Hydrolysis of fully esterified alcohols containing from one to eight hydroxyl groups by the lipolytic enzymes of rat pancreatic juice

F. H. Mattson and R. A. Volpenhein

Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio 45239

Abstract The enzymatic hydrolysis in vitro of the esters of methanol, ethylene glycol, glycerol, erythritol, pentaerythritol, adonitol, sorbitol, and sucrose in which all alcohol groups were esterified with oleic acid was studied. Various preparations of rat pancreatic juice, including pure lipase, were used as the sources of enzymes. Lipase (EC 3.1.1.3) did not hydrolyze compounds that contained more than three ester groups. Compounds containing four and five ester groups were hydrolyzed by certain preparations of pancreatic juice; this activity is attributed to the enzyme, nonspecific lipase. This enzyme also hydrolyzed esters of primary alcohols. The compounds containing six (sorbitol) and eight (sucrose) ester groups were not hydrolyzed.

Supplementary key words fat digestion · methyl oleate · ethylene glycol dioleate · erythritol tetraoleate · pentaerythritol tetraoleate · adonitol pentaoleate · sorbitol hexaoleate · sucrose octaoleate · pancreatic lipase · pancreatic nonspecific lipase

In 1966 we offered evidence that rat pancreatic juice contains at least three carboxylic ester hydrolases (1). Besides the already recognized lipase (glycerol ester hydrolase, EC 3.1.1.3), which hydrolyzes water-insoluble esters of primary alcohols (2), these are an enzyme capable of splitting esters of secondary alcohols and another enzyme that hydrolyzes water-soluble esters. The enzyme that split secondary esters appeared to have little substrate specificity; consequently, we suggested the trivial name nonspecific lipase (3). Although this earlier work distinguished nonspecific lipase from lipase and from the esterase responsible for the hydrolysis of water-soluble substrates, it was not certain whether this enzyme was identical with sterol ester hydrolase (EC 3.1.1.13). Subsequently, Hyun et al. (4) offered evidence that sterol ester hydrolase was indeed a separate enzyme. Nonspecific lipase may be the enzyme of approximately 60,000 molecular weight that later was partially purified by Morgan et al. (5). These authors reported this enzyme to be capable of cleaving micellar solutions not only of monoglycerides, triglycerides, and β-naphthyl acetate but also of cholesterol esters.

Two characteristics that allow one to distinguish the activity of lipase from nonspecific lipase are their response to the presence or absence of bile salts and their relative susceptibility to digestion by proteolytic enzymes. Thus, lipase is inhibited by sodium taurocholate in a simple system consisting of water–enzyme–substrate and is relatively stable to proteolytic hydrolysis. On the other hand, nonspecific lipase has an absolute requirement for sodium taurocholate and is readily inactivated by proteolytic enzymes (1). These two properties make it possible to measure the activity of these enzymes independently. The specificity of these two enzymes could, of course, be determined by using the pure enzymes. However, of the two, only lipase has been isolated.

In the experiments described here, we have studied the hydrolysis of the complete esters of alcohols containing from one to eight hydroxyl groups by the lipolytic enzymes of the pancreas. The compounds used as substrate were the fully esterified oleate esters of methanol, ethylene glycol, glycerol, erythritol, pentaerythritol, adonitol, sorbitol, and sucrose. The results not only reveal another class of compounds that cannot be hydrolyzed by pancreatic lipase but also demonstrate another role for nonspecific lipase in the digestion of fat.

MATERIALS AND METHODS

Chemicals

Oleic acid was isolated from olive oil by low temperature solvent crystallization and urea adduction. The purified acid was 99% pure by gas–liquid chromatography. The alcohols were obtained from the designated companies: methanol, Mallinckrodt Chemical Works, St. Louis, Mo.; ethylene glycol, Allied Chemical, General Chemical Division, Morristown, N.J.; erythritol, pentaerythritol, sorbitol, and sucrose, Eastman Kodak Co., Rochester, N.Y.; adonitol, K & K Laboratories, Inc., Jamaica, N.Y.; glycerol, Procter & Gamble Co., Cincinnati, Ohio.

The alcohols, except ethylene glycol, were transesterified with an excess of methyl oleate to form the complete esters (6). Ethylene glycol dioleate was prepared by acylation of the alcohol with oleoyl chloride (6). The resulting complete esters were purified by crystallization from acetone at −35°C and silica gel column chromatography until homogeneous by thin-layer chromatography. The developing solvent was petroleum ether–ethyl ether 85:15 for both column and thin-layer chromatography. The ester preparations had a zero hydroxyl value; TLC showed a single spot with a mobility proper to that for the fully esterified alcohol.

Sodium taurocholate was obtained from the Maybridge Chemical Co., Launceston, Cornwall, U.K. The sample was estimated to be 98% pure by thin-layer chromatography (7).

Enzymes

Combination fluid. The common bile–pancreatic duct was cannulated at a point near its entrance into the duodenum. In the 24-hr period following the cannulation, the combination of bile and pancreatic fluid was collected in a flask at 4°C. The lipolytic enzymes in this preparation retained their activity for at least 48 hr.

Untreated pancreatic juice solids. Pancreatic juice, free of bile, was obtained by cannulating separately the bile and pancreatic ducts as described previously (1). The pancreatic fluid was collected at 4°C and freeze-dried. On the day of use, the solids were reconstituted, 3 mg/ml, in 0.01 M histidine, pH 7.0.

Treated pancreatic juice solids. To inactivate nonspecific lipase in the present study, we dissolved 150 mg of crude pancreatic juice solids and 1 mg of α-chymotrypsin (3 × crystallized, Worthington Biochemical Corp., Freehold, N.J.) in 80 ml of 0.01 M histidine, pH 9.0. This solution was maintained for 1 hr at 40°C, then adjusted to pH 7.0 and brought to a volume of 100 ml. This treatment completely inactivated nonspecific lipase as demonstrated by the inability of this preparation to hydrolyze 2,3-butane dioleate (1). Less than 10% of the lipase activity was lost by this treatment.

We have reported (1) that nonspecific lipase can b inactivated by maintaining reconstituted pancreatic juice at 40°C and pH 9.0 for 1 hr. Subsequently it was observed that the nonspecific lipase in some collections of pancreatic juice was not inactivated by this treatment. Apparently, these differences in stability are related to variations in the active proteolytic enzymes in the pancreatic juice. The addition of α-chymotrypsin overcomes this problem.

Purified lipase. Lipase (EC 3.1.1.3) was isolated from the untreated pancreatic juice solids using the method of Vandermeers and Christophe (8). The purified lipase had an activity of 2900 units/mg as measured by the method of Marchis-Mouren, Sarda, and Desnuelle (9).

Procedure

The esters were digested in a cylindrical, flat-bottomed glass tube 30 mm (I.D.) × 90 mm. Four 3-mm indentations in the side wall prevented vortexing during stirring. Each digest contained 100 mg of substrate, 85 μmoles of $CaCl_2$, 3.5 mg of histidine (final concentration 0.002 M), 152 mg of NaCl (final concentration 0.15 M), and the enzyme in a total volume of 10 ml. Where indicated, 200 mg of sodium taurocholate (final concentration 37 mM) was included in the mixture. Prior to the addition of the enzyme, all the components of the digest were stirred for 5 min. The digestions were carried out at pH 9.0 and at 27°C. All substrates were liquids at this temperature. The pH was maintained with the aid of a pH stat by the addition of 0.02 N KOH. The rate of addition of alkali was linear during the first several minutes of digestion and is reported here as the rate of hydrolysis: μmoles of free fatty acid released per minute per milligram or per milliliter of enzyme preparation. The values were reproducible within 5%. The amount of enzyme used in the digestions was selected so as to realize a rate of hydrolysis within the limits of response of the pH stat. In some experiments, large amounts of enzyme were added to show that under certain conditions no hydrolysis occurred. For all substrates and for all enzyme preparations, the rate of the reaction, if it occurred, was enzyme-limited.

The digestion conditions described here differ from those used in our earlier work in the total volume of the digest and in the taurocholate and NaCl concentrations. Unlike the previous study, the taurocholate used here was essentially pure. The concentration, 37 mM, which gives maximum enzyme activity, is considerably higher than that we found necessary for the impure taurocholate used earlier (1). With this change and the reduction in volume of the digest, 0.15 M NaCl was sufficient for maximum activity of both enzymes.

RESULTS

The rates of hydrolysis of the esters of the eight different alcohols by the various preparations of rat pancreatic juice are given in Table 1. The numbers in parentheses are the volume or weight of enzyme preparation that was used in that particular digest. The combination of bile–pancreatic fluid digested all substrates with the exception of sorbitol hexaoleate and sucrose octaoleate. This failure of hydrolysis was obtained in spite of using 400 times as much combination bile–pancreatic fluid as was used when triolein was the substrate.

When pancreatic juice without bile was used as a source of the enzymes, the esters that were hydrolyzed depended on the presence or absence of added sodium taurocholate. In the absence of sodium taurocholate, only those substrates that contained less than four ester groups were hydrolyzed. The addition of sodium taurocholate to the digest permitted the hydrolysis also of the substrates containing four and five ester groups. There were marked differences in the rates of hydrolysis of the oleate esters of methanol, ethylene glycol, and glycerol if taurocholate was not present, but these differences disappeared if this bile salt was added to the digest.

In the absence of sodium taurocholate, the pattern of digestion by treated pancreatic juice was similar to that seen with the untreated pancreatic juice. However, in the presence of added sodium taurocholate, pancreatic juice that had been treated with the proteolytic enzyme could not digest any of the substrates.

The final set of results was obtained with purified pancreatic lipase. If sodium taurocholate was not present, this enzyme hydrolyzed methyl oleate, ethylene glycol dioleate, and triolein, but did not hydrolyze the substrates that contained more than three ester groups. The addition of sodium taurocholate blocked completely the hydrolytic activity of this enzyme.

DISCUSSION

Earlier reports have shown that pancreatic lipase is active only at an oil–water interface (10) and that it hydrolyzes esters of primary alcohols (2) but not esters of secondary alcohols (1, 3). The results reported here for the purified lipase reveal another limitation of this enzyme—it cannot hydrolyze compounds that contain more than three ester groups. This was particularly unexpected, since as shown here, increasing the number of ester groups from one (methanol) to two (ethylene glycol) to three (glycerol) progressively increased the rate of hydrolysis. However, the addition of a fourth ester group (erythritol) resulted in a compound which was not hydrolyzed.

Although the substrates containing more than three ester groups were not hydrolyzed by lipase, those containing four or five ester groups were hydrolyzed by untreated pancreatic juice if it contained bile or added sodium taurocholate. We have reported similar observations for the complete ester of butane-2,3-diol (1). As in the studies with the butane dioleate ester, the enzyme that hydrolyzed compounds containing more than three ester groups was inactivated by exposure to a proteolytic enzyme. Because of these similarities, we believe the same enzyme is responsible for the hydrolysis of these two classes of substrate, namely esters of secondary alcohols and compounds containing more than three but less than six ester groups.

None of the enzymes in pancreatic juice hydrolyzed sorbitol hexaoleate or sucrose octaoleate in vitro. We have confirmed this observation in rats by the fat balance technique. These results will be reported separately.

We do not have an explanation for the inability of lipase to hydrolyze compounds containing more than three ester groups. All of these contained esters of primary alcohols, so this failure cannot be attributed to the absence of the proper type of ester linkage. It is, of course,

TABLE 1. Relative rates of hydrolysis by rat pancreatic juice enzymes of the complete oleate esters of the listed alcohols

	Enzyme Preparation									
	Pancreatic–Bile Juice		Untreated Pancreatic Juice				Treated Pancreatic Juice[a]		Purified Lipase	
	No TC[b]		No TC		TC Added		No TC	TC Added	No TC	TC Added
	μmoles FFA/min/ml		*μmoles FFA/min/mg*				*μmoles FFA/min/mg*		*μmoles FFA/min/mg*	
Methanol, 1[c]	54	(0.05)[d]	2.6	(1.2)	4.0	(1.2)	2.5 (1.2)	0 (1.2)	63 (0.02)	0 (0.3)
Ethylene glycol, 2	160	(0.025)	10	(0.3)	4.3	(0.3)	7.7 (0.3)	0 (0.3)	200 (0.01)	0 (0.1)
Glycerol, 3	2100	(0.005)	73	(0.075)	6.0	(0.15)	70 (0.06)	0 (0.3)	1900 (0.002)	0 (0.02)
Erythritol, 4	1.9	(1)	0	(6)	1.4	(3)	0 (6)	0 (6)	0 (0.1)	0 (0.1)
Pentaerythritol, 4	1.1	(1)	0	(6)	1.1	(3)	0 (6)	0 (6)	0 (0.1)	0 (0.1)
Adonitol, 5	0.53	(2)	0	(6)	0.25	(3)			0 (0.1)	0 (0.1)
Sorbitol, 6	0	(2)	0	(6)	0	(12)	0 (6)	0 (12)	0 (0.1)	0 (0.1)
Sucrose, 8	0	(2)	0	(6)	0	(12)	0 (6)	0 (12)	0 (0.1)	0 (0.1)

[a] Nonspecific lipase was inactivated by treatment with α-chymotrypsin (see text for details).
[b] TC, sodium taurocholate.
[c] Number of ester groups.
[d] The number in parentheses is the volume or weight of the enzyme preparation that was used.

tempting to invoke steric hindrance as an explanation for these observations. Besides the structure of the substrates, this would depend also upon the orientation of the compound at the oil–water interface. Adam and Dyer (11) have studied the orientation of pentaerythritol tetrapalmitate by physical methods at an air–water interface. They found the orientation to be similar to that of a triglyceride; the fatty acids of the complete esters of both pentaerythritol and glycerol extended into the air phase. Thus, neither the structure of the ester group nor the orientation of the molecule at the interface seems a likely explanation for the inability of lipase to hydrolyze pentaerythritol tetraoleate. The possibility remains that steric hindrance by these compounds prevents the formation of the enzyme–substrate complex. However, since the nature of this complex is not known, even where triglyceride is the substrate, this explanation can only be speculative.

Brockerhoff (12) has examined the rate of hydrolysis of the esters of a number of alcohols, although not those studied here. Besides steric hindrance, he proposed that the rate of hydrolysis of a compound depends on the electrophilicity of the alcohol moiety. It is difficult to see how this latter explanation could account for the ability of lipase to hydrolyze glycerol esters and its inability to hydrolyze esters of erythritol. Furthermore, as will be reported subsequently, the triester of erythritol is hydrolyzed by pancreatic lipase.

With one exception, the results reported here, where the complete esters of methanol, ethylene glycol, or glycerol were the substrates, are the same as those reported earlier for triolein (1, 3). The exception is the complete inhibition of lipase activity by added sodium taurocholate. In the earlier studies, bile salts caused approximately an 80% inhibition of this enzyme. This difference could be due to the higher concentration of bile salts employed in the studies reported here or to different sources of sodium taurocholate. The taurocholate used in the present studies was pure, whereas in the earlier studies at least one other bile salt, as well as other contaminants, was present. Although the inhibition of pancreatic lipase by bile salts under these conditions has been reported (13, 14), the mechanism is not understood. The solution to this problem awaits an adequate method for resolving the nature of the oil–water interface in these systems. In the case of another surface-active agent, normal alcohols, we have proposed (15) that the mechanism of inhibition is competition between the substrate and the surface-active agent for location at the oil–water interface.

The complete inhibition of lipase activity occurred regardless of whether taurocholate was added to the system containing pure enzyme or to one in which lipase was a component of the treated pancreatic juice. Consequently, the addition of this bile salt to the untreated pancreatic juice also would bring about complete inhibition of the lipase in that preparation. Therefore, the hydrolysis of the esters of methanol, ethylene glycol, and glycerol that took place with the untreated pancreatic juice in the presence of taurocholate must be attributable to the enzyme, nonspecific lipase. Since methyl oleate and ethylene glycol contain only esters of primary alcohols, this enzyme can hydrolyze such esters. The ability of this enzyme to split esters of primary alcohols, esters of secondary alcohols, and esters of compounds containing more than three but less than six ester groups supports our suggestion that an appropriate trivial name for this enzyme is nonspecific lipase.

Manuscript received 22 September 1971; accepted 7 December 1971.

REFERENCES

1. Mattson, F. H., and R. A. Volpenhein. 1966. Carboxylic ester hydrolases of rat pancreatic juice. *J. Lipid Res.* **7:** 536–543.
2. Mattson, F. H., and L. W. Beck. 1956. The specificity of pancreatic lipase for the primary hydroxyl groups of glycerides. *J. Biol. Chem.* **219:** 735–740.
3. Mattson, F. H., and R. A. Volpenhein. 1968. Hydrolysis of primary and secondary esters of glycerol by pancreatic juice. *J. Lipid Res.* **9:** 79–84.
4. Hyun, J., H. Kothari, E. Herm, J. Mortenson, C. R. Treadwell, and G. V. Vahouny. 1969. Purification and properties of pancreatic juice cholesterol esterase. *J. Biol. Chem.* **244:** 1937–1945.
5. Morgan, R. G. H., J. Barrowman, H. Filipek-Wender, and B. Borgström. 1968. The lipolytic enzymes of rat pancreatic juice. *Biochim. Biophys. Acta.* **167:** 355–366.
6. Mattson, F. H., and R. A. Volpenhein. 1962. Synthesis and properties of glycerides. *J. Lipid Res.* **3:** 281–296.
7. Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *J. Lipid Res.* **3:** 127–128.
8. Vandermeers, A., and J. Christophe. 1968. α-Amylase and lipase of the pancreas of the rat. Chromatographic purification, molecular weight determination and amino acid composition. *Biochim. Biophys. Acta.* **154:** 110–129.
9. Marchis-Mouren, G., L. Sarda, and P. Desnuelle. 1959. Purification of hog pancreatic lipase. *Arch. Biochem. Biophys.* **83:** 309–319.
10. Sarda, L., and P. Desnuelle. 1958. Action of pancreatic lipase on emulsified esters. *Biochim. Biophys. Acta.* **30:** 513–521.
11. Adam, N. K., and J. W. W. Dyer. 1924. The molecular structure of thin films. VI. *Proc. Roy. Soc. Ser. A.* **106:** 694–709.
12. Brockerhoff, H. 1968. Substrate specificity of pancreatic lipase. *Biochim. Biophys. Acta.* **159:** 296–303.
13. Mattson, F. H., and R. A. Volpenhein. 1966. Enzymatic hydrolysis at an oil/water interface. *J. Amer. Oil Chem. Soc.* **43:** 286–289.
14. Benzonana, G., and P. Desnuelle. 1968. Action of some effectors on the hydrolysis of long chain triglycerides by pancreatic lipase. *Biochim. Biophys. Acta.* **164:** 47–58.
15. Mattson, F. H., R. A. Volpenhein, and L. Benjamin. 1970. Inhibition of lipolysis by normal alcohols. *J. Biol. Chem.* **245:** 5335–5340.