Purification and Partial Characterization of a Retinyl Ester Hydrolase from the Brush Border of Rat Small Intestine Mucosa: Probable Identity with Brush Border Phospholipase B†

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ABSTRACT: Retinol esterified with long-chain fatty acids is a common dietary source of vitamin A, that is hydrolyzed prior to absorption. An intrinsic brush border membrane retinyl ester hydrolase activity had previously been demonstrated for rat small intestine [Rigtrup, K. M., & Ong, D. E. (1992) *Biochemistry 31*, 2920–2926]. This activity has now been purified to apparent homogeneity by a three-column procedure to obtain a protein of apparent molecular weight of 130 000. The purified protein retained the pattern of bile salt stimulation, specificity for the acyl moiety of the retinyl ester, and the K_m values previously observed for the activity present in the isolated brush border membrane. This protein also had a potent phospholipase activity, while having little measurable ability to hydrolyze triacylglyceride and cholesteryl ester substrates. The retinyl ester hydrolase enzyme was localized to the distal two-thirds of the small intestine. A polyclonal antiserum against rat brush border phospholipase B reacted with the purified retinyl ester hydrolase, strongly suggesting that this enzyme was the same as that previously purified and characterized as a calcium-independent brush border phospholipase B [Pind, S., & Kuksis, A. (1991) *Biochem. Cell Biol. 69*, 346–357]. Detailed kinetic studies revealed lower K_m values for retinyl palmitate substrate compared to phosphatidylcholine substrate, with all tested bile salts. The K_m values for each substrate were bile salt dependent and differently altered when bile salts were changed. V_{max} values were also bile salt dependent. Retinyl palmitate was hydrolyzed most rapidly in the presence of deoxycholate and least rapidly in taurocholate. Interestingly, the opposite was true for phospholipid substrate, with taurocholate resulting in the highest V_{max} and deoxycholate the lowest. These studies indicated a specific bile salt–enzyme interaction that affected the catalytic activity of the enzyme and altered the kinetic parameters differently for the two preferred substrates retinyl ester and phosphatidylcholine.

Vitamin A exists in the diet as either provitamin carotenes, from plants, or retinol (vitamin A alcohol) esterified to long-chain fatty acids, from animal tissue. The retinyl esters must first be hydrolyzed to retinol and free fatty acids in the lumen of the small intestine prior to absorption of the free retinol across the intestinal brush border membrane (Mahadevan *et al.*, 1963a,b). Absorption then occurs by a passive, carrier-mediated process (Hollander & Muralidhara, 1977; Said *et al.*, 1988). This hydrolysis can be catalyzed by lipolytic enzymes secreted by the pancreas or by lipases intrinsic to the brush border membrane. In a previous study (Rigtrup & Ong, 1992), we examined the ability of purified rat intestinal brush border to hydrolyze retinyl esters. We discovered two distinguishable activities. One is of pancreatic origin (possibly cholesterol ester hydrolase) and primarily hydrolyzed esters with fatty acyl chains of less than 10 carbons in length. The other is intrinsic to the brush border membrane and constituted the majority of brush border activity toward long-chain retinyl esters that are typical of those that would be found in the diet.

An intrinsic brush border lipase, a stalked hydrolase characterized as a phospholipase B, has been purified from rat (Pind & Kuksis, 1989) and guinea pig (Gassame-Diagne *et al.*, 1989), after release from the brush border membrane by papain proteolysis. The guinea pig enzyme was shown to hydrolyze tri-, di-, and monoacylglycerides also, while having little activity for cholesteryl esters (Gassame-Diagne *et al.*, 1992). The enzyme from rat has not been tested with those substrates, and neither that from rat or guinea pig was examined for the ability to hydrolyze retinyl esters. Consequently, it was not known if this phospholipase B activity might be responsible for the retinyl ester hydrolytic activity we had observed. However, the relative stimulation by the various bile salts of the rat brush border phospholipase (Pind & Kuksis, 1988) and retinyl ester hydrolase (Rigtrup & Ong, 1992) contrasted sharply. The retinyl ester hydrolase activity was stimulated more strongly by unconjugated bile salts, while the phospholipase activity was stimulated more strongly by their taurine-conjugated analogs.

To examine this question, we have purified the intrinsic rat brush border retinyl ester hydrolase in its native form after detergent solubilization from brush border membranes. The isolated enzyme had a potent phospholipase activity and reacted with antibodies to phospholipase B, strongly suggesting that the retinyl ester hydrolase and the phospholipase B activities are present in the same protein. Detailed kinetic studies revealed differential abilities to hydrolyze these two substrates that varied with the bile salt present. The kinetic parameters determined in the presence of the physiological bile salt taurocholate suggest that this enzyme could contribute significantly to the lumenal hydrolysis of both substrates.

EXPERIMENTAL PROCEDURES

Materials. Phosphate-buffered saline was made using FTA hemagglutination buffer purchased from Becton Dickinson. Retinyl palmitate was purchased from Sigma. All other retinyl esters were synthesized from the acyl chloride as previously described (Huang & Goodman, 1965). The purity and identity

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of all synthesized retinyl esters were confirmed by UV spectra (in ethanol) and reverse-phase (C18) HPLC, monitored at 325 nm. Glycerol tri[1-^{14}C]palmitate (56 mCi/mmol), 1,2-di[1-^{14}C]palmitoylphosphatidylcholine (112 mCi/mmol), and cholesteryl [1-^{14}C]oleate (54 mCi/mmol) were purchased from Amersham. Glycerol tri[1-^{14}C]oleate (110 mCi/mmol) was purchased from NEN. Adult Sprague-Dawley rats (>250 g) were purchased from either Harlan or Sasco. ConA–Sepharose was from Pharmacia. Imidazole was purchased from Sigma and recrystallized from ethyl acetate. The hydroxyapatite used was Biorad Biogel HTP. The DEAE HPLC column [GlasPac TSK DEAE 5PW (8 × 75 mm)] was purchased from Pharmacia LKB. Antiserum to rat brush border phospholipase B was a generous gift of Arnis Kuksis, Ph.D.

Preparation of Rat Brush Border Membranes. Intestinal brush border membranes were prepared from the small intestine of adult (>250 g) rats as previously described (Rigtrup & Ong, 1993). Protein determinations were made using Pierce BCA reagents, with bovine serum albumin as a standard. Papain solubilization of retinyl ester hydrolase (REH)[1] from the membranes followed the procedure previously published for rat brush border phospholipase B (Pind & Kuksis, 1989).

Purification of Detergent-Solubilized Rat Brush Border Retinyl Ester Hydrolase. Two different protocols, each using three chromatographic steps, were used to purify the retinyl ester hydrolase to homogeneity from detergent-solubilized brush border membranes. Brush border membranes were solubilized at an approximate protein concentration of 4 mg/mL. Either 10% octyl β-glucoside or 2.5% Brij 35 was added dropwise while stirring, to a final detergent concentration of either 2% octyl β-glucoside or 0.25% Brij 35. The mixture was gently stirred for 60 min at 4 °C, followed by centrifugation at 100000*g* for 60 min. The first method began with the material (from brush border membranes containing 100 mg of protein) solubilized by Brij 35. After exchange into 0.15 M Tris·HCl (pH 8) buffer containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.35 M NaCl, and 0.25% Brij 35, 8 mL of ConA–Sepharose gel that had been equilibrated in the same buffer was added and gently agitated at 4 °C for 2–4 h. Following sedimentation of the gel, the supernatant liquid was removed. The gel was washed 4 times with 35 mL of 0.005 M imidazole acetate (pH 6). Activity was eluted with 8 mL of a 0.005 M imidazole acetate (pH 6) buffer containing 1.2 M methyl α-D-mannopyranoside and 2% octyl β-glucoside, incubated for 16 h at 4 °C. After collection of the supernatant liquid plus wash, a second elution was then performed, resuspending the gel in 1 volume of 0.1 M Tris·HCl (pH 8) buffer containing 0.9 M NaCl, 10 mM EDTA, and 0.6 M methyl α-D-mannopyranoside with 1% octyl β-glucoside for 16 h at 4 °C.

Following dialysis of the solution from the second elution step against 0.005 M imidazole·acetate (pH 6) with 1% octyl β-glucoside, the two recovered fractions were subjected to ion-exchange chromatography on a 4-mL DEAE HPLC column. After an initial wash with 8 mL of the above buffer with octyl β-glucoside, a gradient from 0.005 to 0.06 M imidazole acetate buffer (64 mL total, flow rate of 0.8 mL/min) was applied. The activity eluted in 5 fractions (1.6 mL each) at a calculated buffer concentration of 0.03 M. The material from the DEAE separation was then applied to a 4-mL hydroxyapatite column that had been equilibrated in 0.1 M Tris·acetate (pH 8) with 1% octyl β-glucoside, at a flow rate of 0.7 mL/min. After 10 mL of Tris–detergent solution that contained 0.05 M $MgCl_2$, then 10 mL of the Tris–detergent solution alone, a 0–0.25 M sodium phosphate (pH 8) gradient, prepared in the Tris–detergent buffer, was applied to the column over 62 mL. The majority of activity eluted in a peak at the end of the gradient. This material exhibited a single band upon SDS–PAGE.

An improved method was later developed for brush border membranes solubilized in 2% octyl β-glucoside. The first step was chromatography on the DEAE HPLC column at pH 6, similar to the step described above, with a peak of activity eluting about halfway through an 80-min gradient from 0.005 to 0.06 M buffer (in 1% octyl β-glucoside). This peak was then applied to a 4-mL hydroxyapatite column, similar to that described above. Activity eluted at 0.28 M potassium phosphate. The recovered material was then submitted to a second chromatography on DEAE at pH 8 in Tris·acetate buffer with 1% octyl β-glucoside. The activity eluted early in a 3-h gradient from 0.122 to 0.176 M buffer at a calculated buffer concentration of 0.130 M.

Enzyme Assays. Retinyl ester hydrolase assays were done as previously published (Rigtrup & Ong, 1992) with one minor modification. The mobile phase for final HPLC separation and quantification of the product retinol was either 89% *n*-hexane/11% dioxane (vol %) or 1.0% dioxane and 0.10% glacial acetic acid in toluene. Phosphatidylcholine hydrolysis was carried out for 15 min at 37 °C with 10–25 ng of purified enzyme by a previously published method for the rat brush border phospholipase B (Pind & Kuksis, 1988). Cholesteryl ester hydrolysis and triglyceride hydrolysis assays were done for 30 min at 37 °C with 10–25 ng of purified enzyme using a previously published method (Harrison, 1988).

Preparation of Anti-Retinyl Ester Hydrolase Antisera. Two rabbits each received about 15 μg of REH (purified by the first method) emulsified with 0.25 mL of TiterMax #R-1 adjuvant (CytRx Corp., Norcross, GA) in a total volume of 0.5 mL. Half the dose was given in five to six intradermal injections on sites on both sides of the abdomen in small volumes, and the rest was injected intramuscularly. The rabbits were boosted after 14 weeks with a similar dose of antigen given as an emulsion again. A second boost (17 μg of antigen in 200 μL) was given 40 days after the first boost, injected intramuscularly. Serum samples were collected at various times after immunization, and antibody presence was determined using Western blots. The antiserum used in the studies here was collected 11 days after the second boost.

Immunoblotting. Brush border membrane equivalent to 12 μg of protein was subjected to SDS–PAGE on a 6% polyacrylamide gel. The proteins were transferred to nitrocellulose paper [TE 50X Transphor Unit (HSI)] in Tris–glycine buffer with 20% methanol using 1-A current for 90 min. The immunoreactive proteins were detected using the ECL Western blotting detection kit from Amersham, according to their protocol. Briefly, after being blocked in 1% normal goat serum, 0.1% BSA, 0.1% Tween 20, and 0.9% NaCl, in 0.1 mM Tris, pH 7.5, the nitrocellulose paper was incubated sequentially with (i) a 1:10 000 dilution of anti-REH in blocking buffer, (ii) 1:2000 dilution of HRP-labeled second antibody in blocking buffer, and (iii) ECL detection reagent, with washing in buffer between each incubation. Excess detection reagent was drained; the blot was covered with plastic wrap and exposed to X-ray film for 2 s.

RESULTS

Purification of Rat Intrinsic Brush Border Retinyl Ester Hydrolase. Retinyl ester hydrolase activity was rapidly

[1] Abbreviation: REH, retinyl ester hydrolase.

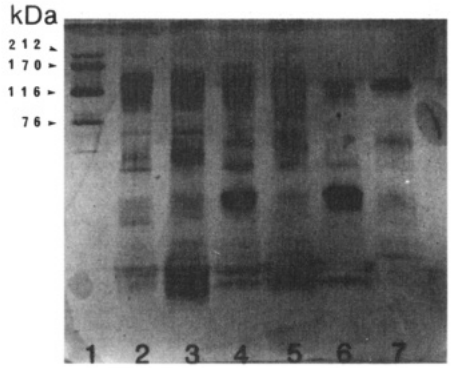

FIGURE 1: SDS–PAGE of samples at different stages of purification of the retinyl ester hydrolase activity. The 8–25% gradient gel was run on the Pharmacia Phast system and developed by silver staining. Lane 1, molecular weight standards; lane 2, brush border membrane (100 μg of protein/mL); lane 3, solubilized brush border membrane proteins (200 μg of protein/mL); lane 4, first eluant from ConA–Sepharose (60 μg of protein/mL); lane 5, second eluant from ConA–Sepharose (60 μg of protein/mL 1); lane 6, pooled peak from DEAE chromatography (15 μg of protein/mL); lane 7, pooled peak from hydroxyapatite chromatography (20 μg of protein/mL). Approximately 1 μL from the samples indicated was loaded per lane.

Table 1: Purification of Retinyl Ester Hydrolase from Rat Intestinal Brush Border Membranes[a]

step	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]	recovery[b] (%)	purification (x-fold)
mucosa	2.2	100	1
BBM	26	56	12
Brij solubilized	29	34	13
ConA–Sepharose	190	16	84
DEAE	370	11	160
hydroxyapatite	2000	6.6	900

[a] All assays were done with 30 μM retinyl palmitate substrate and 0.5% deoxycholate. All other assay details, retinol extraction, and quantification are outlined under Experimental Procedures. [b] Recovery refers to the amount of retinyl palmitate hydrolytic activity present after each step as a percent of the activity present in the mucosal homogenate.

released from brush border membranes by treatment with papain, with over 50% of the total activity recovered in the supernatant fraction after a 5-min exposure. Less than 10% of the activity was recovered in the supernatant fraction in control experiments in which exogenous protease was not added. Although this procedure provided a rapid method to effect significant purification, we elected to purify the uncleaved enzyme. In the first method developed, REH solubilized by 0.25% Brij 35 was then retained on ConA–Sepharose. Generally, 50% of the applied activity was recovered, but this required two lengthy incubations in 0.6 M methyl α-D-mannopyranoside. Chromatography on a DEAE resin at pH 6 gave a preparation of two major bands, as judged by SDS–PAGE. The contaminating band was separated from REH by sodium phosphate gradient elution from hydroxyapatite. Purified REH gave a dominant band on a reducing SDS–PAGE gel, with an apparent molecular weight of approximately 130 000 (Figure 1). Traces of other bands can be seen that became more prominent with storage, suggesting they might have arisen by proteolysis of REH. Approximately 100 μg of purified enzyme was obtained from 50 rat intestines, with an overall yield of 6.6% (Table 1). This gave a 900-fold increase in specific activity, compared to the activity present in homogenized mucosa.

An improved method was developed during the course of this work that gave greater yields of purified enzyme. REH was solubilized in 2% octyl β-glucoside and then purified by DEAE chromatography at pH 6, hydroxyapatite chromatography at pH 8, and a second DEAE chromatographic step at pH 8. Again, a single band on reducing SDS–PAGE gels was observed, at the same position (apparent molecular weight of 130 000) determined from REH from the original method. This procedure had an overall yield of 20%, and the material was of the same specific activity and enrichment as that obtained by the first method. The improved yield was attributed to the deletion of the ConA–Sepharose step that required extended elution times and may have permitted some proteolysis by endogenous proteases. The relatively large amounts of purified enzyme available from these methods allowed for detailed kinetic studies with a variety of substrates. Data presented here on retinyl ester hydrolysis are primarily from REH purified by the first method. Comparative hydrolysis of lipid substrates was studied with REH obtained by the second method. No differences were noted in the enzymatic properties of REH for these two methods of preparation.

Table 2: Relative Hydrolysis[a] of Retinyl Palmitate at the Indicated Bile Salt Concentrations[b]

bile salt	0.5%[c]	1%[c]
cholate	50 ± 0	100 ± 9
taurocholate	29 ± 2	66 ± 10
deoxycholate	210 ± 10	160 ± 0
taurodeoxycholate	54 ± 6	59 ± 3

[a] Relative hydrolysis is presented as the percent of hydrolytic activity observed with 1% cholate [1.31 μmol min^{-1} (mg of protein)$^{-1}$]. [b] All reactions contained 30 μM retinyl palmitate and 15 ng of purified hydrolase. Other assay, retinol extraction, and quantification descriptions are under Experimental Procedures. All determinations were done in triplicate and are expressed as mean ± SD. [c] Weight per volume percent (grams per 100 mL).

Comparison of the Purified Enzyme to Previously Described Intrinsic Brush Border Retinyl Ester Hydrolase Activity. REH had previously been studied only as a component of the isolated intestinal brush border membrane (Rigtrup & Ong, 1992). Such membranes contained not only intrinsic retinyl esterase activity(s) but also activity(s) derived from pancreatic secretions. Consequently, brush border membranes from rats with ligated common ducts were examined in that study to define properties of intrinsic esterases toward retinyl ester substrates. The purified enzyme generated in this study proved to have essentially identical properties to those observed for the activity present on the isolated membranes from the animals that had undergone ligation. The enzyme was strongly stimulated by both dihydroxy and trihydroxy bile salts (Table 2), with the greatest degree of stimulation observed in the presence of 0.5% deoxycholate, a dihydroxy bile salt. Unconjugated bile salts produced greater stimulation of activity than did the taurine-conjugate analogs. The activity of the enzyme toward retinyl esters with different acyl groups (Figure 2) determined in the presence of 1% taurocholate, was greatest for retinyl palmitate, the major dietary ester. This mirrored the pattern previously observed for the activity present with brush border membranes obtained from common duct-ligated animals. Membranes isolated from nonligated animals have their most potent activity with retinyl caproate (C6) and octanoate (C8), with rates about 3-fold greater than seen for retinyl palmitate (Rigtrup & Ong, 1992).

Hydrolysis of Lipid Esters by Purified Enzyme. The purified enzyme was also active in hydrolyzing phosphatidylcholine, as well as retinyl esters, but had little if any activity toward triacylglycerol or cholesteryl ester (Table 3). Hydrolysis of phosphatidylcholine was observed in the presence

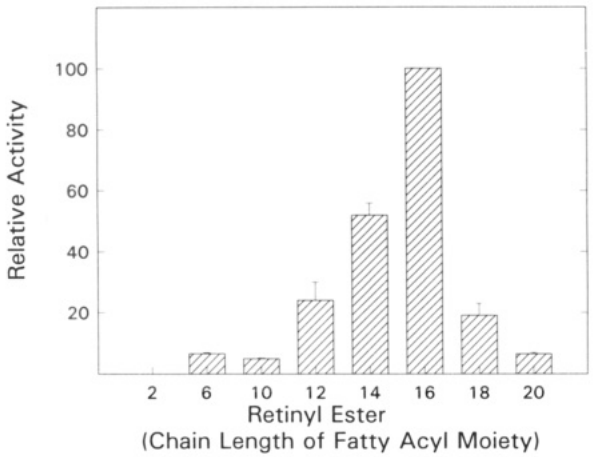

FIGURE 2: Relative rate of hydrolysis of retinyl esters by purified retinyl ester hydrolase. The relative rate was defined as the percent of the rate observed for retinyl palmitate (0.75 μmol min^{-1} mg^{-1}). All esters had saturated acyl chains of the indicated length. Reactions were carried out in the presence of 1% taurocholate with 30 μM retinyl ester and 15 ng of purified REH. Determinations were done in triplicate on two occasions and are reported as the average of the means ± SEM.

Table 3: Hydrolysis of Various Substrates by Purified Retinyl Ester Hydrolase

substrate	concn (μM)	rate of hydrolysis [μmol/(min·mg)][a] 0.5% deoxycholate	1.0% cholate
retinyl palmitate	5	1.2	3.3
	30	3.2	3.1
retinyl oleate	5	0.15	0.82
	30	0.29	0.83
dipalmitoylphosphatidylcholine	5	0.22	2.4
	30	0.63	7.4
cholesteryl oleate	5	0.01	0.01
	30	0.03	0.02
tripalmitoyl glyceride	5	0.01	0.03
	30	0.01	0.02

[a] Values are averages of duplicate or triplicate determinations.

of both a dihydroxy (deoxycholate) and a trihydroxy (cholate) bile salt, with a 10-fold higher rate obtained with the latter. All substrates contained the palmitate acyl group, except for the cholesterol ester, tested as the oleate. This might have some effect on the ability of the enzyme to achieve significant rates of hydrolysis. Retinyl oleate hydrolysis was reduced 10-fold and 4-fold, compared to retinyl palmitate hydrolysis, in deoxycholate and cholate, respectively. However, triolein was hydrolyzed at about the same rate as was found for tripalmitin. The differences in hydrolytic rates observed with this change in fatty acid moiety were less dramatic than the differences observed when the alcohol moiety was changed.

Regional Distribution of Retinyl Ester Hydrolytic Activity and Retinyl Ester Hydrolase in the Small Intestine. Retinyl palmitate hydrolytic activity was observed for brush border membrane preparations from the proximal, middle, and distal thirds of rat small intestine, when examined in the presence of taurocholate, with a 2-fold increase noted from the proximal to the distal third (Figure 3). In deoxycholate, little activity was detected in the proximal third, but activity was present in the middle and distal thirds, with the latter about 3-fold higher than the former. Because purified REH was stimulated by both dihydroxy and trihydroxy bile salts, while the pancreatic-derived retinyl ester hydrolytic activity(s) is(are) stimulated only by trihydroxy bile salts (Rigtrup & Ong, 1992),

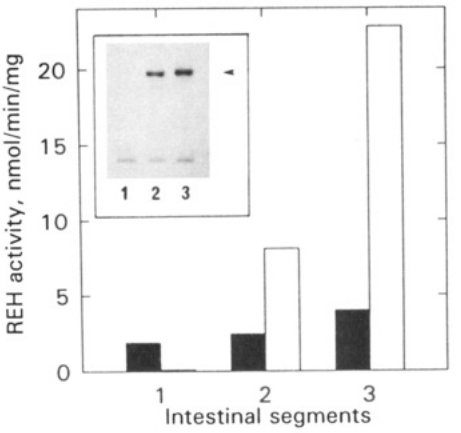

FIGURE 3: Regional distribution of retinyl ester hydrolytic activity and REH in the small intestine. The rate of hydrolysis of retinyl palmitate (30 μM) was determined for preparations of brush border membranes from the (1) proximal, (2) middle, and (3) distal thirds of the small intestine, assayed in 1% taurocholate (filled bars) or 0.5% deoxycholate (open bars). Western blot analysis of the presence of REH in these membranes is shown in the inset. The arrow indicates the migration position of pure REH. The bands at the bottom of the gel are at the dye front and are an artifact of this procedure. Each lane contained 12 μg of brush border membrane protein.

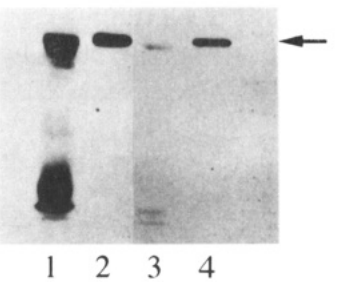

FIGURE 4: Recognition of REH by antiserum raised against phospholipase B. Preparations of brush border membranes and pure REH were analyzed by the Western blot procedure with antisera produced against either pure REH (lanes 1 and 2, 1/10 000 dilution) or phospholipase B (lanes 3 and 4, 1/10 000 dilution). Lanes 1 and 3 contained approximately 17 μg of brush border membrane protein. Lanes 2 and 4 contained 75 ng of purified REH. The arrow indicates the migration position of REH.

this suggested that REH was not present in the proximal third of the small intestine, the activity present probably due to the binding of pancreas-derived enzyme(s) by the brush border membrane.

The absence of REH in the proximal gut was confirmed by immunoblotting with polyclonal antibodies raised against purified retinyl ester hydrolase. Detectable levels of immunoreactive material were found only in preparations of brush border membranes from the middle and distal thirds of the small intestine, when examined by the Western blot procedure (Figure 3, inset). The migration position was identical to that of pure REH, not shown in this figure. This distribution is essentially the same as that found by Western analysis of phospholipase B in rat small intestine (Pind & Kuksis, 1991). An aliquot of that antiserum against phospholipase B was kindly provided to us. It was found to give a single band on Western analysis of our preparations of brush border membranes that was identical in position to the major band observed with our antiserum (equal dilutions were used) and also to react with our preparation of pure REH (Figure 4). This greatly strengthened the evidence that these two hydrolase activities were indeed due to a single protein. The immunostaining seen below the band for REH appears to be from proteolyzed REH, because it was not observed if the brush

Table 4: Kinetic Parameters[a] for the Hydrolysis of Phosphatidylcholine and Retinyl Palmitate in the Indicated Detergent

	phosphatidylcholine		retinyl palmitate	
detergent	K_m (μM)	V_{max} [μmol/(min·mg)]	K_m (μM)	V_{max} [μmol/(min·mg)]
taurocholate (1.0%)	11.3 ± 1.9	13.9 ± 1.5	0.65 ± 0.2	0.74 ± 0.04
cholate (1.0%)	21.9 ± 1.4	13.3 ± 0.7	0.9 ± 0.2	3.5 ± 0.3
deoxycholate (0.5%)	18.1 ± 3.9	0.9 ± 0.1	11.9 ± 1.6	4.4 ± 0.4

[a] Parameters were calculated by the K-cat program (BioMetallics, Inc.) with the choice of constant relative error and a robust fit of 3.

border membranes were processed more rapidly for this procedure (compare to Figure 3).

Bile Salt Dependence of the Kinetic Parameters for Hydrolysis of Retinyl Ester and Phosphatidylcholine. In the presence of taurocholate, a physiological bile salt, typical Michaelis–Menten kinetics were observed for the hydrolysis of both retinyl palmitate and 1,2-dipalmitoylphosphatidylcholine. For retinyl palmitate, the K_m was 0.65 ± 0.2 μM, which compared well with the K_m value of 0.72 μM previously determined for brush border membranes assayed under the same conditions (Rigtrup & Ong, 1992). The V_{max} was 0.74 ± 0.04 μmol min^{-1} mg^{-1}, 100-fold higher than that measured for the brush border membranes, consistent with the fold enrichment realized during the purification.

For phosphatidylcholine, much higher values for both K_m (11.3 ± 1.9 μM) and V_{max} [13.9 ± 1.5 μmol min^{-1} (mg of protein)$^{-1}$] were obtained.

The values for both K_m and V_{max} were significantly altered for both substrates when kinetic studies were carried out in the presence of cholate and deoxycholate (Table 4). For retinyl palmitate, a similar K_m (0.9 μM) but a 5-fold higher V_{max} was observed with cholate, compared to values obtained in the presence of its taurine-conjugated analog (taurocholate). An even higher V_{max} was obtained in the presence of deoxycholate, but this was accompanied by a 10-fold increase in its K_m value (11.9 μM).

The kinetic parameters for 1,2-dipalmitoylphosphatidylcholine hydrolysis were altered differently than were these parameters for retinyl palmitate hydrolysis when the bile salt was changed. The K_m value obtained with cholate was about 2-fold greater than that determined in taurocholate, but the V_{max} value was unaltered, which contrasts markedly with the 5-fold increase in V_{max} and unaltered K_m observed for retinyl palmitate. Deoxycholate did not increase the K_m for phosphatidylcholine over that measured in cholate, but a dramatic 15-fold *decrease* in V_{max} accompanied this change in bile salt, in contrast to the moderate *increase* seen for retinyl palmitate with the same bile salt shift. It should be noted that phosphatidylcholine had K_m values greater than those for retinyl palmitate in all tested bile salts.

DISCUSSION

The rapid solubilization of the retinyl ester hydrolytic activity by papain treatment is consistent with the idea that this protein is a stalked brush border protein. Such proteins consist of a short N-terminal cytoplasmic sequence, a single transmembrane-spanning domain, a connecting segment, and a large luminal portion that includes the catalytic site [reviewed in Alpers (1987)]. Treatment with papain will frequently release the luminal domains from the membrane, while maintaining catalytic activity. Sucrase, leucine aminopeptidase, folate conjugase, and the phospholipase B of particular interest here are all examples of stalked brush border proteins. Previously, phospholipase B was purified from rat intestine after such proteolytic release (Pind & Kuksis, 1991) or by a process that involved SDS–PAGE. The two purification procedures developed here began with activity solubilized from the membrane preparation by detergent. Thus, we assume we have recovered the native protein, including the natural N-terminus. At this time, we have not been successful in obtaining reliable sequence data from the native protein, suggesting the N-terminus may be blocked. However, an advantage to the development of a procedure for purification of the native, active form was the relatively good yield of active REH that allowed us to conduct considerable characterization of the enzymic properties of the purified protein.

The retinyl ester hydrolase activity(s) present in preparations of brush border membrane is (are) stimulated by both di- and trihydroxy bile salts (Rigtrup & Ong, 1992) as was the purified REH examined here. The acyl chain specificity pattern was also the same as that attributed to the intrinsic activity previously: a strong preference for retinyl palmitate, the most common natural retinyl ester, and little ability to hydrolyze short acyl chain retinyl esters. Thus, this enzyme can account for the majority of the retinyl palmitate hydrolysis carried out by the membrane preparations. It should be noted that the ratio of stimulation of pure enzyme by dihydroxy compared to trihydroxy bile salts was 2; for brush border membranes, the ratio was 1.3. This difference can be explained by the presence on the membranes of activity of pancreatic origin, which is not active in the presence of dihydroxy bile salts. This presence was particularly noticeable for brush border membranes from the proximal third of the gut. Although it has greater activity for short acyl chain retinyl ester substrates, it may well have activity toward retinyl palmitate. Pure bovine cholesterol ester hydrolase exhibited a pattern of retinyl ester hydrolysis that was greatest for short acyl chains, but still with substantial activity for retinyl palmitate (Rigtrup & Ong, 1992).

Our previous suggestion that the intrinsic retinyl ester hydrolase activity was separate from phospholipase B was based primarily on the patterns of stimulation by different bile salts that did not match for the two activities, when assayed with their separate substrates (Pind & Kuksis, 1988; Rigtrup & Ong, 1992). However, that pattern was reproduced here with the single enzyme, and it is now almost certain that the two activities reside in the same protein. First, the purified retinyl ester hydrolase was found to have a potent phospholipase activity. Second, the presence of REH only in the lower two-thirds of the small intestine was identical to the location reported for rat phospholipase B (Pind & Kuksis, 1991). Finally, the fact that antibodies raised to phospholipase B reacted with purified retinyl ester hydrolase strongly indicates that these two activities originate from the same enzyme.

However, the enzyme is not simply a nonspecific lipase. For example, pancreatic cholesterol ester hydrolase is also known as carboxyl ester hydrolase (or lipase) because it has a broad specificity, capable of significant hydrolysis of long- and short-chain triacylglycerols, phosphatidylcholines, retinyl esters, and nonphysiological esters such as *p*-nitrophenyl acetate, as well as cholesterol esters [e.g., see Rudd and Brockmann (1984)]. Here, we observed little ability to

hydrolyze either cholesterol esters or triacylglycerols under conditions appropriate for the other two substrates.

The properties and distribution of the enzyme are consistent with a physiological role in retinyl ester hydrolysis. The V_{max}/K_m values, measures of the catalytic efficiencies of the enzyme, were 1.2 for both retinyl palmitate and dipalmitoylphosphatidylcholine, but the substrate much less abundant in the diet, retinyl palmitate, had a substantially lower K_m, perhaps permitting it to compete effectively with phosphatidylcholines for the enzyme. Hydrolysis in the proximal third of the small intestine, where the intrinsic enzyme is absent, is most likely carried out by the trihydroxy bile salt-requiring activity of pancreatic origin (Rigtrup & Ong, 1992). REH expression began distally, where the level of the pancreatic-derived activity would be decreasing, resulting in a relatively constant ability to hydrolyze retinyl esters (in the presence of taurocholate) throughout the length of the small intestine. This point has previously been noted for the hydrolysis of phosphatidylcholines (Pind & Kuksis, 1991).

The distinct kinetic behaviors with each substrate that were quite dependent on which bile salt was present suggest specific interaction of the two different bile salts with the enzyme, producing two different active forms. In support, preliminary studies of the rates of hydrolysis of increasing amounts of retinyl palmitate in the presence of a constant level of both taurocholate and deoxycholate produced biphasic velocity plots (data not shown). The plots were similar to what would be observed if two separate enzymes were present that had differences in K_m and V_{max} values similar to those determined for the pure enzyme in the individual bile salts alone. Thus, the altered kinetic properties cannot be explained by differences in detergent micelles or substrate solubilization, but must reflect different specific interactions of the two bile salts with REH. Specific bile salt–enzyme interactions have been documented for cholesterol ester hydrolase, activated by trihydroxy, but not dihydroxy, bile salts (Jacobson *et al.*, 1990). As assessed by changes in circular dichroism spectra, the enzyme alters its conformation in the presence of cholate at concentrations far below its critical micelle concentration. Similar concentrations of deoxycholate also change the CD spectra, but in a way that was suggested to indicate that the conformational changes are transient and unstable. Here, both classes of bile salts produced stable, activated enzyme.

In summary, the properties of this intrinsic enzyme of the brush border of the rat small intestine, when activated by the presence of the physiological bile salt taurocholate, suggest it may contribute to the necessary hydrolysis of both retinyl esters and phosphatidylcholines, but not of triacylglycerols or cholesterol esters. This dual ability poses a problem in selecting an appropriate name for the enzyme. Neither of the two names currently assigned, REH and phospholipase B, would appear to accurately describe this activity. Perhaps the development of a relatively convenient purification procedure reported here will permit more extensive studies of the interesting properties of this enzyme and lead to an appropriate name.

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