Purification and Characterization of a Porcine Liver Microsomal Triacylglycerol Hydrolase†

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ABSTRACT: We have purified an enzyme from porcine liver microsomes which catalyzes hydrolysis of triacylglycerols. The enzyme was solubilized from the membranes by the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and was purified to apparent homogeneity by sequential chromatography on Q-Sepharose, hydroxyapatite, Affi-Gel heparin, and Mono-Q. The purified hydrolase migrated in SDS−polyacrylamide gel electrophoresis (PAGE) as a single polypeptide band of an apparent molecular mass of 60 kDa. The enzyme hydrolyzed long-, medium-, and short-chain triacylglycerols, as well as a chromogenic lipase substrate, 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester. The highest specific activity was obtained with tributyroylglycerol (240 μmol·min^{-1}·mg^{-1}). The reaction rate was maximal at pH 8.5. Sulfhydryl-directed reagents, such as *N*-ethylmaleimide (NEM), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and dodecyldithio-5-(2-nitrobenzoic acid) (C_{12}-TNB) had no effect on the hydrolase activity; however, the enzyme was sensitive to $HgCl_2$. Serine reagents, such as diethyl-*p*-nitrophenyl phosphate (E_{600}) and diisopropyl fluorophosphate (DFP), used in 100-fold molar excess completely inhibited the activity, suggesting that it is a serine esterase. These results suggest that the enzyme may participate in the intracellular neutral lipid metabolism since the enzyme is located in the endoplasmic reticulum, an organelle where *de novo* triacylglycerol synthesis and assembly of lipoproteins take place.

The biosynthesis of very low density lipoprotein (VLDL)[1] in liver and chylomicrons in intestine is a complex process requiring concomitant neutral lipid, phospholipid, and protein synthesis. A simplified model where *de novo* synthesized triacylglycerols are directly transferred to nascent VLDL has been compromised by recent data demonstrating that the majority of triacylglycerol formed via the glycerol 3-phosphate pathway is not directly secreted but undergoes a lipolysis/re-esterification cycle prior to incorporation into plasma lipoproteins (Francone et al., 1989; Yang & Kuksis, 1991; Wiggins & Gibbons, 1992; Gibbons et al., 1994; Yang et al., 1995, 1996; Gibbons, 1995). In addition, accumulated triacylglycerol droplets in liver cells appear to be hydrolyzed, reacylated, and secreted as VLDL triacylglycerol (Hoyumpa et al., 1975; Mooney & Lane, 1981). The intracellular location and properties of lipase(s) that may participate in mobilization of triacylglycerol for VLDL assembly and secretion remain to be determined. It had been initially suggested that an acid lipase was responsible for the hydrolysis (Francone et al., 1989). The acid lipase was reported to be present in lysosomes of hepatic (Goldstein & Brown, 1977; Schmitz & Assman, 1989) and intestinal tissues (Rao & Mansbach, 1990) where it was thought to act on stored (cytosolic) and endocytosed triacylglycerols. The enzyme has been purified from secretions of human fibroblasts (Sando & Rosenbaum, 1985) and from human liver (Ameis et al., 1994), and its amino acid sequence has been deduced from cDNA (Ameis et al., 1994: Anderson & Sando, 1991). Subjects deficient in the enzyme (Wolman's disease) accumulate lipid droplets in the cytoplasm (Schmitz & Assman, 1989). The acid lipase activity could be blocked by the administration of chloroquine. Chloroquine treatment, however, does not inhibit the bulk of intracellular hydrolysis of triacylglycerols; it appears that other lipase(s) may be involved in the mobilization of triacylglycerols for VLDL/chylomicron synthesis and secretion. This has been supported by recent experiments in fibroblasts isolated from patients afflicted with Wolman's disease, cholesteryl ester storage disease (CESD), and neutral lipid storage disease (NLSD). It has been demonstrated (Hilaire et al., 1993, 1994) that the lysosomal acid lipase is involved in the metabolism of receptor-mediated endocytosed triacylglycerol (low-density lipoprotein) but not of triacylglycerols present in endocytosed high-density lipoprotein or of endogenously formed triacylglycerol. Hydrolysis of the latter two pools of triacylglycerols was impaired in the NLSD fibroblasts which contain normal acid lipase activity, indicating a defect in lipolytic processing distinct from lysosomes. The presence of an alkaline lipase activity in porcine (Di Nella et al., 1960)

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[1] Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; C_{12}-TNB, dodecyldithio-5-(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); E_{600}, diethyl-*p*-nitrophenyl phosphate; NaTDC, sodium taurodeoxycholate; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; *p*-CMB, *p*-chloromercuribenzoic acid; TBS, Tris-buffered saline; TC4, tributyroylglycerol; TC8, trioctanoylglycerol; TC18:1, trioleoylglycerol; VLDL, very low density lipoprotein.

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and rat (Rao & Mansbach, 1993) intestine has been reported. A nonlysosomal triacylglycerol lipase activity was also demonstrated to be present in rat liver microsomes (Coleman & Haynes, 1983). A long-chain diacylglycerol transacylase activity in rat liver and intestinal microsomes has also been documented, and the 52 kDa enzyme has been purified from the intestine (Lehner & Kuksis, 1993a). The enzyme utilizes diacylglycerol substrates, yielding monoacylglycerol and triacylglycerol products; however, it is not capable of hydrolysis of triacylglycerols.

Here we report the isolation, purification, and characterization of a triacylglycerol hydrolase activity from porcine liver microsomes. We demonstrate that the purified enzyme is capable of hydrolysis of long-chain triacylglycerols and therefore may play a role in early steps of lipoprotein assembly.

EXPERIMENTAL PROCEDURES

Materials. Tributyroylglycerol (TC4, purum) and trioctanoylglycerol (TC8, puriss) were from Fluka Chemie AG (Buchs, Switzerland). [9,10-^{3}H(N)]Trioleoylglycerol (18.4 Ci/mmol) (TC18:1) was purchased from DuPont-NEN (Les Ulis, France). Oleoyl-CoA, *p*-nitrophenyl palmitate and laurate, oleic acid, trioleoylglycerol, phosphatidylcholine (egg), phosphatidylserine (beef brain), 1(3)- and 2-oleoylglycerol, 1,2-dioleoylglycerol, 1,3-dioleoylglycerol, 1,2-didecanoyl-*sn*-glycerol, 1,2-dilauroyl-3-phosphorylcholine, CoA, bovine serum albumin (essentially fatty acid free), sodium taurodeoxycholate, CHAPS, anti-rabbit IgG (whole molecule) peroxidase conjugate, glycerol, β-mercaptoethanol, *p*-choromercuribenzoate, *N*-ethylmaleimide, and diethyl-*p*-nitrophenyl phosphate (E_{600}) were obtained from Sigma Chimie (Fallavier, France). Affi-Gel heparin, Econo-pac Heparin, and Q and HIC cartridges were from Bio-Rad S.A. (Paris, France). Mono-Q, protein A−Sepharose, HA-Ultrogel, SDS−polyacrylamide gel electrophoresis molecular mass standards, and the isoelectric focusing calibration kit were from Pharmacia LKB (Uppsala, Sweden). Silica gel 60H plates were purchased from Merck. DTNB and 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester were obtained from Boehringer-Mannheim (Meylan, France). ECL Western blotting reagents and Hyperfilm-ECL were from Amersham France SA (Les Ulis, France). Cytoscint was purchased from ICN Biomedicals (Orsay, France). Tetrahydrolipstatin was a generous gift from Dr. H. Lengsfeld (Hoffmann-La Roche Ltd., Basel, Switzerland). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Isolation of Porcine Liver Microsomes. Liver tissues were washed extensively with ice-cold Tris-buffered saline, cut in small pieces, and homogenized in 250 mM sucrose, 25 mM Tris-HCl, pH 7.4, and 5 mM EDTA to yield 20% (w/v) crude extract. The homogenate was filtered through a single layer of gauze (Nu Gauze, Johnson and Johnson), and the microsomal membranes were prepared by centrifugation as described earlier (Lehner & Kuksis, 1993a). Crude microsomes were washed with 50 mM Tris-HCl, pH 7.4, 0.5 M KCl. Washed microsomes were suspended in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl to give a final concentration of protein of 10 mg/mL. All centrifugation steps were carried out at 4 °C.

Solubilization of Microsomal Membranes. The zwitterionic detergent CHAPS (1%, detergent:protein weight ratio 1:1) was added to the washed microsomal membranes, and the mixture was gently stirred for 30 min on ice. Solubilized proteins were recovered in the supernatant following centrifugation at 106000*g* for 1 h. The extract was then dialyzed overnight against 20 mM Tris-HCl, pH 7.4, and recentrifuged at 106000*g* for 1 h.

Purification of Triacylglycerol Hydrolase. All chromatographic steps were carried out using FPLC at ambient temperature. All buffers contained 0.1% CHAPS. Column eluants were placed immediately on ice.

(A) High-Q. One hundred milligrams of solubilized and dialyzed microsomal extract was loaded on two 5 mL Econo-Pac High Q cartridges connected in tandem. The columns were washed with 20 mL of 20 mM Tris-HCl, pH 7.4, 10% glycerol and subsequently eluted with a 0−1 M NaCl gradient.

(B) Hydroxyapatite. Active fractions eluted from High-Q chromatography were combined and dialyzed overnight against 5 mM sodium phosphate, pH 7.1. The protein mixture was loaded onto a 5 mL Econo-Pac HTP cartridge preequilibrated with 5 mM sodium phosphate, pH 7.1. The column was eluted with a 5−250 mM phosphate gradient.

(C) Heparin. Combined active fractions were mixed with 4 mL of Affi-Gel heparin preequilibrated with 20 mM Tris-HCl, pH 7.5, containing 10% glycerol. Bound proteins were eluted with 1 M NaCl.

(D) Mono-Q. Active fractions recovered after heparin chromatography were loaded onto Mono-Q and eluted with a 0−250 mM NaCl gradient. Purified triacylglycerol hydrolase was concentrated to give a final protein concentration of 1 mg/mL.

(E) Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gels (10% and 12%) (Laemmli, 1970) were run under reducing conditions and were silver-stained (Rabilloud et al., 1988). Phast (Pharmacia) native and isoelectric focusing gels were run according to manufacturer's instructions.

Triacylglycerol Hydrolase Assays. *(A) Potentiometric Assay*. The hydrolysis of short- and medium-chain triacylglycerols was determined at pH 8.0 and 37 °C with pH-stat (TTT 80 Radiometer) by titrating liberated fatty acids with 0.1 N NaOH. The standard mixture using tributyroylglycerol contained the following in a final volume of 15 mL: 14.9 mL of 150 mM NaCl and 100 μL of tributyroylglycerol. With trioctanoylglycerol, the assay mix was composed of 14.5 mL of 150 mM NaCl, 2 mM sodium taurodeoxycholate, 0.1 mg/mL bovine serum albumin, and 0.5 mL of substrate. Assay with egg-yolk lipoprotein was performed as described previously (de Haas et al., 1968).

(B) Radiolabeled Assay. Long-chain triacylglycerol hydrolysis was assayed using gum arabic-emulsified radiolabeled trioleoylglycerol (250 μM, specific activity 1 mCi/mmol) in a final volume of 0.2 mL of 50 mM Tris-HCl, pH 8.0, 4 mM $CaCl_2$, and 150 mM NaCl containing 1 mg/mL BSA as a fatty acid acceptor. The reaction was terminated by addition of 3.25 mL of methanol/chloroform/heptane (3.85:3.42:2.73 by volume); 0.3 mL of 150 mM NaCl, lipid carriers (100 μg of unlabeled oleic acid, mono-, di- and trioleoylglycerol), and 50 μL of 1 N NaOH were added, the mixture was vortexed and centrifuged, and 1 mL of the upper phase (fatty acids) was mixed with 5 mL of Cytoscint and counted (Ghosh et al., 1990). Alternatively, the reaction was stopped by the addition of 4 mL of chloroform/methanol (2:1 v/v), total lipids were extracted (Folch et al., 1957), and the

distribution of radioactivity in acylglycerols and free fatty acids was determined following thin-layer chromatography by scintillation counting (Lehner & Kuksis, 1992).

(C) Spectrophotometric Enzyme Assays. Hydrolysis of 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester (100 μM) was determined at 37 °C in final volume of 1 mL as follows: Substrate solution was prepared by mixing 40 μL of the ester (2 mg/mL dioxane) with 160 μL of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Triton X-100. To this was added 790 μL of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.53% sodium taurodeoxycholate, and 1.33 mM $CaCl_2$, and the reaction was started by addition of enzyme (10 μL). The release of resorufin was monitored at 572 nm.

Liberation of *p*-nitrophenol from *p*-nitrophenyl laurate or palmitate was measured at 405 nm. The standard 1 mL assay mixture consisted of 100 μM substrate in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.01% Triton-X100.

Hydrolysis of oleoyl-CoA was determined by reaction of released CoA with DTNB. The 1 mL assay contained 100 μM substrate, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM DTNB. The colored product was measured at 412 nm (Berge, 1979). The values were blank-corrected (incubations without the enzyme). Product standard curves were constructed for all spectrophotometric assays.

(D) Lipid Monolayer Assay. Measurements were performed with a KSV 2200 Barostat (KSV, Helsinki) using a "zero-order trough" as described for pancreatic lipases (Verger & de Haas, 1973). 1,2-Didecanoyl-*sn*-glycerol or *sn*-1,2-dilauroyl-3-phosphorylcholine was spread on the surface of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA. Enzyme was injected into the subphase, and the reaction was monitored at a constant surface pressure of 30 mN/m by moving the mobile barrier.

Detection of Activity on Polyacrylamide Gels. Native and isoelectric focusing gels were overlaid with a solution of bromochloro-3-indolyl butyrate (0.2 mg/mL) and nitro blue tetrazolium (0.4 mg/mL) in 0.1 M Tris-HCl, pH 7.4.

Effect of Inhibitors. Purified triacylglycerol hydrolase (20 μg) was preincubated with selected inhibitors for 30 min at 37 °C in a final volume of 100 μL. The remaining activity was then determined by the potentiometric assay procedure as described above using tributyroylglycerol as substrate.

Antibody Preparation. Purified triacylglycerol hydrolase (200 μg in 0.5 mL of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) was combined with 0.5 mL of Freund's complete adjuvant, and the emulsion was injected subcutaneously into two rabbits. Booster intramuscular injections were given 4 and 6 weeks later with 50 μg of antigen in Freund's incomplete adjuvant. Rabbits were bled prior to (preimmune) and 7 weeks after the initial immunization; sera were prepared and stored at −80 °C.

Western Blotting. Proteins separated on SDS−polyacrylamide gel electrophoresis were electroblotted onto a poly(vinylidene difluoride) (PVDF) membrane in ice-cold 25 mM Tris-HCl, 192 mM glycine, pH 8.3, at 100 V constant voltage for 1 h. Following transfer, the membrane was blocked with 5% skim milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) for 1 h at room temperature or overnight at 4 °C and then incubated for 1 h with a 1:2000 dilution of rabbit serum in TBS containing 1% skim milk and 0.1% Tween 20 (T-TBS-M). The membrane was washed 3 times for 10 min with blocking buffer and subsequently incubated for 1 h with a 1:10 000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Sigma) in T-TBS-M. The membrane was then washed 3 times for 10 min with TBS−0.1% Tween 20 (T-TBS). The bound antibody was detected by the ECL detection system according to instructions from the manufacturer.

Immunoprecipitation of Triacylglycerol Hydrolase. A 20% porcine liver homogenate was prepared by homogenizing 10 g of liver in 50 mL of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, and 1 mM benzamidine. CHAPS was added to the homogenate to a final concentration of 0.5%. Solubilized supernatant containing triacylglycerol hydrolase activity was recovered after centrifugation of the mixture for 1 h at 106000*g*; 50 μL (1 mg of protein) of the detergent extract was incubated overnight at 4 °C with 5 μL of either immune or preimmune sera, and then with 10 μL of protein A−Sepharose at 4 °C for 1 h. Beads were washed 4 times with T-TBS, and bound proteins were analyzed by gel electrophoresis under nonreducing conditions.

Other Methods. Protein concentration was determined by the Bio-Rad Protein Assay kit using BSA as a protein standard.

RESULTS

Purification of Triacylglycerol Hydrolase. Total porcine liver homogenate contains alkaline triacylglycerol acylhydrolase activity of approximately 200 nmol (mg of protein)$^{-1}$ min^{-1} using trioctanoylglycerol as substrate. More than 65% of the activity remained associated with the microsomal fraction upon subcellular fractionation. Less than 10% of the activity was found in the 106000*g* supernatant (cytosol). The activity could not be removed from the membranes by washing with 0.5 M KCl or by incubation with 1 mM Tris-HCl, pH 8.8, which releases soluble luminal proteins (Wettereau et al., 1991). Treatment with a low concentration of CHAPS (0.1%) resulted in recovery of only 10−15% of the activity in the supernatant following ultracentrifugation. These results suggested that the enzyme is tightly associated with the membranes. Increased concentration of CHAPS to 1% (detergent:protein weight ratio 1:1) solubilized more than 80% of the lipolytic activity without having any apparent inhibitory effect. On the other hand, nonionic detergents, although capable of solubilizing the enzyme, substantially inhibited the activity (results not shown). The solubilized extract was first chromatographed on a High-Q anion exchange medium. The cartridges were eluted with a NaCl gradient. The triacylglycerol hydrolase activity interacted with the matrix and was displaced at approximately 100 mM salt concentration. The active fractions were combined, dialyzed, and loaded onto a hydroxyapatite cartridge. The hydrolase activity was eluted between 100 and 150 mM phosphate concentration. The activity did not bind to heparin matrix; however, this step resulted in removal of other contaminating proteins. Although SDS−polyacrylamide gel electrophoretic analysis of triacylglycerol hydrolase after chromatography on heparin showed apparently homogeneous enzyme preparation (Figure 1), we have routinely carried out an additional chromatography on Mono-Q to obtain highly purified enzyme. Overall, the alkaline triacylglycerol hydrolase activity was purified 50-fold starting from the microsomal detergent extract with a recovery of 50% of the original solubilized activity (Table 1). The purified enzyme was stable for several months without apparent loss of activity when stored at −70 °C in 20 mM Tris-HCl, 150

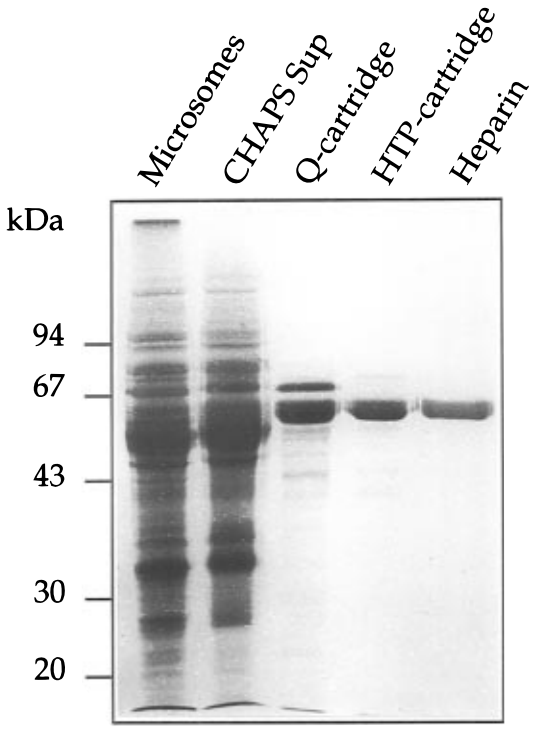

FIGURE 1: SDS−polyacrylamide gel electrophoretic profile of porcine liver TG hydrolase at various stages of purification. Twenty micrograms of microsomal and solubilized protein and 1 μg of the HTP- and heparin-purified fractions were analyzed. The gel was silver-stained. Molecular mass standards are indicated in the left margin.

Table 1: Purification of Porcine Triacylglycerol Hydrolase[a]

step	protein (mg)	sp act [μ_{mol} (mg of protein)$^{-1}$ min^{-1}]	purification (*n*-fold)	recovery (%)
CHAPS Sup	160	1.6	1	100
Q-Cartridge	11	18	11	76
HTP-Cartridge	2.5	65	41	62
Affi-Gel heparin	2.0	73	46	55
Mono-Q	1.6	80	50	49

[a] The hydrolytic assays were performed in a total volume of 15 mL containing 10−500 μg of protein, 100 mM trioctanoylglycerol, 150 mM NaCl, 2 mM NaTDC, and 0.1 mg/mL BSA. Assay was carried out by the pH-stat method as described under Experimental Procedures.

mM NaCl, 10% glycerol, and 0.1% CHAPS. The activity of the purified enzyme remained also unchanged after 24 h at room temperature.

Characterization of Triacylglycerol Hydrolase. A silver-stained SDS−polyacrylamide gel electrophoresis profile of the purified enzyme yielded a polypeptide of apparent molecular mass of 60 kDa (Figure 1). The enzyme migrated as a single band under nondenaturing conditions (Figure 2). Isoelectric focusing gel electrophoresis showed that the enzyme is made up of several isoforms (Figure 2). At least six distinct bands could be identified, all in the acidic region (p*I* 5.3−5.8). All six bands reacted with bromochloroindolyl butyrate in an overlay assay (data not shown). The origin of the observed heterogeneity is currently unknown. It may reflect modifications acquired through posttranslational processing (glycosylation/phosphorylation).

The purified enzyme hydrolyzed various lipid substrates (Table 2). The highest specific activity was obtained with tributyroylglycerol. Medium-chain triacylglycerols were

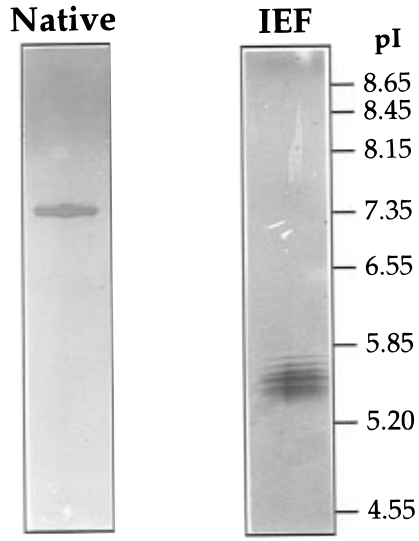

FIGURE 2: Native and isoelectric focusing gel electrophoresis of purified porcine liver TG hydrolase. Phast system (Pharmacia) was used to analyze isoforms of the purified enzyme. Conditions were selected to resolve proteins with isolelectric points from pH 3 to 9. Gels were silver-stained. Migration of p*I* standards is indicated in the right margin.

Table 2: Substrate Specificity of Triacylglycerol Hydrolase[a]

substrate	sp. act. [μmol (mg of protein)$^{-1}$ min^{-1}] +NaTDC (2 mM)	−NaTDC
tributyrin (C4:0)	240	240
tricaprylin (C8:0)	80	65
triolein (C18:1)	0.2	0.15
resorufin ester (diC12:0 ether)	ND[b]	0.2
p-nitrophenyl laurate (C12:0)	7	7
p-nitrophenyl palmitate (C16:0)	0.15	0.13
oleoyl-CoA (C18:1)	0.008	0.08
1,2-dilauroyl-3-phosphorylcholine	ND	0
egg-yolk lipoprotein	0	0

[a] Enzyme activity was determined by pH-stat (tributyroylglycerol, trioctanoylglycerol, egg-yolk lipoprotein), radiolabeled (triolein), chromogenic (resorufin ester, *p*-nitrophenyl palmitate/laurate, oleoyl-CoA), and monolayer (*sn*-1,2-dilauryl-3-phosphorylcholine) methods as described under Experimental Procedures. [b] ND, not determined.

utilized less efficiently. The hydrolase also displayed hydrolytic activity toward gum arabic-emulsified radiolabeled trioleoylglycerol, yielding monoacylglycerol and 1,2(2,3)-dioleoyl-*sn*-glycerol products. No 1,3-dioleoylglycerol was produced, indicating that the hydrolase was specific for the primary ester groups. The chromogenic lipase substrate 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester was also utilized by the purified enzyme. Long-chain fatty acyl *p*-nitrophenyls and acyl-CoA were hydrolyzed with low specific activity when compared to other purified acyl-CoA hydrolases (Lehner & Kuksis, 1993b; Alexson et al., 1993; Mukherjee et al., 1993). Phospholipids were not hydrolyzed by the enzyme.

The enzyme exhibited Michaelis−Menten saturation kinetics with respect to tributyroylglycerol with an apparent K_m value of 8 μM (Figure 3, panel A). The maximal velocity was reached with 50 μM substrate concentration and remained constant up to 100 mM tributyroylglycerol concentration. This result shows that the enzyme does not undergo interfacial activation and is able to hydrolyze both water-soluble and insoluble esters. Enzyme activity was

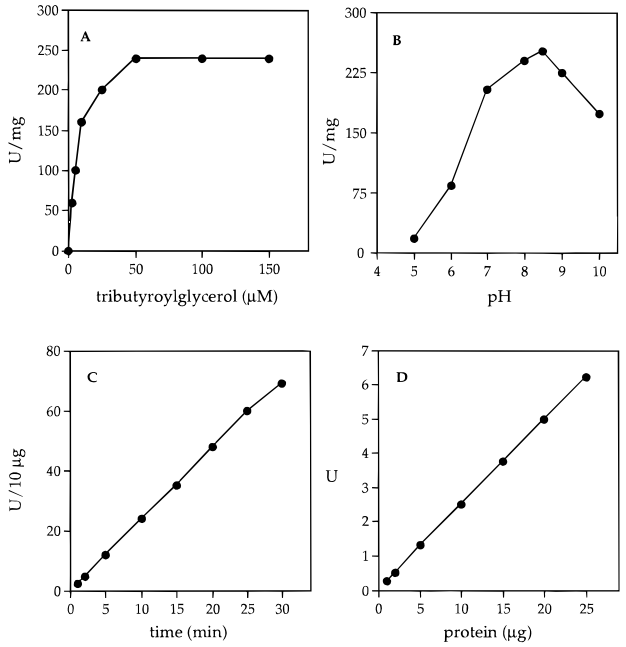

FIGURE 3: Kinetics of tributyroylglycerol hydrolysis by purified porcine liver TG hydrolase. Dependence on (A) substrate concentration, (B) pH, (C) time, and (D) protein concentration. Assays were performed as described under Experimental Procedures in a final volume of 15 mL of 150 mM NaCl using a pH-stat. All incubation mixtures, except (A), had 100 mM tributyroylglycerol; all, except (B), were incubated at pH 8. Panels A, B, and C had 10 μg of enzyme, and incubations in panels A, B, and D were carried out for 10 min.

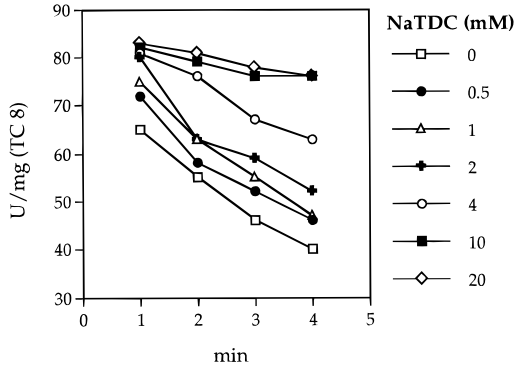

FIGURE 4: Effect of bile salt on porcine liver TG hydrolase activity. The hydrolysis of trioctanoylglycerol in the presence of various concentrations of sodium taurodeoxycholate was monitored using a pH-stat method as described under Experimental Procedures. The incubation mixture contained the following in a final volume of 15 mL: 150 mM NaCl, 100 mM trioctanoylglycerol, 10 μg of purified enzyme, and the detergent.

maximal at pH 8.5 (Figure 3, panel B), and the lipolytic reaction was linear with time and the amount of enzyme employed (Figure 3, panels C and D, respectively).

The presence of ionic (sodium taurodeoxycholate) detergent in the assay mixture was necessary for optimal hydrolytic activity of medium-chain triacylglycerols (Figure 4). In the absence or at low concentration (below critical micellar concentration) of the detergent, the activity diminished rapidly, presumably due to the lack of desorption of the lipolytic products from the lipid/water interface. For instance, at 0.5 mM detergent concentration, the specific activity of the enzyme at 4 min reaction was only 56% of that obtained after 1 min incubation, while at 10 mM concentration of the detergent the specific activity of the

Table 3: Effect of Inhibitors and Cofactors on Triacylglycerol Hydrolase Activity[a]

inhibitor	activity (% control)
standard	100
E_{600} (10 mM)	0
DFP (10 mM)	0
tetrahydrolipstatin (0.5 mM)	80
thiol reagents (1 mM NEM, DTNB, C_{12}-TNB, *p*-CMB)	100
$CaCl_2/MgCl_2/MnCl_2$ (10 mM)	100
$HgCl_2$ (10 mM)	70
$HgCl_2$ (20 mM)	50
BSA (1 mg/mL)	100
enzyme boiled for 5 min	0

[a] Purified triacylglycerol hydrolase was preincubated at 37 °C for 30 min with the indicated inhibitors and cofactors. Enzyme activity was subsequently determined using the pH-stat method as described under Experimental Procedures. The standard assay medium contained the following in 15 mL: 15 μg of purified enzyme, 25 mM tributyroylglycerol, and 150 mM NaCl.

enzyme decreased only by 6%. On the other hand, assays with tributyroylglycerol produced linear kinetics even in the absence of detergents due to the complete solubility of the released butyric acid in the aqueous phase (Figure 3, panel C). Ionic detergent (NaTDC) could be substituted by a zwitterionic bile salt detergent analog (CHAPS) in the reaction medium; however, incubations in the presence of the nonionic detergent Triton X-100 at the same concentration (1%) resulted in a 60% decreased of the lipolytic activity. Sodium dodecyl sulfate (SDS) completely inactivated the enzyme.

Preincubation of the purified triacylglycerol hydrolase with the serine modifiers diethyl-*p*-nitrophenyl phosphate (E_{600}) and diisopropyl fluorophosphate (DFP) resulted in complete inhibition of the activity (Table 3). The enzyme was also sensitive to tetrahydrolipstatin, which has been demonstrated to inhibit several mammalian lipases (Hadvary et al., 1988; Borgström, 1988; Gargouri et al., 1991: Lookene et al., 1994). Classical thiol-directed reagents did not affect the hydrolase activity; however, the enzyme was inhibited by $HgCl_2$, and this inhibition could be partially reversed by mercaptoethanol. The presence of divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}), cofactors for optimal activities of lipases and acyltransferases, had no effect on the specific activity of the enzyme.

Immunocharacterization of Triacylglycerol Hydrolase. The purified enzyme preparation was used to immunize rabbits. The polyclonal antiserum reacted with purified triacylglycerol hydrolase on Western blots and did not show any apparent cross-reactivity with other porcine cellular proteins. The antiserum (5 μL) immunoprecipitated all triacylglycerol hydrolase present in 50 μL of detergent extract of total homogenate (20 mg/mL) containing approximately 1 μg of triacylglycerol hydrolase based on the observed activity. Gel electrophoresis of the immunoprecipitate showed that the antiserum was specific for the enzyme (Figure 5). The polyclonal antibodies also cross-reacted with a 60 kDa protein in total homogenates and microsomes isolated from porcine kidney and intestine (Figure 6). These microsomal membranes also possessed triacylglycerol hydrolase activity, indicating that the same or closely related enzyme is expressed in other tissues. On the other hand, the protein and the activity are absent in heart tissue. The antibodies also recognized a protein of apparently identical

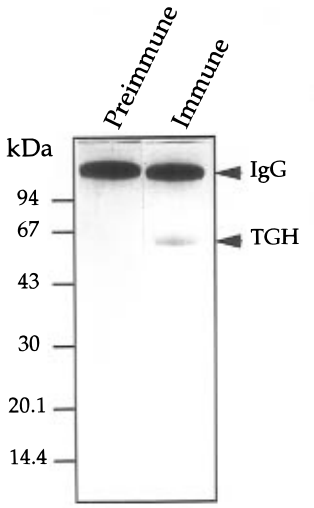

FIGURE 5: Immunoprecipitation of porcine liver TG hydrolase. One milligram of detergent extract of total liver homogenate was immunoprecipitated with 5 μL of anti-hydrolase serum. After addition of protein A−Sepharose and centrifugation, the immunoprecipitated proteins were analyzed by SDS−polyacrylamide electrophoresis performed under nonreducing conditions. The gel was stained with Coomassie blue. Molecular mass standards are indicated in the left margin.

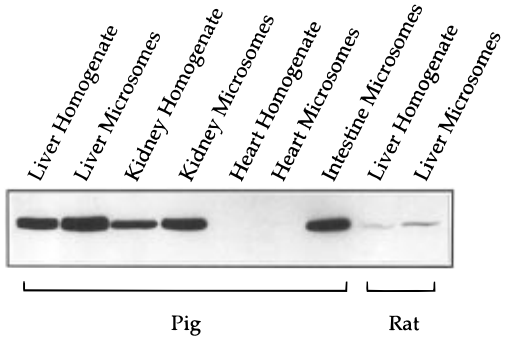

FIGURE 6: Tissue and species distribution of TG hydrolase. Proteins present in total homogenates (50 μg) and microsomes (10 μg) isolated from various porcine and rat tissues were electrophoresed in a 10% SDS−polyacrylamide gel, transferred onto a nitrocellulose membrane, and reacted with anti-TG hydrolase serum as described under Experimental Procedures.

molecular mass in rat liver microsomes, although with not the same intensity as in porcine tissues, suggesting the presence of a related protein.

Sequence Analysis. The amino acid sequence of the first eight N-terminal residues of the purified enzyme, GQ-PASPPV, is identical to that of porcine proline-β-naphthylamidase (Matsushima et al., 1991) which shares a high degree of sequence homology with rat liver (Robbi et al., 1990) and rabbit (Korza & Ozols, 1988; Ozols, 1989) carboxylesterases. Furthermore, N-terminal sequences of 30 and 20 kDa peptides obtained by limited chymotrypsin digestion of triacylglycerol hydrolase were SFVK and KSYPI, corresponding to amino acid residues 58−61 and 365−369 of the proline-β-naphthylamidase, respectively. The fifth amino acid residue in the 30 kDa chymotryptic peptide did not give a clear response in the HPLC analysis, suggesting a modified residue. In the deduced amino acid sequence of proline-β-naphthylamidase, this residue is an asparagine followed by two threonine residues denoting a potential N-glycosylation site. This site is also conserved in three rat carboxylesterases (Ozols, 1989; Takagi et al., 1988; Long et al., 1988; Yan et al., 1994).

DISCUSSION

The mechanisms that regulate hepatic and intestinal triacylglycerol synthesis and their assembly and secretion into plasma as part of lipoprotein particles are not yet fully elucidated. Recent *in vivo* studies (Francone et al., 1989; Yang & Kuksis, 1991; Wiggins & Gibbons, 1992; Gibbons et al., 1994; Yang et al., 1995, 1996; Gibbons, 1995) are consistent with an intracellular hydrolysis of triacylglycerols synthesized in the endoplasmic reticulum of hepatocytes and jejunum prior to reacylation and secretion as lipoproteins. Previous studies in the intestine suggested the presence of both cytosolic and particulate lipase activity (Di Nella et al., 1960; Rao & Mansbach, 1993). A nonlysosomal Mn^{2+}-stimulated triacylglycerol lipase activity was also described in rat liver microsomes (Coleman & Haynes, 1983). The properties of a porcine liver triacylglycerol hydrolase presented here appear to be different from the above characterized lipolytic activities. Unlike the intestinal cellular lipases, the purified 60 kDa enzyme is insensitive to *p*-CMB and albumin, and its activity is not affected by the presence of divalent cations. The hydrolase also differs from the rat liver microsomal activity as Mn^{2+} neither stimulates nor inhibits the enzyme and more importantly is fully active in the presence of bile salts at concentrations that were inhibitory for the rat lipase. The 60 kDa porcine liver triacylglycerol hydrolase could not be released from the microsomes by osmotic disruption, salt wash, or mild detergent treatment, which is consistent with tight association with membranes. Ionic bile salt detergents (Na TDC, sodium cholate) and their zwitterionic derivative (CHAPS) were effective in solubilizing and preserving the hydrolase activity. On the other hand, nonionic detergents (Triton X-100 and *n*-octyl glucoside) above their critical micellar concentrations had an inhibitory effect (results not shown). Medium-chain triacylglycerols were utilized much more effectively by the enzyme in the presence of a detergent, indicating the importance of the physical state of the substrate. In the absence of detergents, the hydrolase activity decreases sharply probably due to the impaired desorption of the reaction products. This was not the case with tributyroylglycerol because butyric acid released during the enzymatic reaction is a water-soluble compound. The enzyme hydrolyzed gum arabic-emulsified long-chain triacylglycerols at a much lower rate than that obtained for detergent-emulsified medium- and short-chain triacylglycerol. At present, it is not known whether the low specific activity toward the natural triacylglycerol substrate can be attributed to the chain-length specificity of the enzyme, the physical state of the substrate, or the impaired desorption of the long-chain lipolytic products from the interface. Much lower specific specific activity with long-chain triacylglycerols was also observed with other well-characterized lipases (Rogalska et al., 1993). For example, porcine pancreatic lipase exhibits a specific activity of 6000 μmol mg^{-1} min^{-1} toward tributyroylglycerol but only 90 μmol mg1^{-1} min^{-1} with trioleoylglycerol, and rabbit gastric lipase exhibited a 1000-fold decrease of specific activity from tributyroylglycerol to trioleoylglycerol. These results suggest impaired desorption of the long-chain lipolytic products from the lipid/water interface. It is also possible that the low microsomal

triacylglycerol hydrolase activity on oil droplets may be attributed to the fact that the enzyme may attack the unimolecular form of long-chain triacylglycerols. The membrane-associated enzyme may thus act on newly synthesized di- and/or triacylglycerols dissolved in the phospholipid bilayer. It has been previously reported that the kinetic behavior of lipases can be drastically affected by the presence of nonhydrolyzable co-lipids such as phosphatidylcholine (Pieroni & Verger, 1979).

The enzyme possesses properties characteristic of a serine esterase. It is inhibited by the serine modifiers DFP, E_{600}, and tetrahydrolipstatin. Organic sulfhydryl-directed compounds had no effect on the activity while a substantial decreased of activity was observed by preincubation of the enzyme with $HgCl_2$. This inhibition was reversible by addition of mercaptoethanol, indicating that a cysteine or perhaps a histidine residue at or near the active site of the enzyme was modified. Protease inhibitors, benzamidine (1 mM), pepstatin (0.5 μg/mg of enzyme), leupeptin (0.7 μg/mg of enzyme), and PMSF (1 mM), had no effect on the enzymatic activity (results not shown).

Sequence analysis of N-terminal amino acid residues and of peptides obtained from proteolytic digestion with chymotrypsin showed identity with a proline-β-naphthylamidase. This enzyme has been initially purified from porcine intestine (Takahashi et al., 1989) and later from liver (Matsushima et al., 1991) and reported to form a homotrimer (Takahashi et al., 1991). The specific activity toward amide substrates (Takahashi et al., 1989) gave generally much lower values than those we obtained with lipid substrates. The deduced amino acid sequence of the enzyme shares homology with rat liver (Robbi et al., 1990), rat kidney (Yan et al., 1994), and rabbit liver (Korza & Ozols, 1988; Ozols, 1989) carboxylesterases. The activity of the rat liver carboxylesterases toward long-chain triacylglycerols has not been reported. A carboxylesterase purified from rat adipose tissue exhibited 40-fold lower specific activity toward trioleoylglycerol (0.005 μmol mg^{-1} min^{-1}) than the porcine triacylglycerol hydrolase reported here. Unlike the rat and rabbit carboxylesterases with known amino acid sequences (Alexson et al., 1993; Robbi et al., 1990; Korza & Ozols, 1988; Ozols, 1989; Takagi et al. 1988; Takahashi et al., 1989; Yan et al., 1994), the porcine enzyme contains within its sequence a proposed neutral lipid binding site motif in the C-terminal domain (residues 399−406) composed of an octapeptide, FLDLMGDV. This octapeptide conforms to a consensus sequence, F(C)LXLXXXn, where n corresponds to a nonpolar residue (Au-Young & Fielding, 1992). The same motif is present in human cholesteryl ester transfer protein (Drayna et al., 1987), lecithin:cholesterol acyltransferase (McLean et al., 1986), cholesterol esterase (Kissel et al., 1989), liposacharide binding protein (Schumann et al., 1990), cholesterol 7α-hydroxylase (Noshiro & Okuda, 1990), and rat hormone sensitive lipase (Holm et al., 1988). All the above enzymes have been suggested to bind molecular nonpolar lipid.

Immunoblot analyses revealed that the microsomal triacylglycerol hydrolase is expressed in liver, in intestine, and in kidney but it is absent from heart. Of the two rat microsomal carboxylesterases for which sequences are known, one is present in liver, kidney, heart, and lung but is absent from intestine while the other is expressed in liver, lung, testis, adipose tissue, and heart but is not present in kidney and intestine (Mentlein et al., 1987). The triacylglycerol hydrolase expression coincides with ontogeny of lipoprotein secretion by the liver (Lehner et al., 1996). Immunocytochemical studies localize the hydrolase expression exclusively to liver cells surrounding the capillary vessels, an area that is likely to be active in lipoprotein secretion (Lehner et al., 1996). Moreover, the enzyme is absent from liver-derived HepG2 and McA RH7777 hepatoma cells which have impaired VLDL assembly and secretion (Gibbons et al., 1994; Wu et al., 1996). These results suggest that this triacylglycerol hydrolase may participate in the lipolysis/reesterification cycle of triacylglycerol assembly into VLDL.

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