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Pancreatic Cholesterol Esterases. 1. Pancreatic Cholesterol Esterase Induction during Maturation

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ABSTRACT: The activities of pancreatic cholesterol esterase from calf and cow pancreas were examined in detail. A 1300-fold enhancement of enzymatic activity was found after maturation, even though cholesterol esterase activity levels in other organs did not change from the juvenile to the adult species. Radioimmunoassays also showed that the calf pancreas contained at least 100-fold less cholesterol esterase protein. Decreased amounts of protein were not due to enhanced proteolysis, since cytosol from cow pancreas degrades exogenously added cholesterol esterase faster than that from calf pancreas. Rather, enhancement of pancreatic cholesterol esterase activity associated with bovine maturation was the result of specific, increased synthesis of a 72-kDa enzyme. This labile 72-kDa cholesterol esterase species was purified to homogeneity by a two-step process in 75% yield and is the major form of bovine pancreatic cholesterol esterase (99%). A much less abundant 67-kDa species, accounting for less than 1% of total pancreatic cholesterol esterase activity, was also purified to homogeneity in a similar two-step process. These results demonstrate that a specific form of pancreatic cholesterol esterase is induced during maturation, and they bear importantly on understanding juvenile cholesterol metabolism as related to dietary absorption of this sterol.

Coronary artery disease is caused by progressive occlusion of the coronary arteries, and its incidence and severity can be correlated with the plasma concentration of cholesterol (Kannel et al., 1971; Goldbourt et al., 1985; Goodman, 1988). Since the biochemical mechanism for the induction of atherosclerosis is not known, dietary manipulation has been the only available recourse for early treatment or prevention. There is increasing evidence that the disease begins early in life, and many of the dietary recommendations proposed for adults have been suggested for children as well, on the assumption that the mechanism of cholesterol uptake is the same in adults as in children (Kwiterorich, 1986; McNamara et al., 1974).

One of the critical steps in cholesterol absorption is the pancreatic cholesterol esterase catalyzed cleavage of cholesteryl esters, one of the dietary forms of ingested cholesterol (Vahouny & Treadwell, 1964). This lipolytic enzyme has been extensively investigated from a variety of adult species, including man, pig, rat, and cow (Lombardo et al., 1978; Rudd et al., 1987; Calame et al., 1975; Van Den Bosch et al., 1973), but there have been no studies from the corresponding juvenile animals or children. Thus, biochemical details of cholesterol absorption have remained largely speculative in children, without comparative knowledge of enzyme structure, function, and regulation.

In this work, we examine the pancretic cholesterol esterase activities in cow and calf and find a nearly 1300-fold enhancement of enzymatic activity during maturation. Moreover, this effect seems to be a characteristic of pancreas, since the cholesterol esterase levels in other organs do not change from the juvenile to the adult species. Using purification methods developed in this paper, we show that induction is selective for the 72-kDa form of the enzyme. Study of the regulation of this enhancement by growth factors, hormones, or other moelcules is feasible now that the enzyme has been cloned (Kyger et al., 1989), and it bears further scrutiny since such induction will influence the pattern of cholesterol ab-

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sorption in adolescents and adults.

Materials and Methods

[^{14}C]Cholesteryl oleate and [^{14}C]oleic acid were purchased from Amersham. Heparin–agarose and bovine cholesterol esterase were purchased from Sigma.

Preparation of Pancreatic Cytosol. Pancreas from either cow or calf was obtained at the abattoir and immediately plunged into liquid nitrogen.

Method A. The frozen tissue was broken into small pieces, and the cytosol was prepared with a polytron by mincing 30 g of frozen tissue with 50 mL of 50 mM acetate buffer, pH 5.1, containing 0.5% digitonin. The mixture was centrifuged at 100000*g* for 1 h, and the supernatant was collected and dialyzed against 50 mM acetate buffer, pH 5.1.

Method B. The frozen tissue was lyophilized. Calf cytosol was prepared with a polytron by mincing 50 g of lyophilized tissue in 400 mL of 25 mM acetate buffer, pH 5.1. The mixture was centrifuged at 100000*g* for 1 h, and the supernatant was collected. Cow cytosol was prepared similarly, but 5 g of lyophilized tissue and 125 mL of buffer were used.

Enzyme Assays. Cholesterol esterase activity was determined by measuring the release of [^{14}C]oleic acid from vesicles containing cholesteryl [1-^{14}C]oleate. Vesicles were prepared by drying under nitrogen a solution of 1.00 mL of 1.33 mM egg phosphatidylcholine in hexane and 1.27 mL of 1 mM cholesteryl oleate containing 10 μL of cholesteryl [1-^{14}C]oleate (2.2×10^6 cpm) in chloroform. The precipitate was resuspended in 10 mL of 150 mM Tris[1] buffer, pH 7.5, vortexed vigorously for several seconds, and then sonicated on ice for 20 min under nitrogen. Following sonication, the solution was centrifuged at 48000*g* for 60 min, and the vesicle preparation was carefully decanted and stored at 4 °C.

In a typical assay, 75 μL of cholesteryl [^{14}C]oleate vesicles, 25 μL of 100 mM taurocholate, and 175 μL of 150 mM Tris buffer, pH 7.5, were mixed in a test tube, and hydrolysis was initiated by adding 25 μL of enzyme to the reaction mixture at 37 °C. After a known time, usually 5 min, the reaction was quenched by addition of 600 μL of 0.3 N NaOH and 3 mL of benzene/methanol/chloroform (1:1.2:0.5) (Brecher et al., 1977). After mixing, the samples were centrifuged and 1 mL of the clear aqueous phase was removed and counted for radioactivity. Since only part of the sample was removed for counting, an efficiency sample was prepared by adding 100 μL of [^{14}C]oleic acid vesicles of known specific radioactivity to 200 μL of 150 mM Tris buffer, pH 7.5. The same manipulations were performed on this sample as those described above for assay. The efficiency of transfer was then determined by dividing the number of counts in the 1-mL aqueous phase by the dpm in 100 μL of starting [^{14}C]oleic acid vesicles. Activity is expressed as nanomoles of oleic acid released per milliliter per hour and was less than 0.1 nmol mL^{-1} h^{-1} in the absence of added enzyme.

Preparation and Purification of Rabbit Anti-Cholesterol Esterase Antibodies. Commercially available bovine pancreatic cholesterol esterase (purity <1%) in 10 mM Tris buffer, pH 7.2, was applied to heparin–agarose (1.5 cm × 10 cm) equilibrated with the same buffer. The resin was developed further by washing with 100 mM NaCl and 10 mM Tris buffer, pH 7.2, and as shown in Figure 1, little or no activity was found in any of these preliminary steps, even though virtually all of the applied protein was eluted. When the absorbance at 280 nm returned to zero, the resin was washed with a solution of 20 mM sodium taurocholate containing enough sodium chloride to give the same conductivity as that of the previous washing buffer. All the activity was eluted in several fractions. This single purification step typically provides a 60–80% yield with a 50–100-fold purification and gives a single band at 67 kDa on SDS–PAGE (Figure 2). No additional activity was found when the resin was washed with higher concentrations of salt, and the resin could be regenerated by washing with 2.0 M NaCl and 10 mM Tris buffer, pH 7.2.

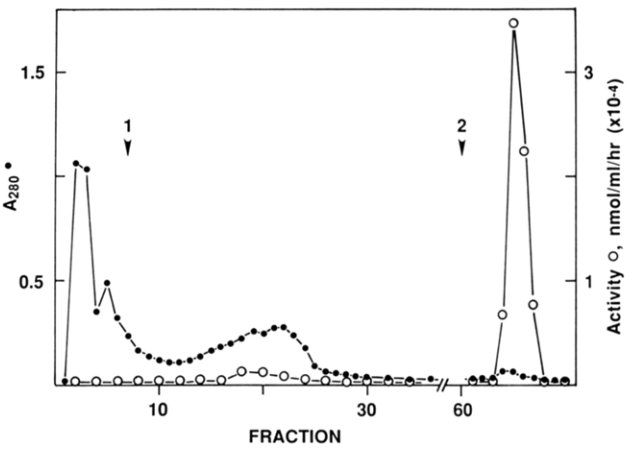

FIGURE 1: Heparin–agarose chromatography of commercial bovine cholesterol esterase. Enzyme was applied to the resin in 10 mM Tris, pH 7.2, and then washed with the following buffers: (1) 100 mM NaCl, 10 mM Tris, pH 7.2; (2) 80 mM NaCl, 20 mM taurocholate, 10 mM Tris, pH 7.2.

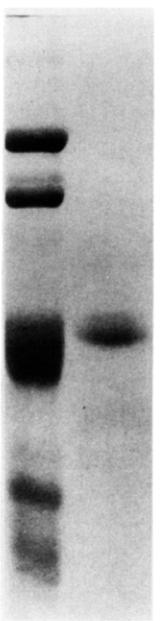

FIGURE 2: (Left) SDS–PAGE (7.5%) molecular mass standards: 116, 97, 67, 45, and 22 kDa. (Right) Purified bovine cholestrol esterase.

Five hundred micrograms of homogeneous 67-kDa protein was emulsified in Freund's complete adjuvant and injected subcutaneously into a New Zealand White rabbit. Twenty-one days later the rabbit was boosted with intraperitoneal injections of 250 μg of protein dissolved in 1 mL of 10 mM sodium phosphate and 150 mM NaCl buffer, pH 7.1. The rabbit was bled 10 days later and the presence of anti-cholesterol IgG was determined on Ouchterlony plates. Rabbit IgG was purified by passing 20 mL of rabbit serum over a protein A–Sepharose column equilibrated with 20 mM Tris and 20 mM NaCl

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography.

buffer, pH 8.0. The resin was washed with equilibration buffer, followed by 20 mM Tris, 0.5% deoxycholate, and 500 mM NaCl buffer, pH 8.0, and then equilibration buffer. Finally, the IgG was eluted with 100 mM glycine buffer, pH 2.8.

Cholesterol Esterase Anti-Cholesterol Esterase Assay. Two hundred microliters of pancreatic cytosol or purified cholesterol esterase was added to a microtiter well and incubated with 7-mm nitrocellulose disks overnight at 4 °C. The solution was removed by aspiration and the disks were incubated at 4 °C for 3 h with bovine serum albumin (5 mg/mL) in PBS. Each disk was then washed with 250 μL of PBS and transferred to a new microtiter well, and 200 μL of 5% rabbit anti-cholesterol esterase serum in PBS was added. After incubation for 1 h at 37 °C, the serum was removed, the disks were washed four times with PBS, and 200 μL of ^{125}I protein A (30 820 cpm/pmol) in PBS was added. After 1 h at 37 °C, the ^{125}I protein A solution was removed and the disks were washed four times with PBS. The disks were then sealed to the bottom of the wells with hot agar, the well was cut away from the plate, and ^{125}I dpm were determined on an LKB 1282-Compugamma.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed by the method of Laemmli (1970), using either 10% or 7.5% acrylamide, pH 8.8, as the separating gel and 4.5% acrylamide, pH 6.8, as the stacking gel. Protein was visualized with 0.2% Coomassie Brilliant Blue and the molecular weight determined from a plot of R_f versus log M_r.

Western Blotting. Immediately following electrophoresis, the polyacrylamide gel was soaked for 30 min in 20 mM Tris, 150 mM glycine, and 20% methanol buffer, pH 8.3. Nitrocellulose, equilibrated with the same buffer, and the gel were sandwiched between heavy filter paper and Scotch-Brite pads, and electroelution was performed for 16 h at 30 V and 0.1 A. The nitrocellulose sheet was washed four times with 285 mL of PBS containing 0.3% (v/v) Tween 20 and then incubated for 3 h with rabbit anti-cholesterol esterase antibody and 1% ovalbumin. After extensive washing with PBS, the sheet was incubated with ^{125}I protein A (100 μL, 10^5 cpm/pmol) containing 1% ovalbumin. The nitrocellulose sheet was then washed again with PBS, dried, and exposed to X-ray film (6–72 h).

Results

Comparison of Cholesterol Esterase Activity in Calf and Cow Pancreas. Calf and cow cytosols were prepared from freshly obtained pancreas in 10 mM phosphate and 50 mM benzamidine buffer, pH 6.0, a solvent condition that minimizes proteolytic degradation of cholesterol esterase. When an aliquot of each pancreatic cytosol was assayed for hydrolytic activity toward cholesteryl oleate, there was more than 1300-fold greater activity per gram of tissue in the mature cow preparation than in that from calf, 9130 nmol mg^{-1} h^{-1} and 6.7 nmol mg^{-1} h^{-1}, respectively (n = 3) (Table I). Although the calf is well past infancy, it is possible that this reduced cholesterol esterase activity reflects a general down-modulation of cholesterol metabolism. To examine this possibility, the level of cholesterol esterase activity in other organs from calf was quantitated. Compared to those in the mature cow, activities in aorta, kidney, liver, lung, adrenal, and brain were essentially the same in calf; only pancreas had diminished activity (Table I).

Quantitation of Cholesterol Esterase by Radioimmunoassay. To investigate the molecular basis for the diminished pancreatic activity in calf, cytosol was prepared from calf and cow pancreas and chromatographed over immobilized heparin, equilibrated with 10 mM phosphate buffer, pH 6.0. After the resin was washed with 50 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2, to remove protein impurities, a concentrated protein peak was removed with 500 mM NaCl, 50 mM benzamidine, and 10 mM Tris buffer, pH 7.2. This material was dialyzed against 10 mM phosphate buffer, pH 6.0, and then analyzed by SDS–PAGE and immunoblotting. As shown in Figure 3, in cow pancreas, several cross-reacting species were found, with prominent bands at 72, 67, and 52 kDa. On the other hand, for calf pancreas, no cross-reacting proteins were present.

Table I: Cholesterol Esterase Activity Found in Cow and Calf Organs

organ	activity[a] (nmol mg^{-1} h^{-1}) cow	calf
pancreas	9130 ± 500	6.7 ± 0.7
adrenal	10.2 ● 1.5	7.4 ± 0.6
kidney	8.4 ● 0.7	7.4 ± 0.7
liver	7.9 ● 0.7	6.7 ± 0.8
lung	4.4 ● 0.7	6.1 ± 0.8
brain	0.1 ● 0.03	0.1 ± 0.03
aorta	0.1 ± 0.03	0.1 ± 0.03

[a] Measured as hydrolysis of cholesteryl oleate.

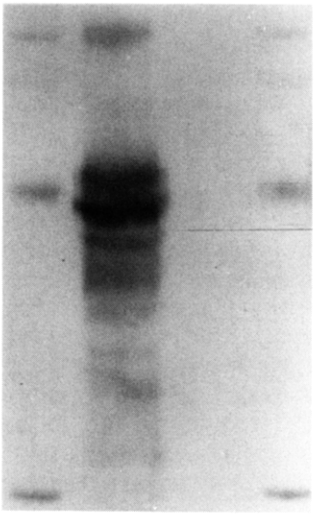

FIGURE 3: Western blot of bovine pancreas with antibody to 67-kDa bovine cholesterol esterase. Lanes 1 and 4, molecular mass standards, 116, 97, 67, and 29 kDa; lane 2, 20 μg of cow pancreatic cytosol; lane 3, 20 μg of calf pancreatic cytosol.

To quantitate this phenomenon, a radioimmunoassay was developed to measure the total amount of enzyme that cross-reacts with anti-bovine 67-kDa cholesterol esterase antibody, which recognizes all these proteins. This radioimmunoassay was linear over a 1000-fold protein concentration range, from 0.18 to 180 μg of enzyme/mL. Cow pancreatic cytosol, either undiluted or diluted 10-fold, contained a significant amount of cross-reactive material; 15 μg/mL enzyme was present in cytosol. On the other hand, cross-reactive material was undetectable even in undiluted calf cytosol, indicating that the juvenile pancreas contains less than 0.18 μg/mL of any form of cholesterol esterase. Both these experiments indicate that the calf pancreas contains much less immunoreactive protein than the cow pancreas.

Comparative Catabolic Rates for Calf and Cow Esterase. The low level of activity and the small amount of immunoreactive protein in calf pancreas could be due to enhanced proteolytic degradation of cholesterol esterase(s) in calf pancreatic cytosol compared to cow cytosol. Degradation rates were therefore quantitated by adding 8.1 μg of homogeneous

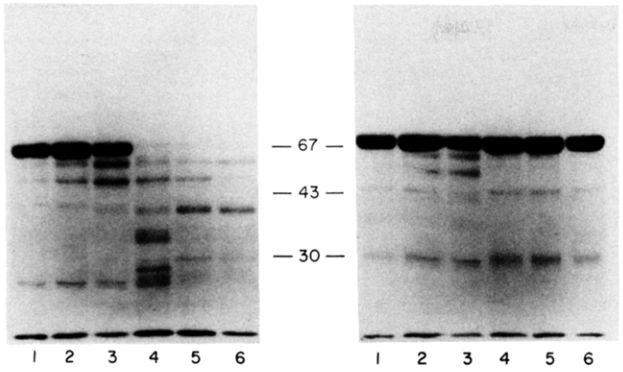

FIGURE 4: Degradation of ^{125}I cholesterol esterase (67 kDa) by cow cytosol (left) and calf cytosol (right). Labeled enzyme (8.1 μg) was incubated with cytosol at pH 7.4, and aliquots were removed at 0 (lane 1), 30 (lane 2), 60 (lane 3), 90 (lane 4), 120 (lane 5), and 180 min (lane 6) for SDS–PAGE (7.5%) and subsequent autoradiography.

^{125}I bovine cholesterol esterase (67 kDa) to either calf or cow pancreatic cytosol in 10 mM sodium phosphate buffer, pH 7.4. Aliquots were removed at various times and SDS–PAGE was performed, followed by autoradiography. Almost complete proteolytic degradation of the 67-kDa species occurred in cow cytosol after 2 h (Figure 4, left), but very little degradation was evident in calf cytosol, even after 3 h. Thus, these proteolytic results coupled with the radioimmunoassay studies indicate that the large enhancement of pancreatic cholesterol esterase activity associated with bovine maturation is due to increased synthesis of either the 67- or 72-kDa enzyme form or both.

Purification Strategy. Since cholesterol esterase consists of at least two polypeptide forms, a purification scheme was needed to identify and quantitate the amount of each of these forms in cow and calf pancreas. Because cow pancreas contains large amounts of cholesterol esterase (Figure 3), its purification is straightforward, but the purification of calf cholesterol esterase is far more difficult. In this case, the presence of pancreatic proteases and the small number of hydrolytic units initially present dictated the need to develop a method that would preserve activity under adverse conditions.

The degree of proteolytic degradation of the various enzymatic forms was determined by monitoring both the total number of cholesterol esterase activity units and the ratio of the amount of 72- to 67-kDa enzyme in cow cytosol. These studies indicated that the 72-kDa form was far more labile toward proteolysis than was the 67-kDa enzyme. For example, overnight dialysis of cytosol at pH 5.1 reduced the cholesterol esterase units by over 70%, and Western blots indicated that this loss was due almost entirely to proteolytic degradation of the more abundant and labile 72-kDa species (data not shown). This degradation did not result in the appearance of more 67-kDa species; thus the 67-kDa enzyme apparently is not derived from the 72-kDa form by proteolytic cleavage.

Therefore, two methods for cytosol preparation were devised. In method A (see Materials and Methods), cytosol was prepared from frozen tissue in the presence of digitonin and was dialyzed at pH 5.1. This method is similar to conditions previously reported (Calame et al., 1975; Rudd et al., 1987), and it is now known that this does not preserve the more labile 72-kDa enzyme. Hence, method A facilitates the isolation and purification of the 67-kDa form. On the other hand, for the purification of the labile 72-kDa form or for the isolation of calf cholesterol esterase, instances where proteolytic degradation had to be eliminated or minimized, the cytosol was prepared from lyophilized tissue and was not dialyzed (method B under Materials and Methods).

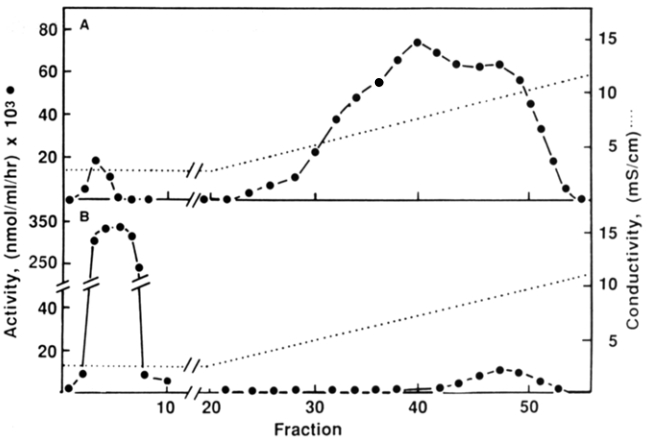

FIGURE 5: SP-Sephadex chromatography of cow cytosol. (A) Cytosol was prepared in 50 mM acetate buffer, pH 5.1, by method A (see Materials and Methods), which facilitates the isolation of the 67-kDa form. (B) Cytosol was prepared in 25 mM acetate buffer, pH 5.1, by method B (see Materials and Methods), which facilitates the isolation of the more labile 72-kDa form.

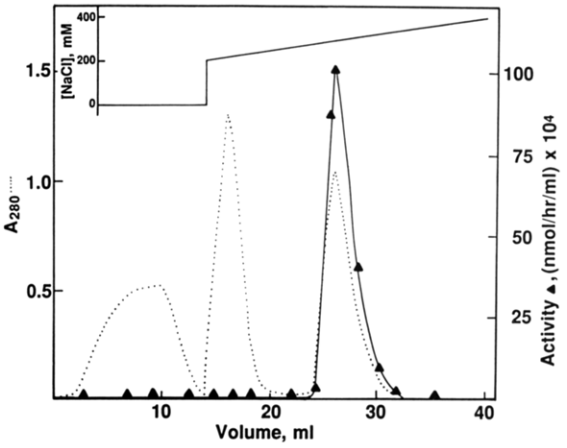

FIGURE 6: Mono S chromatography of bovine cholesterol esterase in 10 mM acetate buffer, pH 5.1.

Separation of Two Different Homogeneous Forms of Bovine Cholesterol Esterase. When 25 mL of cow pancreatic cytosol prepared by method A was applied to SP-Sephadex (1.5 cm × 20.0 cm) in 50 mM acetate buffer, pH 5.1, 97% of the esterolytic activity was bound. Elution with a linear sodium chloride gradient (0–150 mM) produced two overlapping peaks of activity (Figure 5A), and subsequent SDS–PAGE (7.5%) and Western blotting revealed two cholesterol esterase species, 72 kDa (centered at fraction 40) and 67 kDa (centered at fraction 49). With the Western blot as a guide, two pools were collected; pool I (72 kDa) contained 21% (6.6-fold purification) of the initial activity, whereas pool II (67 kDa) contained 12% (5.5-fold purification) of the initial cholesterol esterase activity. Those fractions that contained both 67- and 72-kDa cholesterol esterase were discarded.

Final purification was achieved with FPLC, with a Mono S cation-exchange chromatography column. Application of pool I in 10 mM acetate buffer, pH 5.1, followed by elution with a linear sodium chloride gradient (200–400 mM), produced one peak of activity, which eluted at 295 mM NaCl (Figure 6). A similar elution profile was found when pool II was purified in this way, but the activity eluted at 320 mM NaCl.

As shown in Figure 7, SDS–PAGE and Western blotting of pool I and pool II revealed a single protein band in each case, of molecular mass 72 and 67 kDa, respectively. The 72-kDa product was purified 50-fold in 37% yield, whereas

Table II: Purification of Bovine Cholesterol Esterases from Unproteolyzed Pancreatic Tissue

enzyme form	step	protein (mg)	total act.[a] ($\times 10^6$)	sp act. (units/mg $\times 10^3$)	recovery (%)	purification (x-fold)
72 kDa	cytosol	1146	33.9	29.6	100	1
72 kDa	SP wash	759	31.1	41.0	92	1.4
72 kDa	Mono S	28	25.3	903.6	75	30.0
67 kDa	cytosol	1146	33.9	29.6	100	1
67 kDa	SP bound	3.2	1.0	314.0	3[b]	10
67 kDa	Mono S	0.14	0.08	571.4	0.3	20

[a] Measured as hydrolysis of cholesteryl oleate. [b] The recovery is low because most of the activity is removed in the SP wash as the 72-kDa form. Based on an estimate of the initial amount of 67-kDa enzyme present, the overall yield was approximately 50%.

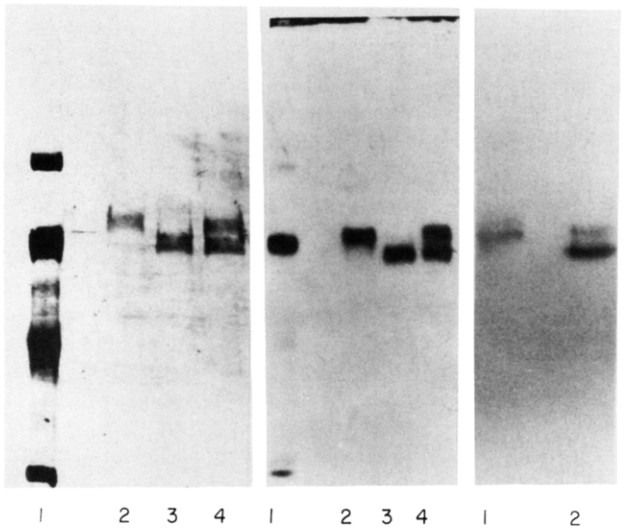

FIGURE 7: (Left) SDS–PAGE of purified 72- and 67-kDa cow pancreatic esterase. Lane 1, molecular mass standards, 94, 67, 43, and 20 kDa; lane 2, 72-kDa cow cholesterol esterase (10 μg); lane 3, 67-kDa cow cholesterol esterase (15 μg); lane 4, mixture of 72- and 67-kDa enzymes. (Middle) Corresponding Western blot of SDS–polyacrylamide gel with antibody to 67-kDa bovine enzyme. (Right) Lane 1, Western blot of calf cholesterol esterase; lane 2, Western blot of a mixture of 72- and 67-kDa cow cholesterol esterase.

the 67-kDa product was purified 46-fold in 21% yield.

Purification and Quantitation of the Relative Amounts of 67- and 72-kDa Cholesterol Esterase in Cow Pancreatic Cytosol. One hundred milliliters of cow cytosol (method B) in 25 mM acetate buffer, pH 5.1, was applied to SP-Sephadex (2.5 cm $\times$ 25 cm) equilibrated with the same buffer. Over 92% of the activity washed through, while in the purification by method A, 97% of the activity bound to the resin. The active fractions from the washthrough were pooled and lyophilized (1.33 g, pool III), and the SP-Sephadex column was developed further with a linear sodium chloride gradient (0–150 mM) (Figure 5B). This time, only one peak was found (pool IV) and it contained only 3% of the total applied activity.

Each of these pools was applied to Mono S and the column was developed as described above. It was found that only 25–50 mg of lyophilized pool III could be loaded onto the Mono S column (5 mm $\times$ 50 mm) before overloading occurred with significant loss of recovered protein. When 24 mg of pool III was applied, a single peak of activity was eluted in 90% yield (0.51 mg) at 270 mM sodium chloride. The enzyme was purified 38-fold and gave a single band on SDS–PAGE at 72 kDa. Application of all of pool IV gave a broad peak of activity, centered at 280 mM sodium chloride. SDS–PAGE of the active fractions revealed a homogeneous 72-kDa protein contaminated by only a small amount (<15%) of the 67-kDa enzyme.

In summary (Table II), when care is taken to eliminate proteolysis during the initial tissue solubilization, greater than 99% of the cholesterol esterase activity is found in a 72-kDa protein, and this can be rapidly prepared in homogeneous form. By calculation, in unproteolyzed bovine pancreas there is approximately 5 mg of 72-kDa cholesterol esterase and only 0.03 mg of 67-kDa cholesterol esterase per gram of lyophilized tissue.

Purification of Undegraded Calf Cholesterol Esterase. Similarly, when calf cytosol (519 mL) in 25 mM acetate buffer, pH 5.1 (method B), was applied to SP-Sephadex, 49% of the activity washed through, indicating that the calf enzyme may be similar to the 72-kDa cow cholesterol esterase. The activity was pooled and lyophilized (5.30 g, pool V), and a linear sodium chloride gradient (0–150 mM) was applied to the SP-Sephadex resin, but no activity was eluted from the column.

Chromatography of pool V utilizing FPLC Mono S, as described above, yielded a single peak of activity that eluted at 315 mM NaCl and corresponded to 71% yield. All of the isolated protein was subjected to a Western blot, which revealed only a 72-kDa species (Figure 7, right). SDS–PAGE was not performed due to the small quantity of protein obtained.

Discussion

Absorption of dietary cholesterol in the small intestine requires cholesterol esterase, an enzyme synthesized and secreted by the pancreas (Vahouny & Treadwell, 1964; Dietschy & Wilson, 1970). Recently it has been shown that an increased level of serum cholesterol is a major risk factor for myocardial infarction, giving new importance to the mechanism of cholesterol absorption (Grundy, 1986; Kannel et al., 1971; Goodman, 1988). While it is now recognized that high cholesterol levels lead to an increase in atherosclerotic plaques, it is not known if this process begins at birth and it is not clear how it is regulated by developmental or hormonal factors. To investigate this process, we have studied the level and nature of cholesterol esterase in cow and calf, using isolation and purification techniques that have been developed in our laboratory.

Calf pancreatic cytosol contains about 1300-fold less hydrolytic activity than cow pancreatic cytosol, yet in a comparison of other organs, no significant differences in the level of this activity were found. Since cholesterol esterase activity is influenced by a number of effectors such as bile salt (Treadwell & Vahouny, 1968; Vahouny et al., 1965), this discrepancy between the two organs could be due to assay conditions. Therefore, using antibody to the bovine 67-kDa pancreatic cholesterol esterase, we developed a radioimmunoassay to measure the amount of enzyme protein present in calf and cow cytosols. This method clearly indicates that calf pancreas contains at least 100-fold less immunoreactive protein. Importantly, this decrease is not due to enhanced proteolysis, since the cow pancreas actually contains higher levels of proteolytic activity toward the cholesterol esterase poly-

peptide. Therefore, these data indicate that calf pancreas contains much less cholesterol esterase than cow pancreas.

Recently we have shown that cholesterol esterase binds to the brush border membrane through a reversible interaction with heparin (Bosner et al., 1988). As shown here (Figure 1), this interaction can be used to isolate the enzyme in high yield from a standard commercial preparation. Immunoblots of cow pancreas using antibody to this heparin-purified 67-kDa bovine cholestrol esterase showed that the enzyme is actually a group of cross-reacting forms of molecular mass 67, 72, and possibly 52 kDa [Figure 3; see also Riley et al. (1990)]. Because there are multiple forms for this enzyme, a purification scheme was developed in order to unravel which molecular weight species may be affected by the maturation process. As shown in Figures 5 and 6 and Table II for cow pancreas, the 67-kDa enzyme can be purified and separated from the 72-kDa form by using a simple, two-step purification scheme. This larger molecular weight species is far more abundant in both adult and calf pancreas as well as in pancreatic juice (unpublished observations).

Using the same purification scheme devised for bovine pancreas, we isolated cholesterol esterase from calf tissue. Even though there was insufficient enzyme present to allow complete physical and chemical characterization, an enriched sample of calf enzyme was analyzed by immunoblotting; the data indicate that calf pancreas synthesizes very low levels of a 72-kDa cholesterol esterase. Taken together, all these results indicate that calf pancreas synthesizes 72-kDa cholesterol esterase, the same enzyme as that found in cow pancreas but much less of it.

The existence of the 67- and 72-kDa enzyme forms and their different lability to proteolysis bear importantly on previous attempts to isolate and characterize cholesterol esterase. These methods have usually provided a 67-kDa protein (Calame et al., 1975; Van Den Bosch et al., 1973). Most purification schemes (Calame et al., 1975; Rudd et al., 1987; Albro et al., 1976) use digitonin and low concentrations of benzamidine during the disruption of pancreatic tissue, conditions that favor release of the 67-kDa form (unpublished observations) and proteolytic degradation of the 72-kDa enzyme. Under these conditions, as the purification proceeds, not only does the yield diminish, but the final homogeneous enzyme may simply be a minor pancreatic component that is less susceptible to proteolytic degradation and that contributes in our experience less than 10% of the total cholesterol esterase activity.

Our results make it extremely unlikely that the 67-kDa enzyme could be derived from the 72-kDa form. Thus, when purified 72-kDa enzyme is incubated with cytosol, there is no evidence that 67-kDa cholesterol esterase is produced in the mixture. A clue to the function and significance of this smaller cholesterol esterase form may be found from its interaction with heparin. Even though the 67-kDa form binds reversibly to heparin (Figure 1), it is the only enzyme form that can be removed from the affinity column with sodium taurocholate, a bile salt essential for the hydrolysis and uptake of cholesterol (Kyger et al., 1990). Since taurocholate is present in the small intestine during digestion, and since binding of cholesterol esterase to the enterocyte is essential for cholesterol uptake (Bosner et al., 1988), this indicates that the 67-kDa enzyme may play a more limited role in the intestinal uptake of cholesterol at the brush border. It may even be an intrinsic pancreatic enzyme employed in the pancreas itself for some other aspect of lipid metabolism.

The results found here for enhancement of cholesterol esterase activity and enzyme concentration are similar to the inductions reported for the expression of rat pancreatic genes during development (Bradshaw & Rutter, 1972; Han et al., 1986). Thus, the mRNA levels of 10 other pancreatic enzymes were found to increase from 2- to 80-fold in going from the newborn state to the adult animal. Although the mRNA level of cholesterol esterase was not studied, the 1300-fold enhancement of its activity during maturation is certainly consistent with these reported increases found for rat pancreas. The cause of this enhancement remains obscure. While diet has been shown to affect the synthesis of certain exocrine proteins (Reboud et al., 1964, 1966), specific hormones, especially sex hormones, may also cause these dramatic changes.

In conclusion, the results reported here bear importantly on efforts to curb the process of atherogenesis through juvenile diet manipulation. The serum cholesterol level and endogenous rates of cholesterol biosynthesis are determined in part by the intake of cholesteryl ester and thus by the binding of cholesterol esterase to the small intestinal cell through interaction with heparin (Bosner et al., 1988). Since there may be diminished levels of secreted cholesterol esterase in young children, excessive absorption of dietary cholesterol may be prevented and/or modulated by their developmental state in ways imcompletely understood at present.

Registry No. Cholesterol esterase, 9026-00-0.

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

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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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