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Pancreatic Cholesterol Esterases. 2. Purification and Characterization of Human Pancreatic Fatty Acid Ethyl Ester Synthase

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Received November 30, 1988; Revised Manuscript Received December 20, 1989

ABSTRACT: Human pancreatic fatty acid ethyl ester synthase has been isolated and purified 1200-fold to homogeneity, and its activities, binding properties, and N-terminal amino acid sequence indicate that it is a member of the lipase family. This 52-kDa monomeric protein is present at 0.6-1.2 mg/g of pancreas, and it catalyzes the synthesis and hydrolysis of ethyl oleate at rates of 2400 nmol mg^{-1} h^{-1} and 30 nmol mg^{-1} h^{-1}, respectively. Kinetic analyses reveal a pronounced substrate specificity for unsaturated octadecanoic fatty acids, with ethyl ester synthetic rates of 2400 nmol mg^{-1} h^{-1} (linoleic), 2400 nmol mg^{-1} h^{-1} (oleic), 400 nmol mg^{-1} h^{-1} (arachidonic), 300 nmol mg^{-1} h^{-1} (palmitic), and 100 nmol mg^{-1} h^{-1} (stearic). Like cholesterol esterase, the enzyme binds to immobilized heparin, and this property was critical for its purification to homogeneity. Its N-terminal amino acid sequence is virtually identical with that reported for human triglyceride lipase, NH_2-X-Glu-Val-Cys-^{5}Tyr-Glu-Arg-Leu-Gly-^{10}Cys-Phe-Ser-Asp-Asp-^{15}Ser-Pro-Trp-Ser-Gly-^{20}Ile, and it differs by only four residues from that reported for porcine pancreatic lipase. The synthase purified here also cleaves triglycerides, hydrolyzing triolein at a rate of 30 nmol mg^{-1} h^{-1}, and this activity is stimulated by colipase and inhibited by sodium chloride. Conversely, commercially available porcine triglyceride lipase exhibits fatty acid ethyl ester synthase activity (1530 nmol mg^{-1} h^{-1}) and hydrolyzes triolein at a rate of 23 nmol mg^{-1} h^{-1}. Thus, the predominant source of pancreatic fatty acid ethyl ester synthase activity is the exocrine product triglyceride lipase. These data indicate that triglyceride lipase and the cholesterol esterases belong to a family of enzymes whose members recognize both a fatty acid and an alcohol and bind specifically to heparin.

Fatty acid ethyl esters (FAEE), the products of nonoxidative ethanol metabolism, have been found in those organs commonly damaged by ethanol abuse. In acutely intoxicated subjects, pancreas was especially rich in these lipid metabolites with a level of 175 nmol of FAEE/g, about 50% higher than that found in liver. Moreover, normal human pancreas was also shown to synthesize these esters at the highest rate of all the organs tested (Laposata & Lange, 1986). In this work, the enzyme responsible for this high level of pancreatic fatty acid ethyl ester synthase activity was purified to homogeneity and found to be a 52-kDa protein. A critical step in this purification scheme was heparin-agarose affinity chromatography, indicating that the enzyme binds to heparin like the various molecular weight forms of cholesterol esterase (Cox et al., 1990; Kyger et al., 1990). Characterization of the homogeneous protein showed that its 20 amino acid N-terminus is identical with that reported for human pancreatic triglyceride lipase (DeCaro et al., 1981b). Moreover, the purified synthase also hydrolyzes triolein, and this activity is enhanced by colipase and inhibited by sodium chloride. Thus, the purified 52-kDa enzyme responsible for pancreatic FAEE synthesis is triglyceride lipase.

Materials and Methods

[^{14}C]Oleic acid (52 mCi/mmol) was obtained from Amersham; [^{3}H]arachidonic acid (191 mCi/mmol), [^{14}C]palmitic acid (58 mCi/mmol), [^{14}C]stearic acid (59 mCi/mmol), and [^{14}C]linoleic acid (55.6 mCi/mmol) were purchased from New England Nuclear.

Ethyl [^{3}H]oleate was synthesized by acid-catalyzed esterification of [^{3}H]oleic acid in ethanol, with isolation by thin-layer chromatography (Mogelson & Lange, 1984).

Porcine triglyceride lipase and colipase were purchased from Sigma. The pH of all Tris[1] buffers was determined at 4 °C.

Pancreatic Homogenates. Human pancreas was obtained from autopsy specimens and sliced into 1 cm × 2 cm × 3 cm portions after trimming away surrounding connective tissue. To preserve enzymatic activity, it was necessary either to include benzamidine in the buffers at physiologic pH or to maintain the pH at 6.0 (Cox et al., 1990). Typically, 48 g

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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of trimmed tissue was added to 80 mL of 0.5% digitonin, 50 mM benzamidine, and 10 mM sodium phosphate buffer, pH 6.0, and the mixture was homogenized with a polytron (Brinkman Instruments, Ontario, Canada) at half-maximum speed. The homogenate was then spun at 100000*g* for 60 min at 4 °C, the pellet discarded, and the supernatant dialyzed overnight against 5 L of 10 mM Tris and 20 mM benzamidine buffer, pH 8.2. After centrifugation at 27000*g* at 4 °C for 30 min, the supernatant was ready for purification.

Enzyme Assays. Fatty acid ethyl ester synthase activity was measured by the method of Mogelson and Lange (1984). Briefly, enzyme samples (100 μL) were incubated with 0.4 mM [^{14}C]oleate (20000 dpm/nmol), 1.5 M ethanol, and 60 mM sodium phosphate buffer, pH 7.2 (total volume = 170 μL) in capped tubes at 37 °C. The reaction was quenched by the addition of 2 mL of acetone, and a known amount of ethyl [^{3}H]oleate in 0.3 mM carrier ethyl oleate was added. The liquid was removed by evaporation under nitrogen, and the residue was dissolved in acetone and chromatographed on silica gel 0 plates (Analabs, North Haven, CT), developed with petroleum ether/diethyl ether/acetic acid (75:5:1) (Lange et al., 1981). Fatty acid ethyl ester spots were visualized with iodine vapor and scraped, and the radioactivity in each sample was determined by addition of 10 mL of Aquasol 2. Recovery of ^{3}H was used to adjust ^{14}C counts for yield calculations, and after blanks were subtracted, results were expressed as nanomoles of fatty acid ethyl ester produced per milligram of protein per hour (or nmol mL^{-1} h^{-1}, in the steps prior to final purification).

Fatty acid ethyl ester hydrolysis assays were performed by adding 50 μL of 10 mM Tris buffer, pH 7.2, to 75 μL of 318 μM ethyl [^{3}H]oleate in phosphatidylcholine vesicles (457 cpm/nmol), prepared by sonication as described elsewhere (Cox et al., 1990). After addition of enzyme (50 μL), the mixture was incubated at 37 °C for 45 min and the reaction was quenched with acetone. The reaction volume was reduced under a stream of nitrogen, and the samples were resuspended in acetone and then spotted on Type 0 silica gel plates, along with cold ethyl oleate and oleic acid as reference standards. The plates were developed with petroleum ether/diethyl ether/acetic acid (97:52:3), and fatty acid was quantitated as described above.

Triglyceride lipase activity was assayed by using tri[1-^{14}C]olein in phosphatidylcholine vesicles (1600 cpm/nmol). Vesicles (150 μL) were combined with 100 μL of 10 mM phosphate buffer, pH 7.4, and 50 μL of enzyme, and the capped tubes were incubated for 45 min at 37 °C. The reaction mixture was then treated in the same manner as that for cholesterol ester hydrolysis (Cox et al., 1990), with results expressed in nanomoles of [^{14}C]oleate released per milligram of protein per hour. All assays were linear with respect to expended time and added protein (Lowry et al., 1951).

Cholesterol ester synthesis was quantitated as described elsewhere (Kyger et al., 1990).

Hydrolysis of cholesterol esters was assayed by using [^{14}C]oleate-containing vesicles as described (Cox et al., 1990).

Electrophoresis. SDS–PAGE was performed according to the method of Laemmli (1970). The stacking gel (4.5% polyacrylamide) and separating gel (10% polyacrylamide) were run at 150 and 200 V, respectively. These were stained with 0.1% Coomassie Brilliant Blue or used directly for Western blots. The molecular weight of the enzyme was determined from a plot of R_f versus M_r of the following proteins: bovine serum albumin (monomer, 66000), phosphorylase *b* (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100), and α-lactalbumin (14400).

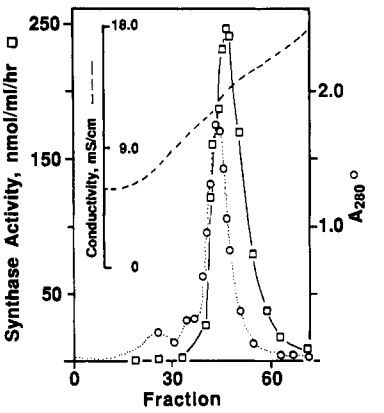

FIGURE 1: Hydroxylapatite chromatography of fatty acid ethyl ester synthase. The column (2.5 cm × 10 cm) was equilibrated with 10 mM sodium phosphate and 20 mM benzamidine buffer, pH 7.2, at a flow rate of 14 mL/h. The enzyme was eluted (2.3-mL fractions) with a linear gradient increasing from 50 mM phosphate and 20 mM benzamidine, pH 7.2, to 350 mM phosphate and 20 mM benzamidine, pH 7.2.

Western blotting (Burnette, 1981) was performed with ^{125}I-labeled staphylococcal protein A (specific radioactivity of 100000–200000 cpm/pmol) and rabbit anti-cholesterol esterase antibody, following procedures described elsewhere (Cox et al., 1990). Radioimmunoassay was performed with rabbit anti-bovine 67-kDa cholesterol esterase antibody as reported elsewhere (Cox et al., 1990).

Amino Acid Analysis. Amino acid analysis was performed by D. J. Strydom of the Harvard Medical School (Center for Biochemical and Biophysical Sciences and Medicine) and by the Protein Chemistry Laboratory, Washington University Medical Center, St. Louis, MO. Samples were hydrolyzed in evacuated sealed ampules for 24 h in 6 N HCl at 110 °C and analyzed on an automated amino acid analyzer (Model 6300, Beckman). N-Terminal amino acid sequencing was completed by D. J. Strydom.

RESULTS

Isolation and Purification of a Cationic Pancreatic FAEE Synthase. Fatty acid ethyl ester synthase activity from pancreatic cytosol (described under Materials and Methods) was applied at 15 mL/h to DEAE-cellulose (2.5 cm × 20 cm) equilibrated with 10 mM Tris and 20 mM benzamidine buffer pH 8.2. Although some activity (25%) bound under these conditions, most synthase activity was recovered in the wash fractions with a 20-fold purification. The active fractions were pooled, dialyzed against 10 mM sodium phosphate and 20 mM benzamidine buffer, pH 7.2, and applied to hydroxylapatite (2.5 cm × 10 cm) equilibrated with the same buffer. The enzyme was bound, and the resin was then washed with 20 mM benzamidine and 50 mM phosphate buffer, pH 7.2, and developed with a linear gradient increasing in phosphate concentration to 20 mM benzamidine and 350 mM sodium phosphate, pH 7.2. As shown in Figure 1, a single peak of enzyme activity emerged at 11–14 mS/cm, resulting in a 65-fold purification and 620% yield. The pH of pooled fractions was lowered to 6.0 by adding 1 N HCl, and the sample was dialyzed against 10 mM sodium phosphate buffer, pH 6.0.

The enzyme was then applied to heparin–agarose (1.5 cm × 14 cm) at pH 6.0, and the column was washed with 5 column volumes of each of the following buffers: 10 mM sodium phosphate, pH 6.0; 10 mM Tris, 50 mM benzamidine, and 50 mM sodium chloride, pH 7.2; 10 mM Tris, 50 mM

Table I: Purification of Human Pancreatic Fatty Acid Ethyl Ester Synthase

	total protein (mg)	total act. (nmol h^{-1})	sp act. (nmol mg^{-1} h^{-1})	purification (x-fold)	yield (%)
starting material	1130	2620	2		
DEAE wash	310	12900	40	20	490
hydroxyl-apatite	90	16250	130	65	620
heparin–agarose	30	64980	2400	1200	2480

Table II: Amino Acid Compositions of Human Pancreatic Fatty Acid Ethyl Ester Synthase, Human Triglyceride Lipase, and Porcine Triglyceride Lipase

amino acid	52 kDa (%)	human lipase[a] (%)	porcine lipase[b] (%)
Cys (half)	2.8	3.3	3.1
Asx	12.1	13.0	13.8
Thr	5.8	6.0	5.6
Ser	7.6	8.4	6.9
Glx	9.8	7.9	8.5
Pro	5.2	4.8	5.6
Gly	12.4	9.8	8.7
Ala	7.5	5.7	4.7
Val	7.5	7.2	7.8
Met	1.4	1.0	0.9
Ile	3.9	4.8	5.8
Leu	6.6	5.7	6.5
Tyr	2.8	3.1	3.6
Phe	5.2	5.7	5.6
His	2.1	2.6	2.2
Lys	5.6	5.5	4.9
Arg	3.4	3.6	4.7
Trp	nd[c]	1.9	1.3

[a] Vandermeers et al. (1974). [b] Calculated from primary sequence (DeCaro et al., 1981a). [c] Not determined.

benzamidine, 30 mM sodium chloride, and 20 mM taurocholate, pH 7.2; 10 mM Tris, 50 mM benzamidine, and 50 mM sodium chloride, pH 7.2. A linear salt gradient increasing from 10 mM Tris, 50 mM benzamidine, and 50 mM sodium chloride, pH 7.2, to 10 mM Tris, 50 mM benzamidine, and 500 mM sodium chloride, pH 7.2, was applied, and fatty acid ethyl ester synthase activity emerged over a conductivity range of 10–17 mS/cm. Heparin–agarose chromatography gave a further 18-fold purification and 450% yield.

A summary of the purification scheme is outlined in Table I; the resulting fatty acid ethyl ester synthase was purified 1200-fold with a specific activity of 2400 nmol mg^{-1} h^{-1} and yield of 2480%. From these results, the amount of this synthase in human pancreas can be calculated to be at least 1.0 mg of enzyme/g of pancreas.

Enzyme Characterization. Molecular weight and purity were determined by SDS–PAGE and fast protein liquid chromatography (FPLC) utilizing Superose 12. SDS–PAGE (10%) of each purification step demonstrates enrichment in a 52-kDa polypeptide, which is the final homogeneous product

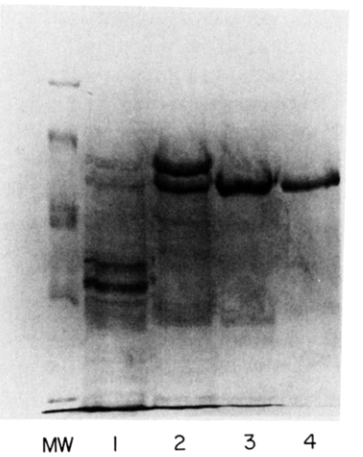

FIGURE 2: 10% SDS–PAGE of the purification of fatty acid ethyl ester synthase from human pancreas: (1) pancreatic cytosol, (2) DEAE-cellulose, (3) hydroxylapatite, and (4) heparin. Molecular mass standards are designated MW and are 94, 67, 43, 30, and 20 kDa. Each sample lane contained 15–40 μg of protein.

(Figure 2). Elution of the purified enzyme from Superose 12 occurred after the bovine serum albumin standard, and a semilog plot of molecular weight versus elution volume demonstrated that the molecular weight of the active enzyme was between 50 000 and 55 000, indicating that it is a monomer (data not shown).

Amino Acid Composition/Sequences. The amino acid composition of the 52-kDa protein is shown in Table II, together with those reported for human (Vandermeers et al., 1974) and porcine (DeCaro et al., 1981a) pancreatic triglyceride lipases. The greatest differences between the two human enzymes occur in the relative amounts of glycine, glutamyl, and alanine residues. The porcine enzyme also differs from the 52-kDa protein in the relative amounts of these residues, as well as in isoleucine residues. The N-terminal sequences of the 52-kDa human synthase, human pancreatic triglyceride lipase (DeCaro et al., 1981b), and the 50-kDa porcine triglyceride lipase (DeCaro et al., 1981a) are given in Table III. The striking correspondence between the first 20 amino acids of the 52-kDa synthase and human triglyceride lipase suggests that these independently described enzymes are likely the same protein. In addition, two previously unknown residues (positions 15 and 18) in the N-terminus of the human triglyceride lipase are shown to be, in fact, serine.

Enzymatic Activities. Relative enzymatic activities of the 52-kDa protein are summarized in Table IV. Ethyl oleate is synthesized by the 52-kDa enzyme at a rate of 2400 nmol mg^{-1} h^{-1} and hydrolyzed at 30 nmol mg^{-1} h^{-1}, resulting in a synthesis to hydrolysis ratio of 80:1. Because differences exist between the fatty acid donor pool and the fatty acid ethyl esters formed in vivo (Lange et al., 1981), we evaluated the 52-kDa synthase for fatty acid chain length and saturation specificity by incubating the purified enzyme with different fatty acids ranging in concentration from 0.025 to 1.6 mM. This enzyme exhibits a marked preference for unsaturated, 18-carbon fatty

Table III: N-Terminal Amino Acid[a] Sequences of Human Pancreatic Synthase and Human and Porcine Pancreatic Triglyceride Lipases

	1	10	20
human 52-kDa synthase	NH_2-X E V C Y E R L G C F S D D S P W S G I-		
human triglyceride lipase[b]	NH_2-K E V C Y E R L G C F S D D X P W X G I-		
porcine triglyceride lipase[c]	NH_2-S E V C F P R L G C F S D D A P W A G I-		

[a] Conventional, one-letter symbols used for amino acids; X indicates unknown amino acid. [b] DeCaro et al. (1981b). [c] DeCaro et al. (1981a).

Table IV: Enzymatic Activities of Human Pancreatic Synthase and Porcine Triglyceride Lipase

enzyme	activity: triolein hydrolysis[a]	activity: ethyl ester synthase[b]
human fatty acid ethyl ester synthase	30 ± 3	2400 ± 150
porcine triglyceride lipase	23 ± 1	1530 ± 130

[a] Nanomoles of fatty acid released per milligram per hour in the absence of sodium chloride and colipase, pH 7.2. [b] nmol mg^{-1} h^{-1}, pH 7.2.

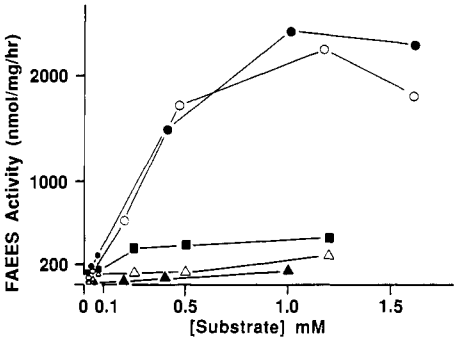

FIGURE 3: Rates of ethyl ester synthesis as a function of the concentration of linoleic acid (●), oleic acid (○), arachidonic acid (■), palmitic acid (△), and stearic acid (▲). Reactions were performed at 37 °C in 60 mM phosphate, pH 7.2, containing 1.5 M ethanol.

acids (Figure 3). Thus, linoleate and oleate are converted into ethyl esters at similar rates of 2400 nmol mg^{-1} h^{-1}, while arachidonate, palmitate, and stearate have rates of 400 nmol mg^{-1} h^{-1}, 300 nmol mg^{-1} h^{-1}, and 100 nmol mg^{-1} h^{-1}, respectively.

Because the amino acid composition of the 52-kDa enzyme is similar to that of pancreatic triglyceride lipase (porcine and human) and because the N-terminal sequences are homologous, the synthase was also investigated for triglyceride lipase activity. As shown in Figure 4, in the absence of sodium chloride and colipase, the 52-kDa enzyme hydrolyzes triolein at a rate of 30 nmol mg^{-1} h^{-1}, indicating that the enzyme has triglyceride lipase activity. This activity is also inhibited by sodium chloride and stimulated by colipase, effects similar to those found with pancreatic triglyceride lipase. Thus, in the absence of colipase, addition of 60 mM sodium chloride decreases the activity 3–4-fold.

Since fatty acid ethyl ester synthase hydrolyzed triolein, porcine pancreatic triglyceride lipase was examined for fatty acid ethyl ester synthase activity. As shown in Table IV, under standard assay conditions, triglyceride lipase can synthesize these esters at a rate comparable to that found for the homogeneous 52-kDa enzyme. The rates of fatty acid ethyl ester synthesis and triolein hydrolysis are slightly lower for the commercially available porcine triglyceride lipase than those found for the 52-kDa protein. The probable explanation for this disparity is that on SDS–PAGE (data not shown) the porcine pancreatic triglyceride lipase is only 30–50% pure.

Discussion

Human pancreas contains high levels of fatty acid ethyl ester synthase activity, an important pathway for the metabolism of ingested ethanol (Laposata & Lange, 1986). Since cholesterol esterase catalyzes the synthesis of cholesterol esters, a similar reaction requiring fatty acid and an alcohol (cholesterol), it was presumed that this enzyme was the most likely source of the activity (Kyger et al., 1990). However, an important clue to the true source of this activity was discovered

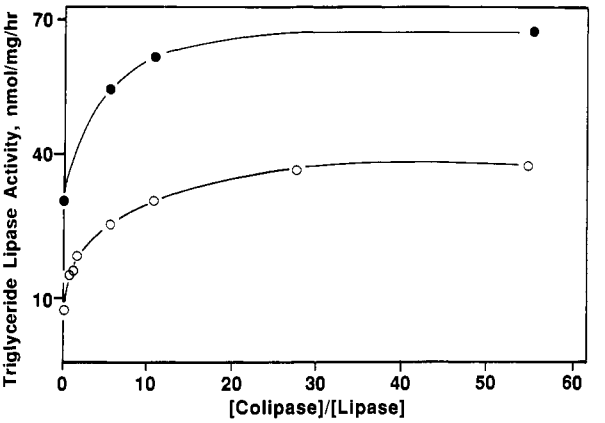

FIGURE 4: Effect of colipase on the hydrolysis of triolein by the 52-kDa enzyme in the absence (●) or presence (○) of 60 mM sodium chloride. Assays were performed at 37 °C in 10 mM phosphate, pH 7.4.

when it was found that DEAE-cellulose separated cholesterol ester hydrolase activity from fatty acid ethyl ester synthase activity and, moreover, that this separation was accompanied by an enhancement on SDS gels of a 52-kDa protein. Therefore, using this fatty acid ethyl ester synthase assay, we now describe the isolation, purification, and characterization of the human pancreatic 52-kDa protein and show that it is an enzyme involved in the metabolism of fatty acids, triglycerides, and ethanol.

The rapid purification scheme summarized in Table I results in the isolation of milligram quantities of homogeneous fatty acid ethyl ester synthase, and the amount of activity in the purified enzyme is 25 times greater than that found in the starting material. Most of this increase is due to the removal of large amounts of contaminating proteins that probably interfere with the fatty acid ethyl ester synthase assay; however, a specific inhibitor may also be present in the crude homogenate and this possibility is currently being investigated. Even though a zymogen has not been observed, activation of such a precursor throughout the purification also cannot be ruled out as an explanation for the enhancement of activity.

An essential step in this purification scheme is heparin chromatography, a procedure that has been successfully used in the purification of the human 100-kDa cholesterol esterase (Kyger et al., 1990). From the high purification achieved by this step, the 52-kDa enzyme must contain a specific heparin-binding site, and thus, like cholesterol esterase, this enzyme may also function while bound through heparin to the enterocyte membrane (Bosner et al., 1988).

Since the homogeneous 52-kDa enzyme has high levels of fatty acid ethyl ester synthase activity, its amino acid composition and N-terminal sequence were determined to compare to those for the intracellular synthase that has been described in myocardium (Mogelson & Lange, 1984). Surprisingly, the 20 N-terminal amino acids were virtually identical with those found in human pancreatic triglyceride lipase. Comparison of their respective amino acid contents demonstrates further similarity, with differences noted primarily in the relative amounts of glycine.

The activity of the 52-kDa enzyme toward triglycerides provided further evidence that the synthase is really triglyceride lipase. The homogeneous synthase not only hydrolyzed triolein, but the activity was strongly influenced by colipase and sodium chloride, hallmarks of the enzymatic activity ascribed to porcine triglyceride lipase (Borgstrom & Erlanson, 1973). In addition, commercially available porcine triglyceride lipase has comparable levels of fatty acid ethyl ester synthase activity, a heretofore unknown property of this lipolytic enzyme.

Taken together, all these structural and activity data indicate that this human 52-kDa fatty acid ethyl ester synthase which is unique to pancreas is most likely human triglyceride lipase.

Recently, porcine pancreatic lipase, bovine lipoprotein lipase, and rat hepatic lipase have been shown to be members of a lipase gene family (Komaromy & Schotz, 1987). Thus, all three enzymes have an extended central region of homology, and moreover, the pattern of disulfide bridges is conserved, indicating that the three lipases have a similar overall conformation (Kirchgessner et al., 1987). In this work, human pancreatic triglyceride lipase is now shown to share some of the same kinetic properties with cholesterol esterase. Thus, human triglyceride lipase (52 kDa) and human cholesterol esterase (100 kDa) both have fatty acid ethyl ester synthase activity (Mogelson & Lange, 1984), they both hydrolyze and synthesize cholesterol esters (Kyger et al., 1990), and finally, they both bind to heparin (Cox et al., 1990; Kyger et al., 1990). Because of these similarities, it will be interesting to determine if cholesterol esterase belongs to a separate but related gene family or if all lipolytic enzymes belong to the same gene family. Cloning and sequence studies, currently in progress in our laboratory (Kyger et al., 1989), will answer this question.

In conclusion, our finding that the high level of pancreatic fatty acid ethyl ester synthase activity resides predominantly in triglyceride lipase may have important implications for the pathogenesis of alcoholic pancreatitis. Fatty acid ethyl esters, products of nonoxidative metabolism of ethanol, have recently been identified in organs often damaged by ethanol ingestion (Laposata & Lange, 1986), and in acutely intoxicated humans, the pancreas was found to contain the highest levels of these esters. It has also been shown that altered lipid metabolism may be one of the initial events leading to alcohol-induced pancreatitis (Saharia et al., 1977). Moreover, from morphological studies of the acinar cell, it has been found that the first response of the pancreas to alcoholic injury is fat accumulation within the cell (Bordalo et al., 1977). Since pancreatic triglyceride lipase is synthesized in high concentration as an active enzyme within the cell, and since it has no absolute requirement for an effector such as bile salt or colipase, there is an existing catalytic mechanism for ethyl ester synthesis whenever the organ is exposed to ethanol. Therefore, the data given here may provide at least a partial explanation on the molecular level for the deleterious effect of alcohol on this organ system, in addition to providing a link between alcohol and lipid metabolism.

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