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Purification and Characterization of Bovine Pancreatic Bile Salt-Activated Lipase

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An enzyme with lipase and esterase activity was purified from bovine pancreas. Furthermore, a non-radioactive lipase assay was developed which is 100 times more sensitive than the conventional methods and allowed the characterization of the lipase activity of the enzyme. The lipase activity increased 42 times in the presence of 10 mM sodium taurocholate, which for the first time provides direct evidence that a bile salt-activated lipase (bp-BAL) was isolated from bovine pancreas. This conclusion is further supported by the fact that the N-terminal amino acid sequence of this lipase/esterase is 88% homologous to human milk BAL and human pancreatic BAL. Staining with various lectins showed that bp-BAL is a glycoprotein which contains fucose residues. Previously from bovine pancreas a lysophospholipase has been purified and a gene was cloned and sequenced encoding an enzyme with cholesterol esterase/lysophospholipase activity. Comparison of the N-terminal amino acid sequence of bp-BAL with the deduced amino acid sequence of the latter revealed that they are identical. Furthermore, the molecular weight of the purified bp-BAL of 63,000, as estimated by SDS-PAGE, is very similar to that of the purified lysophospholipase (65,000) and to the theoretical molecular weight of 65,147 of the cholesterol esterase/lysophospholipase. These data suggest that these three enzymes are one and the same.

Key words: bile salt-activated lipase, bovine pancreas, cholesterol esterase, human milk, lysophospholipase, lipase assay.

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Bile salt-activated lipase (BAL) or bile salt-stimulated lipase (BSSL) catalyzes the hydrolysis of carboxyl ester bonds, not only of triacylglycerols, but also of cholesterol esters or fat-soluble vitamin esters (*1, 2*). While BAL shows esterase activity in the absence of bile salt, its triglyceride hydrolyzing (lipase) activity depends on the presence of bile salts.

BAL is present in the pancreas of many mammals (*3-14*), including human, and also in the milk of mammals, such as human (*15-20*), gorilla (*21*), cats (*22*), and dogs (*22*), but it is absent in bovine milk (*21*). At present only BALs of human milk and human pancreas have been well studied. These enzymes have been purified and characterized in detail (*10, 11, 17-19*), and their genes have been cloned and sequenced (*12, 13, 23, 24*). From human pancreas, a cholesterol esterase has also been purified and its gene has been cloned and sequenced (*25, 26*). Comparison of the deduced amino acid sequences of these three enzymes, human milk BAL (hm-BAL), human pancreatic BAL (hp-BAL), and human pancreatic cholesterol esterase, revealed that they are one and the same enzyme in different locations and with different activities (*1, 2*).

The physiological function of BAL has mainly been studied for hm-BAL. It has been shown that the lipase activity of hm-BAL has an important role in fat digestion in infants (*1, 2, 27-34*) because their pancreatic lipase and esterase activities are low (*29*). The specific properties of hm-BAL support this function; the enzyme has no positional specificity for the ester bonds of triglycerides, it is inactive in human milk which contains only micromolar amounts of bile salts and it is activated in the duodenum by the presence of millimolar amounts of bile salts (*29*). In experiments with kittens, it has been shown that the group fed a formula containing purified hm-BAL grew twice as fast as kittens fed the standard formula (*35*).

From all BALs identified so far, hm-BAL has best been characterized because it is present in human milk in high amounts (approx. 100 mg/liter, 1% of total milk protein), and milk whey contains a much lower number of other protein species in comparison to crude extracts of mammalian internal organs. These properties allow for a simple isolation procedure (*1, 17-19*) which yields large amounts of purified enzyme. This was especially important for the characterization of its lipase activity by non-radioactive methods which are carried out at millilitre scale. This

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Abbreviations: AAA, *Anguilla anguilla* agglutinin; BAL, bile salt-activated lipase; bp-BAL, bovine pancreatic bile salt-activated lipase; DAS, *Datura stramonium* agglutinin; hm-BAL, human milk bile salt-activated lipase; PNPA, *p*-nitrophenyl acetate; PVDF, polyvinylidene difluoride; RCA-120, *Ricinus communis* agglutinin; rp-BAL, rat pancreatic bile salt-activated lipase; SSA, *Salvia sclarea* agglutinin.

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requirement has in the past restricted the characterization of the lipase activity of other BALs because highly sophisticated methods using radioactive compounds had to be used. Therefore, for the characterization of BALs, mainly their esterase activity was monitored (*5, 7, 9-12*), because for this measurement a microlitre scale assay using water-soluble *p*-nitrophenol substrates were available.

In bovine pancreas lysophospholipase and cholesterol esterase activities have been identified. The lysophospholipase, a 65 kDa protein, was purified, characterized, and its amino acid composition was determined (*3*). The gene of the bovine pancreatic cholesterol esterase/lysophospholipase has been cloned and sequenced (*12*). The amino acid composition of the purified lysophospholipase is similar to that reported for hm-BAL (*1*). Furthermore, the deduced amino acid sequence of the cholesterol esterase/lysophospholipase is 78% similar to that of hm-BAL (*1*). From these data, Wang and Hartsuck (*1*) speculated that the 65 kDa lysophospholipase purified by Bosch *et al.* (*3*) and the cholesterol esterase/lysophospholipase cloned by Kyger *et al.* (*12*) are the same enzyme and might be the BAL of bovine pancreas. However, there is no direct evidence of the presence of a bp-BAL whose lipase activity is dependent on bile salt. Therefore, we decided to attempt the isolation and purification of the BAL from bovine pancreas and the characterization of its bile salt dependent lipase activity.

The present report describes the isolation, purification, and characterization of bp-BAL. For the first time the lipase activity of this enzyme was characterized and compared with that of hm-BAL, the only BAL species in which lipase measurements could hitherto be carried out (see above). Furthermore, it was shown that this enzyme is probably the same enzyme as the lysophospholipase and as the cholesterol esterase which have previously been characterized from bovine pancreas (*3, 12*). In addition a simplified method was developed for the purification of hm-BAL.

EXPERIMENTAL PROCEDURES

Materials—Fresh bovine pancreas was obtained from Iwate Shokuniku Kako (Iwate, Japan) and human milk was from our institute. Triolein and *p*-nitrophenyl acetate (PNPA) were purchased from Nacalai (Kyoto, Japan). Polyvinylidene difluoride (PVDF) membrane for Western blotting was from Millipore (Bedford, MA, USA) and the silver staining kit for SDS-PAGE gels was obtained from Daiichi Pure Chemicals (Tokyo, Japan). The Hi-Trap heparin column and the molecular weight standards for SDS-PAGE were from Pharmacia (Uppsala, Sweden). The Lectin Sensor Kit for lectin staining was obtained from Honen (Tokyo, Japan) and the 4-chloro-1-naphthol Substrate Kit was from Vector Laboratories (Burlingame, CA, USA). NEFA C-test Wako for measurement of free fatty acids was purchased from Wako Pure Chemicals (Osaka, Japan).

Enzyme Assays—Lipase activity was determined using triolein emulsion as substrate, which was prepared as follows: 2.5 ml of triolein was emulsified with 7.5 ml of 2% (w/v) polyvinyl alcohol solution for 5 min on ice using a Polytron homogenizer PT10/35 (Kinematica, Kriens/Luzern, Switzerland). The lipase reaction mixture contained 200 μl of triolein emulsion, 100 μl of 0.2 M sodium phosphate buffer (pH 7.5), 80 μl of sodium taurocholate solution, and 20 μl of sample. Triolein hydrolysis was carried out at 37°C in a shaking water bath. The reaction was terminated after 10 and 30 min by addition of 5 ml of chloroform-methanol (2:1, v/v) solution, which destroyed the emulsion. Then 25 μl of this solution was transferred into a new tube, which was heated at 50°C for 5 min to evaporate chloroform and methanol. Twenty-five microliter of 0.2% (v/v) triton X-100 solution was added to each tube to dissolve the released oleic acid. Subsequently, oleic acids was measured spectrophotometrically using the NEFA C-test Wako. Finally, the amount of oleic acid which was released during 20 min was calculated. One unit of lipase activity was defined as the amount of enzyme necessary to release 1 μEq of oleic acid per minute.

Esterase activity was determined using *p*-nitrophenyl acetate (PNPA) as substrate (*19*). One hundred millimolar PNPA in acetonitrile was diluted 10-fold with distilled water prior to use. The reaction mixture contained 1.0 ml of 0.2 M sodium phosphate buffer (pH 7.5), 200 μl of 10 mM PNPA solution, 790 μl of sodium taurocholate solution, and 10 μl of sample. The enzyme reaction was performed at 25°C instead of 37°C to minimize the non-enzymatic hydrolysis of the ester. *p*-Nitrophenol release was measured at 418 nm with a Hitachi U-2000 spectrophotometer equipped with 6-cell positioner. The extinction coefficient of *p*-nitrophenol (pH 7.5) is 11.5×10^3 $M^{-1}\cdot cm^{-1}$ (*19*). One unit of esterase activity was defined as the amount of enzyme which is necessary to release of 1 μmol of *p*-nitrophenol per minute.

Purification of bp-BAL—Unless stated otherwise, all purification steps were carried out at 4°C. To obtain a crude extract, 540 g of fresh and defatted bovine pancreas were homogenized in 1,000 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 0.5 mM PMSF and 2 mM benzamidine. Homogenization was performed in an Ace-homogenizer at 10,000 rpm for 2 min. Then, the homogenate was centrifuged at $10,000\times g$ for 10 min. The supernatant was collected as the crude extract. To concentrate the crude extract, an acid precipitation at pH 5.0, adjusted with 1 N HCl, was carried out. The resulting suspension was centrifuged at $10,000\times g$ for 20 min. The pellet was discarded and the supernatant was further concentrated by ammonium sulfate precipitation. Ammonium sulfate was added to the supernatant to saturation to precipitate the proteins. After 1 h, the solution was centrifuged at $10,000\times g$ for 20 min. The precipitate was dissolved with 50 mM sodium phosphate buffer (pH 7.5) and then dialyzed against the same buffer.

Further purification of the concentrated crude extract was performed using heparin affinity chromatography. Thirty milliliter of the crude extract per cycle was applied to a Hi-Trap heparin column (1.0×1.0 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) at a flow rate of 0.5 ml/min. The column was washed with the same buffer and the bound proteins were eluted with a linear NaCl gradient from 0 to 0.5 M in 30 ml of 50 mM sodium phosphate buffer (pH 7.5). Fractions containing lipase/esterase activity were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.5). An anion exchange chromatography was performed as the next separation step. A Mono Q column (0.5×5.0 cm) was equilibrated

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with 50 mM sodium phosphate buffer (pH 7.5) and 10 ml of the pooled fractions per cycle were applied at a flow rate of 1.0 ml/min. The column was washed with the same buffer and the bound proteins were eluted with a linear NaCl gradient from 0 to 1.0 M in 40 ml of 50 mM sodium phosphate buffer (pH 7.5). Fractions containing lipase/esterase activity were pooled. As the final step of this purification, hydroxyapatite chromatography was performed. The pooled fractions were directly applied to a hydroxyapatite column (PENTAX GH-0810F, 8×100 mm) at a flow rate of 2.0 ml/min by using a HPLC system (Hewlett Packard 1050 system). The column was washed with 2 mM sodium phosphate buffer (pH 7.5) and the retained proteins were eluted with a linear phosphate gradient from 0.01 to 0.4 M in a 20 ml of phosphate buffer (pH 7.5).

Purification of Hm-BAL—Hm-BAL was purified by using heparin-sepharose chromatography and gel filtration chromatography based on the method of Wang and Johnson (*19*). In brief, human milk was centrifuged at 25,000 × *g* for 30 min to obtain milk whey. Ten milliliters of the whey were applied to a Hi-Trap heparin column by the same method as described for the bp-BAL purification. Hm-BAL was retained by this column and eluted with a buffer containing 0.5 M NaCl. The final purification step was a gel filtration chromatography using a Superose 6 column in 0.1 M sodium phosphate buffer (pH 7.0) with 0.15 M of NaCl.

N-Terminal Amino Acid Sequencing—N-terminal amino acid sequences were determined with an automated protein sequencer (Applied Biosystems Model-476A). Purified BAL proteins were electroblotted onto PVDF membrane and stained Coomassie Brilliant Blue R-250 according to the method of Matsudaira (*36*). Then, the BAL containing band was cut out and directly applied to the sequencer. The obtained amino acid sequence was used to search for similar proteins in the EMBL database (DNA sequence data bank version 3.0t77 June 2, 1997) using the TFASTA programme (*37*). The respective regions of homology were aligned manually.

Lectin Stain—Bp-BAL and hm-BAL were stained with four biotinylated lectins, *Anguilla anguilla* agglutinin (AAA), *Datura stramonium* agglutinin (DSA), *Ricinus communis* agglutinin (RCA-120), and *Salvia sclarea* agglutinin (SSA). After each 1 μg of the BAL was separated on a SDS-PAGE gel, the protein was electroblotted onto a PVDF membrane and lectin stain was carried out using the Lectin Sensor Kit. PVDF membrane strips were incubated with 4 μg/ml solution of biotinylated lectins for 60 min. After incubation, lectins were visualized using the 4-chloro-1-naphthol Substrate Kit containing horseradish peroxidase conjugated avidin.

RESULTS

Purification of an Enzyme with Lipase and Esterase Activity from Bovine Pancreas—From a crude pancreatic extract, after acid precipitation (pH 5.0) and ammonium sulfate precipitation, an enzyme with lipase and esterase activity was purified by three chromatography steps (Tables I and II). At first the concentrated extract was loaded to a Hi-Trap heparin column to which the lipase and esterase activities were adsorbed and subsequently eluted at the end of the linear 0–0.5 M NaCl gradient. In the second step these activities were adsorbed to a Mono Q column and eluted with a linear NaCl gradient (0–1.0 M) at 0.15 M. Finally, the eluent was applied to a hydroxyapatite column and the lipase and esterase activities were eluted with a linear phosphate gradient (0.01–0.4 M) at 0.26 M as a single peak. In particular, the lipase activity was strongly stimulated by the bile salt sodium taurocholate. Figure 1 shows the SDS-PAGE analysis of this peak. This figure shows a major 63,000 molecular weight band and three minor bands. SDS-PAGE analysis of the fractions next to that containing the enzyme activities after Mono Q and after hydroxyapatite chromatography showed that this

TABLE I. **Purification of lipase activity from bovine pancreas.**

Purification step	Protein (mg)	Sodium taurocholate concentration: 0 mM, Lipase activity (A) (U)	0 mM, Specific activity (U/mg)	10 mM, Lipase activity (B) (U)	10 mM, Specific activity (U/mg)	Activation B/A[a] (times)
Crude enzyme	6,768.0	7,063.2	1.04	15,588.0	2.30	2.2
Ammonium sulfate precipitation	1,860.1	503.7	0.27	1,139.1	0.61	2.3
Hi-Trap Heparin chromatography	84.8	17.8	0.21	1,160.4	13.7	65.2
Mono Q chromatography	19.9	9.4	0.47	657.3	33.0	69.9
Hydroxyapatite chromatography	12.6	15.5	1.23	649.9	51.6	42.0

[a] This value is the ratio of total lipase activity in the presence of sodium taurocholate (10 mM) to that in the absence of sodium taurocholate (0 mM).

TABLE II. **Purification of esterase activity from bovine pancreas.**

Purification step	Protein (mg)	Sodium taurocholate concentration: 0 mM, Esterase activity (A) (U)	0 mM, Specific activity (U/mg)	10 mM, Esterase activity (B) (U)	10 mM, Specific activity (U/mg)	Activation B/A[a] (times)
Crude enzyme	6,768.0	3,326.4	0.49	14,695.2	2.2	4.4
Ammonium sulfate precipitation	1,860.1	321.9	0.17	1,479.7	0.80	4.6
Hi-Trap Heparin chromatography	84.8	481.2	5.7	2,040.3	24.1	4.2
Mono Q chromatography	19.9	152.5	7.7	1,317.9	66.4	8.6
Hydroxyapatite chromatography	12.6	197.2	15.7	1,173.3	93.2	5.9

[a] This value is the ratio of total lipase activity in the presence of sodium taurocholate (10 mM) to that in the absence of sodium taurocholate (0 mM).

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protein (63 kDa) correlated most strongly with the lipase/esterase activity (results not shown).

N-Terminal Amino Acid Sequencing—To determine whether there is homology with known proteins we determined the N-terminal 25 amino acids of the 63 kDa protein: Ala-Lys-Leu-Gly-Ser-Val-Tyr-Thr-Glu-Gly-Gly-Phe-Val-Glu-Gly-Val-Asn-Lys-Lys-Leu-Ser-Leu-Phe-Gly-Asp-. In the amino acid sequence comparison we found high homology with the derived amino acid sequences of cholesterol esterases, lysophospholipases and carboxyester lipases of the pancreas of various mammals and with BAL of human milk. Table III shows the respective sequences and the percentages of homology of the N-termini of these proteins. The fact that the N-terminal amino acid sequence of the 63 kDa protein is identical to that of the deduced amino acid sequence of the gene of cholesterol esterase/lysophospholipase of bovine pancreas, that it has 88% homology with hm-BAL, and that the lipase activity is activated and strongly enhanced by sodium taurocholate indicates that this enzyme is the bovine pancreatic BAL (bp-BAL).

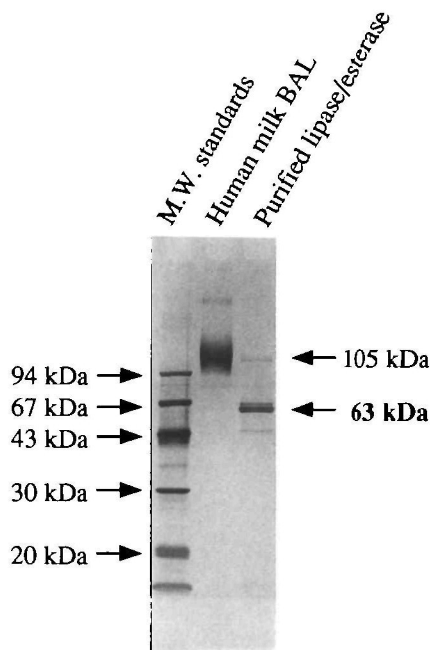

Fig. 1. **SDS-PAGE analysis of the purified enzyme with lipase and esterase activity from bovine pancreas.** It had molecular weight of 63,000, while hm-BAL had one of 105,000.

Purification of Hm-BAL—Hm-BAL was purified with a simplified two step method. The first step was identical to the heparin column chromatography as described for the bovine pancreatic extract above. The second and final step was a gel filtration chromatography using a Superose 6 column. It was easy to separate hm-BAL from the other proteins because hm-BAL has a higher molecular weight than most of the proteins in the partially purified fraction. The purified protein had both lipase and esterase activity (Tables IV and V). SDS-PAGE analysis of the purified hm-BAL shows the typical broad band (*19, 20*) with a molecular weight of 105,000 (Fig. 1).

Effect of Sodium Taurocholate on the Lipase Activity of Bp-BAL and Hm-BAL—During the enzyme purification we observed that the lipase activity of both bp-BAL and hm-BAL were strongly activated by 10 mM sodium taurocholate (Tables I and IV). This phenomenon was investigated in detail with the purified enzyme fractions. Only 2.4% of relative lipase activity was detected with bp-BAL when no sodium taurocholate was present in the reaction mixture. The lipase activity increased with increasing amounts of sodium taurocholate and was highest at 10 mM sodium taurocholate (Fig. 2). In the case of hm-BAL no lipase

TABLE III. **Homology analysis of the enzyme with lipase/esterase activity from bovine pancreas.**

Enzyme	Amino acid sequence	Homology (%)	Ref.
Bp-BAL	**1 AKLGS VYTEG GFVEG VNKKL SLFGD 25**	–	–
Bp-CE	19 ***** ***** ***** ***** ***** 43	100.0	(*12*)
Hm-BAL	51 ****A ***** ***** ***** G*L** 75	88.0	(*37*)
Hp-BAL	19 ****A ***** ***** ***** G*L** 43	88.0	(*38*)
Hp-CE	26 ****A ***** ***** ***** G*L** 50	88.0	(*13*)
Rat-SE	1 ****A ***** ***** ***** **L*G 25	88.0	(*39*)
Rat-CE	26 ****A ***** ***** ***** **L*G 50	88.0	(*40*)
Mouse-CEL	22 ****A ***** ***** ***** **L*G 46	88.0	(*41*)
Mouse-CE	21 ****A ***** ***** ***** **L*G 45	88.0	(*42*)
Rat-LPL	28 ****A L**** ***** ***** **L*G 52	84.0	(*43*)
Hp-CEL	1346 SQ**A ***** ***** ***** G*L** 1370	80.0	(*44*)
Rabbit-CE	25 GD**P ***** ***** E**** **L*A 49	76.0	(*45*)

*Amino acid identical to Bp-BAL. Abbreviations used are: BAL, bile salt-activated lipase; Bp, bovine pancreas; CE, cholesterol esterase; CEL, carboxyl ester lipase; Hm, human milk: LPL, lysophospholipase; Ref, references; SE, sterol esterase.

TABLE IV. **Purification of lipase activity of human milk BAL.**

Purification step	Protein (mg)	Sodium taurocholate concentration 0 mM: Lipase activity (A) (U)	0 mM: Specific activity (U/mg)	10 mM: Lipase activity (B) (U)	10 mM: Specific activity (U/mg)	10 mM: Activation B/A[a] (times)
Human milk whey	115.0	61.2	0.53	112.1	0.97	1.8
Hi-Trap Heparin chromatography	2.1	0	0	57.0	27.1	n.c.
Superose 6 chromatography	0.48	0	0	112.6	234.6	n.c.

[a] This value is the ratio of total lipase activity in the presence of sodium taurocholate (10 mM) to that in the absence of sodium taurocholate (0 mM). n.c., not calculable.

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TABLE V. **Purification of esterase activity of human milk BAL.**

Purification step	Protein (mg)	Sodium taurocholate concentration 0 mM: Esterase activity (A) (U)	0 mM: Specific activity (U/mg)	10 mM: Esterase activity (B) (U)	10 mM: Specific activity (U/mg)	Activation B/A[a] (times)
Human milk whey	115.0	79.4	0.69	240.9	2.1	3.0
Hi-Trap Heparin chromatography	2.1	40.2	19.1	97.4	46.4	2.4
Superose 6 chromatography	0.48	22.9	47.7	47.1	98.1	2.1

[a] This value is the ratio of total lipase activity in the presence of sodium taurocholate (10 mM) to that in the absence of sodium taurocholate (0 mM). n.c., not calculable.

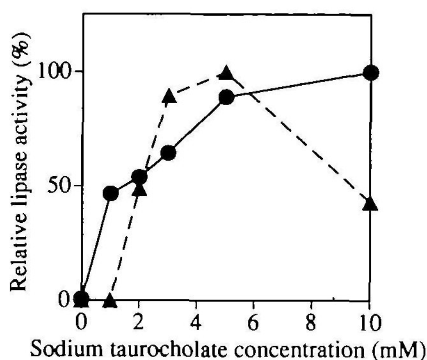

Fig. 2. **Effect of sodium taurocholate concentration on lipase activity of the purified enzyme with lipase and esterase activity from bovine pancreas (●).** Hm-BAL was also tested (▲).

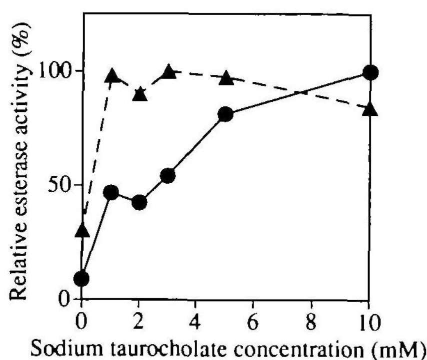

Fig. 3. **Effect of sodium taurocholate concentration on esterase activity of the purified enzyme with lipase and esterase activity from bovine pancreas (●).** Hm-BAL was also tested (▲).

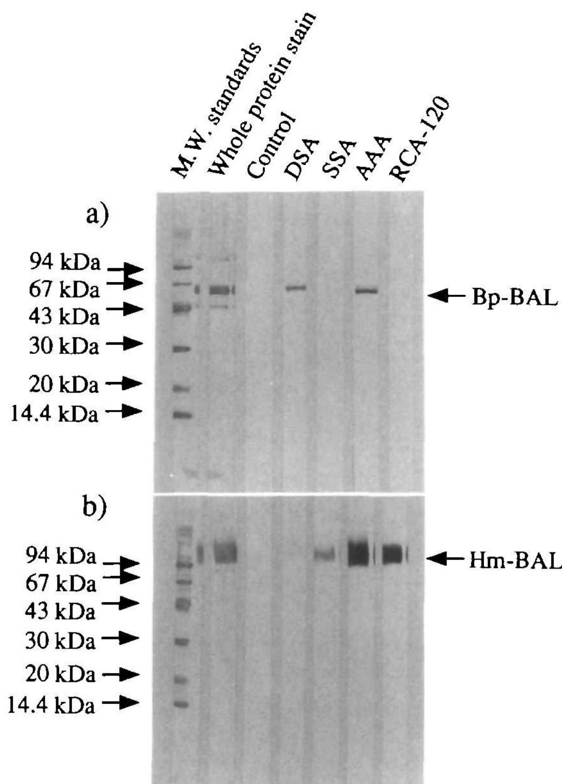

Fig. 4. **Lectin stain analysis of the purified bp-BAL (a) and hm-BAL (b).** Standard proteins for molecular weight were stained by Coomassie Brilliant Blue R-250 (M.W. standards). Whole protein staining was performed by the gold-colloid method (whole protein stain). No lectin was incubated for the control stripes (control).

activity was found in the absence of sodium taurocholate and even in the presence of 1 mM sodium taurocholate. Upon addition of 2-5 mM sodium taurocholate lipase activity increased rapidly and then decreased upon addition 10 mM of this substance (Fig. 2).

Effect of Sodium Taurocholate on the Esterase Activity of Bp-BAL and Hm-BAL—During purification of the enzymes, activation of the esterase activity by sodium taurocholate was observed (Tables II and V), but it was much less than that found for the lipase activity. While the lipase activity was stimulated 42-fold, the esterase activity only increased about 6 times.

Figure 3 shows the effects of the sodium taurocholate concentration on the esterase activities of the purified bp-BAL and hm-BAL. Similar to the activation of the lipase activity, esterase activity of bp-BAL increased with rising sodium taurocholate concentrations up to 10 mM. The esterase activity of the hm-BAL was already strongly activated by 1 mM of sodium taurocholate and decreased slowly with further increasing sodium taurocholate concentrations.

Glycosylation of Bp-BAL and Hm-BAL—To determine whether bp-BAL is glycosylated and to determine the possible composition of the sugar chains, the enzyme was stained after SDS-PAGE by four different biotinylated lectins, DSA, SSA, AAA, and RCA-120. For comparison the same experiment was carried out with hm-BAL. Figure 4a shows that bp-BAL reacted with AAA. This demonstrates that this enzyme is a glycoprotein, which contains fucose residues in its sugar chains. An unrelated protein in the fraction was stained by DSA (Fig. 4a). Hm-BAL was stained by AAA, RCA-120, and SSA, which recognize fucose, galactose, and sialic acid, respectively (Fig. 4b).

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DISCUSSION

Bile salt-activated lipases (BAL) are suggested to have several different enzyme activities such as lipase (triacylglycerol acylhydrolase activity), carboxylesterase, cholesterol esterase, and lysophospholipase (*1, 2*). This suggestion was supported by homology studies of derived amino acid sequences of the cloned genes of enzymes with the mentioned activities, which either show identical or highly homologous amino acid sequences (*1, 2, 12, 13, 38-46*). Only for human milk BAL (hm-BAL), has the lipase activity been characterized in some detail, because large quantities of this enzyme, which are necessary for its characterization, can be easily obtained.

From bovine pancreas enzymes with cholesterol esterase activity and lysophospholipase activity have been isolated and a gene encoding an enzyme with these activities has been cloned and sequenced (*12*). However, a BAL with lipase activity has not yet been isolated, purified and characterized directly from this location (*1*). Recently, Wang *et al.* (*47*) have reported the crystal structure of a supposed BAL from bovine pancreas. In their study they show data about the structure of this bp-BAL, but only limited data of its purification and characterization (especially the employed enzyme assay) were reported. Because of the physiological importance of the multifunctional BAL, it is necessary to determine whether bovine pancreas contains a BAL with lipase activity. The purpose of the present study was to isolate BAL from the bovine pancreas and to characterize its lipase activity.

Nonradioactive lipase assays usually work at millilitre scale (*48*). In the past this has caused limitations in the characterization of the lipase activity of BALs. Often only the esterase activity was measured because for this assay a microliter-scale procedure was available (*4, 5, 7-12, 19*). In the present study we have developed a sensitive non-radioactive microlitre scale lipase assay in order to characterize the lipase activity of bp-BAL and hm-BAL. For this lipase assay we used the NEFA C-test Wako, which is normally used to determine the free fatty acid concentration in human serum. With this method we can now measure free oleic acid (released from triolein) in the range of 10-100 nmol, which is 100 times more sensitive than the conventional non-radioactive titration assays (*48*) and comparable to the radioactive lipase assays. This new assay was used to characterize the lipase activity of BAL from bovine pancreas (see below).

In the present study an enzyme with both lipase and esterase activity was isolated and purified from bovine pancreas. Furthermore, the BAL from human milk was purified as comparison material. During the purification both enzyme activities were monitored in the presence and in the absence of sodium taurocholate. The results show that the enzyme from bovine pancreas has a lipase activity which is strongly activated (42×) in the presence of 10 mM sodium taurocholate. This is comparable to the characteristics found for the lipase activity of hm-BAL (*15-18*), which were confirmed in this study. These data demonstrate for the first time directly the isolation of a bile salt activated lipase from bovine pancreas (bp-BAL). The esterase activity of both enzymes was also activated by sodium taurocholate although to a lesser degree [6×(bp-BAL), 2×(hm-BAL)] (Table III).

Screening for homology with the determined N-terminal amino acid sequence of the bovine pancreatic enzyme shows that this sequence is identical to the deduced N-terminal amino acid sequence of cholesterol esterase/lysophospholipase of bovine pancreas (*12*) and is highly homologous to hm-BAL, hp-BAL (hp-cholesterol esterase), rat pancreatic BAL (rp-BAL), and to comparable enzymes of other mammals (Table III). The high homology with hm-BAL and hp-BAL supports the conclusion that we have isolated bp-BAL. Furthermore, that the N-terminal amino acid sequences of bp-BAL and those of cholesterol esterase/lysophospholipase of bovine pancreas are identical and that the molecular weight of bp-BAL (63,000) is comparable to the deduced molecular weight of the cholesterol esterase/lysophospholipase (65,147) suggest that these three activities belong to one and the same enzyme protein (*3, 12*).

Bile salt activated lipases are glycoproteins. The glycosylation patterns of hm-BAL (*49-51*) and hp-BAL (*52, 53*) have been elucidated and both *N*-linked and *O*-linked oligosaccharides were found. It was shown that hm-BAL has a glycosylation rate of 19-26%, of which the predominant form are *O*-linked oligosaccharides at the proline-rich repeating sequence at the C-terminus of the protein (*1*). The *N*-linked oligosaccharides are mainly of the mono- and disialylated biantennary complex type with or without fucose substitutions and are composed of fucose, galactose, glucosamine, galactosamine, and sialic acid (*18*). Much less is known about the glycosylation of hp-BAL. However, is was found that this protein is *O*- and *N*-glycosylated and that the structure of the *N*-linked oligosaccharides is of the complex-type. Our studies show that also bp-BAL is a glycoprotein. Comparison of the glycosyl moieties of bp-BAL and hm-BAL using four lectins, which can recognize fucose, galactose, sialic acid and *N*-acetyllactosamine, showed that the oligosaccharides of hm-BAL contain fucose, galactose and sialic acid and that the sugar moiety of bp-BAL contains only fucose residues. Furthermore, comparison of the strength of the signal after the lectin stains shows that hm-BAL has a higher sugar content than bp-BAL (see Fig. 4). These results show that bp-BAL is a glycoprotein with fucose residues which are most probably organized in *O*-linked sugar chains.

For humans in particular it has been shown that BAL has an important function in both triacylglycerol and cholesterol ester degradation and therefore for the absorption of fatty acids and cholesterol (*54, 55*). Although BAL shares with other intestinal lipases the hydrolytic activity for triacylglycerol, it is the only intestinal enzyme that hydrolyses cholesterol and vitamin A esters (*47*). Our results show that also in bovine pancreas the multifunctional BAL is present. This finding opens up a way to study the function of this enzyme in mammals with different feeding habits.

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