. Given the fact that these reactions occu simultaneously, the methods involving triglycerides de tect fatty acids released during both reactions. Percent ages of triolein, 1,2-diolein, and monoolein obtained a various time points following the action of purified humai lipase are shown in Table 1. The results show a continuou decrease of triolein, a transient accumulation of 1,2-di olein, and a continuous increase in monoolein withou further hydrolysis. As one would expect from pancreati lipases, no 1,3-diolein was produced.

Because the rate of each reaction depends on sub strate concentration, the rate of production of fatty acid from triolein hydrolysis is different from that from di olein hydrolysis. In the early stages of lipolysis, in whicl the concentration of triolein is far higher than that o diolein, the rate of the first reaction is higher. Thi situation is reversed later as the concentration of tri olein decreases and that of diolein increases (Fig. 2)

Table 1. Percentages of the components of lipolysis at various time intervals.

Time, min	%		
	Triolein[a]	Diolein	Monoolein
0	100	0	0
5	82.3	6.6	11.1
10	70.7	11.0	18.3
20	51.5	11.7	36.8
40	25.3	10.6	46.1
50	20.3	9.9	69.8
70	8.0	8.9	83.1
80	4.0	5.8	90.2
90	3.1	5.5	91.4

[a] Reaction was carried out in the presence of 2.5 mmol/L triolein under th conditions described in *Materials and Methods*.
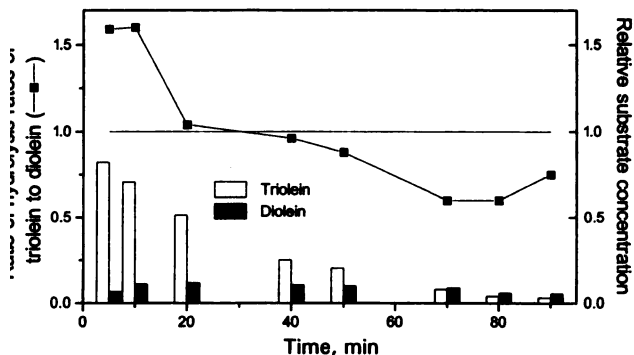
ig. 2. Ratio of the mean hydrolysis rates of triolein to diolein and
ieir corresponding relative concentrations.

he mean hydrolysis rates of triolein or diolein over a certain time period are
alculated from the data of Table 1 as the quotient of the amount of, respec-
/ely, triolein hydrolyzed or monoolein formed between two sequential time
oints to the respective time interval.

ince this reversal occurs already at a triolein:diolein
oncentration ratio of 2:1, hydrolysis of diolein appears
o be faster than that of triolein. We studied the differ-
nce in the reaction rates by using diolein instead of
riolein: The release of fatty acids was ~three times
aster (not shown). However, it is not possible to directly
ompare lipase activities obtained with emulsified tri-
lein and diolein because the latter yields smaller drop-
its and, therefore, more interface is offered to the en-
yme. Triolein at 2.5 mmol/L provides emulsions with
roplets having a mean volume diameter of 3.15 ± 0.12
:m. The respective value for diolein emulsions (same
oncentration as triolein) is 2.80 ± 0.10 μm (A. Avra-
.as, personal communication).

We also determined the contribution of each reaction
o the formation of the final product when the two reac-
ions occur simultaneously. The final result, expressed
s moles of fatty acids, equals the sum of the moles of
riolein hydrolyzed plus the moles of monoolein formed.
he contribution of the first reaction equals the moles of
riolein hydrolyzed as a percentage of the final result,
rhereas the contribution of the second reaction equals
he moles of monoolein formed as a percentage of the
nal result. Fig. 3A shows that, after 10 min, 62% of the
eleased fatty acids are due to the hydrolysis of triolein
o diolein, whereas after 90 min of hydrolysis, 52% of the
atty acids arise from the first reaction. These results
rere obtained with 2.5 mmol/L triolein, a concentration
etween the high concentrations used in titrimetry and
he lowest concentrations used in turbidimetry. In the
resence of 50 mmol/L triolein, however, the accumula-
ion of diglycerides might be further enhanced since the
ate of the reaction is increased fourfold (Fig. 1). We
herefore determined whether increasing the triolein
oncentration enhances the first reaction selectively. In
ict, the contribution of the hydrolysis of triglyceride to
iglyceride reaches 75% with 10 mmol/L triolein and
oes not vary significantly thereafter (Fig. 3B).

Iscussion

Many routine assays of lipase are carried out in the
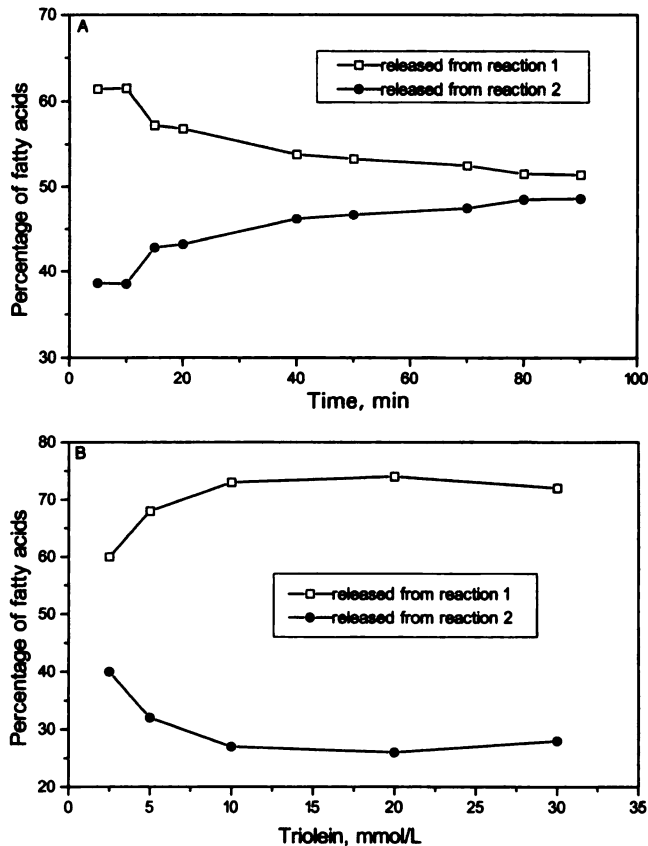resence of low substrate concentrations, either because

Fig. 3. Contribution of each sequential reaction of lipolysis (A) with
respect to time and (B) at different triolein concentrations.

(A) Reaction was performed with 2.5 mmol/L triolein as substrate; calculations
are based on the data of Table 1. (B) Reaction was performed for 10 min. The
total fatty acids released equal the sum of the moles of triolein hydrolyzed
(contribution of first reaction) plus the moles of monoolein formed (contribution
of second reaction). Reaction 1, conversion of triolein to diolein; reaction 2,
conversion of diolein to monoolein.

of the limitations of spectrophotometry or because of the
increased solubility under such conditions. Continuous-
monitoring titrimetric assays allow for use of high sub-
strate concentrations. A peculiarity of lipolysis is that
this reaction, taking place in heterogeneous media, does
not obey classical Michaelis kinetics; i.e., enzyme activ-
ity depends not on substrate concentrations, but rather
on interface area. Another particular feature is the fact
that the initial substrate yields a first product (diglyc-
eride) that serves as substrate for a second reaction
catalyzed by the same enzyme. Lipase assays are most
often optimized with regard to substrate concentrations
and linearity of kinetics, with the assumption that the
enzyme is of the Michaelis type. Such optimizations,
nevertheless, have led to reliable titrimetric assays that
may constitute a basis for the establishment of a refer-
ence method. In such a method it may be preferable to
determine a product arising from one reaction. Given
that the two sequential lipolytic reactions occur simul-
taneously, but at different rates, it appeared to be of
prime importance to determine the contribution of each
reaction to the result obtained after ordinary incubation
times (e.g., 10 min). With high triolein concentrations,
fatty acids are mainly delivered from the first reaction
(triolein to diolein). This is certainly due to the fact that,

under these conditions, the concentration of triglyceride greatly exceeds that of diglyceride.

Recent titrimetric assays fulfill these requirements (high substrate concentrations, short assay times) and, therefore, may be considered to determine products essentially arising from one reaction. Further pertinent studies are needed to determine the influence of the other components of lipolysis (bile salts, colipase, calcium ions) with regard to the first or the second reaction. Such studies may lead to standard assay conditions based almost exclusively on one reaction.

Although the hydrolysis of diglycerides occurs more rapidly than that of triglycerides, when these are used as initial substrates, their use cannot be recommended in such assays for several reasons. Diglycerides are unstable, expensive, and known to be hydrolyzed to some extent by carboxyl esterase. They can be used in routine assays carried out with low substrate concentrations because their increased solubility may cancel discrepancies due to the emulsification procedure. It may be acceptable for routine assays to be optimized by classical means, e.g., following the determination of a linear portion of kinetics regardless of the origin of the product determined, provided that the assays are adequately calibrated and are supplemented with the necessary cofactors (e.g., bile salts, colipase, calcium ions).

Other novel procedures for the determination of lipolytic enzymes, and also of amylase, proteases, and ribonucleases, should be evaluated with regard to the exact reaction(s) involved in the formation of the product measured.

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