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Two-dimensional electrophoretic analysis of vesicular and micellar proteins of gallbladder bile[1]

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Abstract

Proteins associated with lipid vesicles or mixed micelles of human gallbladder bile were separated by Sepharose-2B gel filtration chromatography followed by protein concentration and delipidation. After two-dimensional polyacrylamide gel electrophoresis and silver staining up to 59 and 471 polypeptide spots were counted in vesicular and micellar fractions, respectively. As major components the plasma proteins transferrin, albumin, α-fibrinogen, β-fibrinogen, γ-immunoglobulin G, immunoglobulin light chains, α-1 antitrypsin and haptoglobin α-2 chain were identified in the lipid vesicles by comparison with human protein reference maps. However, most biliary proteins including the anionic polypeptide fraction are associated with mixed micelles. The pathophysiological significance of these proteins associated with lipids needs to be investigated further. © 1997 Elsevier Science B.V.

Keywords: Lipid vesicles; Proteins

1. Introduction

Gallbladder bile is a complex system composed mainly of water, inorganic cations and anions, conjugated bile salts, phospholipids, cholesterol, bilirubin, mucin and proteins. The different biliary lipids are present as vesicles composed of phospholipids and cholesterol and as smaller mixed micelles consisting of bile salts, phospholipids and cholesterol [1]. These particles have been studied by gel filtration chromatography [2–4], quasi-elastic light scattering [5], nuclear magnetic resonance [6,7] and cryotransmission electron microscopy [8] and represent the major carriers of the water insoluble cholesterol. Both the vesicles and mixed micelles in gallbladder bile are associated with proteins.

Anionic polypeptide fraction (APF) is a small biliary polypeptide [9,10] synthesized by hepatocytes and its secretion is stimulated by bile salts. In bile it is associated particularly with lipid vesicles. As APF has some similarities with apolipoprotein A-1, a component of human HDL [11], it may be cosecreted with vesicles. Additional proteins have been isolated from biliary vesicles such as aminopeptidase N, or other glycoproteins which favor the crystallization of cholesterol in model bile [12,13]. The characterization of proteins associated to vesicles or mixed micelles in bile is of particular interest, since these might affect the solubility of cholesterol and by this mechanism the formation of cholesterol gallstones. Recently, it has been shown that annexin VI may interact with phospholipid–cholesterol vesicles in bile and might be involved in the secretion of

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phospholipids by human hepatocytes [14]. Moreover, an association of canalicular membrane enzymes, such as alkaline phosphatase, γ-glutamyltranspeptidase and 5′-nucleotidase, with mixed micelles and lipid vesicles in bile was demonstrated [15]. The heterogeneous but significant association of these surface membrane ectoenzymes with biliary lipids suggested that they might not be mere "contaminants" but might represent specific integral components of these lipid structures.

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a high resolution method for separation of complex protein mixtures and very recently, 2-DE was performed to analyze proteins in gallbladder bile [16]. We used this technique to compare the pattern of proteins associated with mixed micelles or lipid vesicles in selected specimens of gallbladder bile from patients with cholesterol gallstones.

2. Experimental

2.1. Instrumentation

Immobilized pH gradient (IPG) gel strips with pH gradient 4-7 were used for isoelectric focusing (IEF) in Multiphor II connected to a constant power supply EPS 3500 XL. Cooling of the whole system was achieved by MultiTemp II. The IPG dry strips (pH 4–7, 110×3.3 mm), the Multiphor II, the power supply EPS 3500 XL, the MultiTemp, the fraction collector (Redi Frac), the peristaltic pump P-1 and the column for gel chromatography were from Pharmacia (Uppsala, Sweden).

2.2. Samples and chemicals

2.2.1. Bile sample collection

Patients with normal gallbladder function who underwent laparoscopic cholecystectomy because of symptomatic cholesterol gallstone disease were included in the study. During laparoscopic surgery the gallbladder was punctured and a flexible probe with side ports was inserted; bile was aspirated as completely as possible because of the known stratification of human gallbladder bile [17].

2.2.2. Analysis of bile composition

For the analysis of bile composition, aliquots were stored at −30°C prior to determination. Lipids and total protein were determined in duplicate using standard methods [18].

2.2.3. Separation of vesicles and micelles

Vesicles were separated from micelles by gel filtration chromatography. Glass columns (40×1.0 cm) were filled with Sepharose-2B (Pharmacia) using a 20 mmol/l Tris–HCl (pH 8.0), 140 mmol/l NaCl, 3.0 mmol/l sodium azide buffer, which contained 5 mmol/l sodium taurocholate to prevent disruption of micellar lipids. After ultracentrifugation (100 000 *g* for 1 h) of the bile sample, 0.5 ml of the isotropic phase was applied to the column and collected in 1.0 ml fractions. The buffer was run at a flow-rate of 0.5 ml/min using a P-1 pump (Pharmacia). Thirty-five fractions were collected and analyzed for cholesterol, phospholipid and protein content.

2.2.4. Preparation of proteins of gallbladder bile for 2-DE analysis

After the gel filtration chromatography fractions of vesicles and micelles were pooled separately. The vesicular and micellar pools were lyophilized, the dry powders were delipidated and lyophilized again. The two freeze dried samples derived from the vesicular and micellar pools were solubilized in lysis buffer and centrifuged (12×1000 *g* for 5 min) and the supernatants were applied to 2-DE [16].

2.2.5. Chemicals

Pharmalytes, ampholytes, acrylamide, N,N′-methylenebisacrylamide (Bis), ammonium peroxodisulfate, N,N,N′,N′-tetramethylenediamide (TEMED), bromphenol blue, sodium dodecyl sulfate (SDS), glycine and mercaptoethanol were products of Pharmacia Biotech, Uppsala, Sweden. 1,4-Dithioerythritol (DTE) and other chemicals were from Merck (Darmstadt, Germany).

2.3. IEF with IPG and SDS–PAGE

2.3.1. IEF with IPG

IEF with IPG in first-dimensional and sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS–PAGE) in second-dimensional separation was mainly performed as reported previously [16]. For IEF the IPG dry gel strips (pH 4–7) were rehydrated in a rehydration buffer consisting of urea (8 mol/l), Nonidet P-40 (2%), DTE (10 mmol/l) and pharmalyte or ampholine, pH 3–10, (0.2%). The duration for the rehydration time exceeded a period of 7 h to obtain complete rehydration of the dry IPG gel strips. The rehydrated IPG gel strips were rinsed with bidistilled water, blotted with tissue paper and placed on the cooling plate of Multiphore II. The temperature of the cooling plate was 20°C regulated by Multi Temp LKB. For IEF of vesicular and micellar proteins, the samples applied amounted to 0.5 ml bile prepared by ultracentrifugation. Both vesicular and micellar fractions were pooled and lyophilized. The dried powders of vesicular and micellar fractions were solubilized in the lysis buffer and applied at the anodic side of the IPG strips, with a constant application volume of 20–30 μl. The electric limits following sample application were set at 300 V, 1 mA, 5 W for 60 min. Sample application was repeated when large volumes of sample had to be applied onto the IPG strips. Samples were applied beneath a layer of paraffin (relative gravity 0.88), and a trace of bromphenol blue was added to the protein samples as an indicator. The running conditions facilitated sample protein entry into the IPG gel strips. The electrical limits for running conditions were set as in phase A: 300 V and (500 V), 1 mA, 5 W for 2 h, followed by an increase in voltage to 600, 700, 800, 900, 1100 and 1500 V at 60 min intervals with a limit of 1 mA and 5 W. After completion of the running conditions in phase A as described above, IEF was undertaken in phase B at the following electric limit settings: 500 V, 1 mA, 5 W for 5 h, followed by 3500 V, 1 mA, 5 W for 5 h, followed by an increase in the limit settings from 3500 V, 1 mA, 5 W, up to 40 kV h for the IEF of bile samples. The IPG gel strips after IEF were stored at −20°C until the second-dimensional SDS–PAGE separation. Appropriate programs for phases A and B were applied for the electrophoresis power supply EPS 3500 XL. The IEF run reached 40–50 kV h prior to completion.

2.3.2. Second-dimensional run: SDS–PAGE

The IPG gel strips were equilibrated for 2×8 min in 10 ml equilibration buffer containing 0.05 mol/l Tris–HCl buffer, pH 6.8, 6 mol/l urea, 30% glycerol, 2% SDS, 1% DTE and a trace of bromphenol blue. SDS–PAGE was run principally as described by Laemmli [19]. The stacking gel, 5% T, 2.6% C (1×13×0.1 cm) and the separation gel, 12% T, 2.6% C (13×13×0.1 cm) were poured manually and the run was done in a vertical configuration of SDS–PAGE [16,20]. Equilibrated IPG gel strips were embedded in a layer of (1 cm) agarose (m.p. 37°C–42°C) suspended in Tris–HCl, (0.4 mmol/l), pH 6.8 supplemented with DTE (2 mmol/l) in order to avoid air bubbles between the interphases IPG gel strips and SDS gel. The electrical limit settings for the second dimensional separation were as follows: 80 V, 40 mA, for 30 min, and 200 V, 80 mA, for about 4 h, at room temperature. After SDS–PAGE the gels were silver stained according to [21].

3. Results and discussion

3.1. Separation of vesicles from micelles in human gallbladder bile by high resolution Sepharose-2B gel filtration chromatography

Thirty-five fractions were collected by the Sepharose gel filtration chromatography. The localization of the vesicle and mixed micelle fractions was performed by phospholipid and cholesterol determinations (Fig. 1). The vesicle fractions were usually lightly colored but opaque and turbid in appearance, while the micellar fractions were more brown–yellow in color. It has been repeatedly proposed that for the separation of vesicles and mixed micelles by gel filtration chromatography the intermixed micellar/vesicular bile salt concentration in the elution buffer must be employed [22]. However, the determination of this bile salt concentration in individual native bile samples including different bile acid species is extremely difficult to perform in larger numbers of samples. Therefore, we used a completely different approach to overcome this well known problem. In a rigorous attempt to validate the separation step we used different concentrations of sodium taurocholate in the elution buffer covering 4 different concentrations (2.5, 5.0, 7.5 and 10 mmol/l) and a negative control. In multiple samples of gallbladder bile

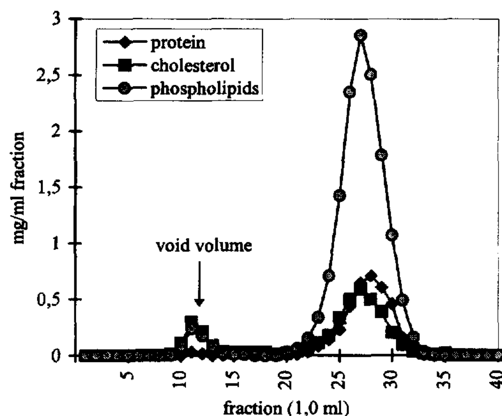

Fig. 1. Gel filtration chromatography of 0.5 ml ultracentrifuged (100 000 g/1 h) gallbladder bile. 5 mmol/l sodium taurocholate in the elution buffer was used to preserve the integrity of vesicles and micelles (Sepharose-2B column; 40×1.0 cm). The vesicles appear in the void volume of the column and were well separated from the mixed micelles.

tested, we found exactly the same distribution of lipids in vesicles and micelles after elution with 2.5, 5.0 and 7.5 mmol/l sodium taurocholate in the elution buffer. However, as expected, gel filtration using the elution buffer without sodium taurocholate slightly favored the amount of lipids in vesicles, while 10 mmol/l sodium taurocholate in the elution buffer resulted in an increase in the amount of lipids eluting in mixed micelles. Therefore, 5 mmol/l of sodium taurocholate in the elution buffer appeared to adequately preserve micellar and vesicular integrity. After the procedures described in Section 2.2.4. such as delipidation, there were still insoluble particles and color in the vesicle and micellar fractions. Therefore the sample volume was used as a basis for this study.

3.2. Identification of proteins with reference maps of human origin

We visualized the vesicular protein pattern of human gallbladder bile by 2-DE separation and attempted an identification of the protein spots on the 2-DE in comparison to human protein reference maps [23–25].

By 2-DE separation up to 59 protein spots from the vesicular fractions (Fig. 2) and up to 471 protein spots from the micellar fractions of human gallbladder bile (Fig. 3) were seen through transmission light. For protein spot matching, relative position of the protein spot, relative intensity of the protein spot and shape of the protein spot were the criteria [23,25]. It should be pointed out clearly that the vesicular and the micellar fractions were separated from the same volume of gallbladder bile.

The vesicular protein amount in gallbladder bile is estimated to be about 1% while the protein amount associated to mixed micelles is estimated to be approximately 99% of the total protein. This distribution is calculated from the sums of protein spot intensities of both fractions on the 2-DE patterns (see Figs. 2 and 3). Among the 59 protein spots from the vesicular fractions of gallbladder bile, we identified eight plasma proteins as major components in comparison with human plasma, red blood and human liver cell protein reference maps [23–25].

These proteins are as follows: (1) transferrin; (2) albumin; (3) α-fibrinogen; (4) β-fibrinogen; (6) γ-immunoglobulin G; (7) immunoglobulin light chains; (8) α-1 antitrypsin; (13) haptoglobin α-2 chain (numbered and named according to Refs. [23–25]. They can be classified in terms of their physiological functions as follows [26]: (1) transport proteins: albumin, transferrin and haptoglobin; (2) immunoglobulins: γ-IgG and Ig light chains; (3) coagulation proteins: fibrinogens; (4) acute phase proteins: α- and β-fibrinogen and α-1 antitrypsin.

Due to the abundance of micellar proteins identification was impaired. However, it is likely that most of the proteins associated to vesicles are also present in micelles. Interestingly, the three isoforms of APF (Fig. 3 arrows) can be easily identified due to their known low-molecular-masses and isoelectric points.

3.3. Establishment of working conditions

2-DE is one of the most powerful separation methods for complex protein mixtures. The underlying principle of the 2-DE is a two-step separation according to the different properties of the protein complex analysed. Protein mixtures are separated in the first dimension according to their isoelectric point (pI). In the second dimension proteins are separated

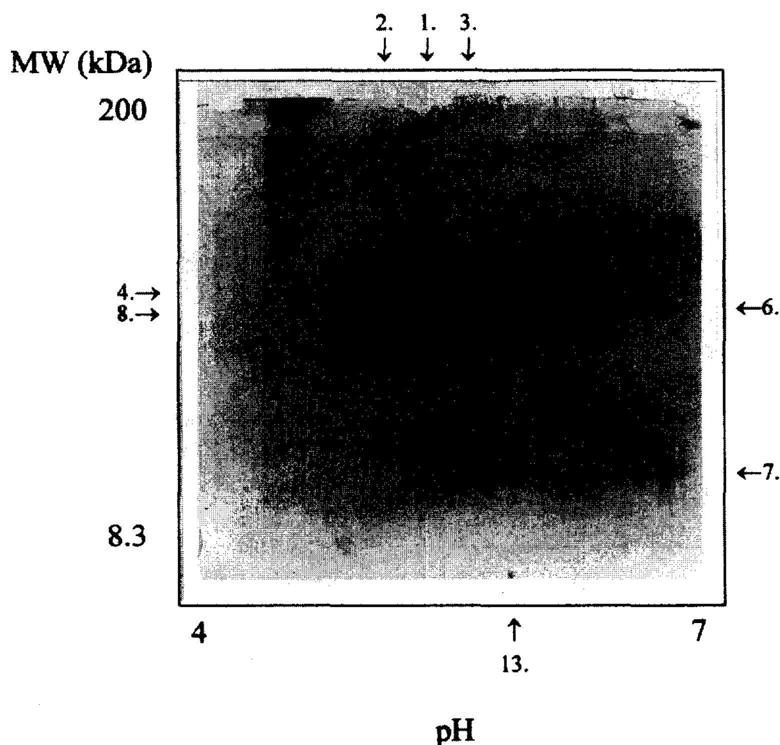

Fig. 2. Pattern of proteins associated to isolated lipid vesicles from human gallbladder bile. (1) Transferrin; (2) albumin; (3) α-fibrinogen; (4) β-fibrinogen; (6) γ-immunoglobulin G; (7) immunoglobulin light chains; (8) α-1 antitrypsin; (13) haptoglobin α-2 chain (numbered and named after Refs. [23–25]). IEF conditions: pH gradient 4–7; 40 kVh. SDS conditions: stacking gel: T=5%; C=2.6%; separation gel: T=12%; C=2.6%; molecular-mass (MW) range of standard proteins from 8000–200 000; pH gradient 4–7; silver stained gels. See Section 2.3.1 Section 2.3.2 for descriptions in detail. kDa=kilodalton.

according to their molecular mass. There are scarcely two identical proteins in both pI and molecular mass and therefore 2-DE, usually, has a higher resolution power for the separation of complex protein mixtures than SDS–PAGE alone.

In our protocol acetone was applied to selectively precipitate proteins from a dispersion of protein–lipid complexes and other biliary constituents. Thus, the vesicular and micellar proteins were in the precipitate after acetone treatment followed by centrifugation. Other biliary components especially lipids should be in the supernatant. After lyophilization, the freeze dried powders of both vesicular and micellar samples were treated with the lysis buffer and followed by the sample preparation method as previously described [16,20]. In view of the biochemical properties of the vesicular and micellar proteins and of the requirements of 2-DE we did not use trichloroacetic acid (TCA).

3.4. Bile sample volume as a basis for comparative studies

The vesicular fractions contain membrane components such as phospholipids, cholesterol and proteins. It is nearly impossible to completely remove lipids from vesicular or micellar proteins without any alteration in the nature of these proteins; however, their primary structure remains unchanged. Furthermore, protein determination in bile samples is difficult due to many interfering substances [1,16]. Therefore, we applied the volume of the original bile samples as a basis for comparing the 2-DE protein patterns of biliary vesicles and mixed micelles [16].

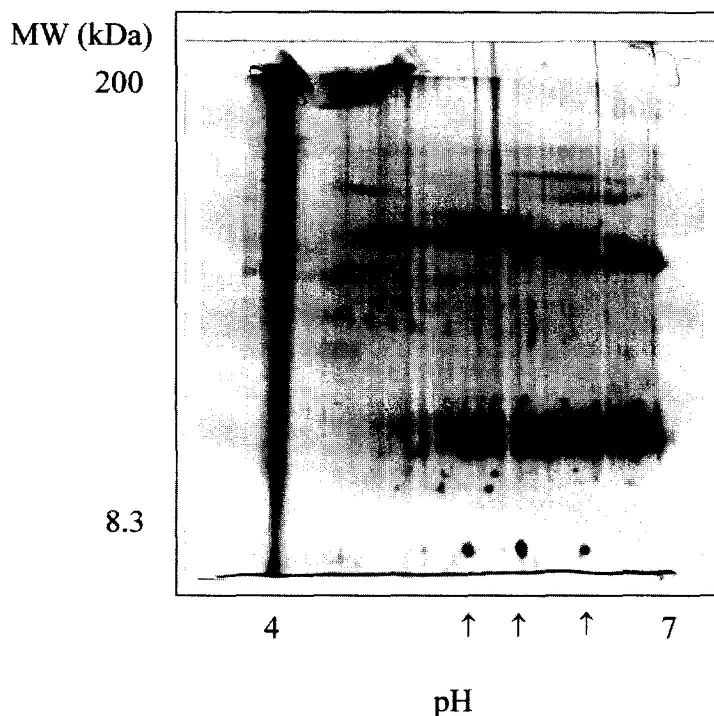

Fig. 3. Pattern of proteins associated to isolated mixed micelles from human gallbladder bile. Original bile sample, working conditions including IEF and SDS–PAGE, molecular-mass standard and protein stain are the same as in Fig. 2. Due to the abundance of different protein spots identification is impaired. However, the three isoforms of APF (↑) are clearly visible.

3.5. Comparison of vesicular and micellar protein patterns

The 2-DE patterns of vesicular and micellar proteins of human gallbladder bile show up to 59 and 471 polypeptide spots (Figs. 2 and 3; $n=3$). Polypeptide spots with nearly equal molecular mass but with different p*I* values represent isoforms of proteins. They are also called "chains of polypeptides", showing nearly the same molecular mass but differences in p*I*. The appearance of these "chains of polypeptides" indicates that posttranslational modifications such as glycosylation including sialylation, or phosphorylation, of vesicular and micellar proteins have occurred. In the case of posttranslational modifications, molecules with the same negative charge for example may run toward the anode faster than related polypeptides with the same molecular mass, while positively charged molecules move more slowly. These p*I* shifts of polypeptides can be seen as "chain of polypeptide" spots with almost the same molecular mass but a different p*I* on the isoelectric point–molecular mass plane of the 2-DE maps. According to publications surveyed to date biliary proteins are glycosylated [27]. Furthermore, sialylation and desialylation [28] as well as phosphorylation and dephosphorylation [29] of the vesicular or micellar proteins are possible.

Our preliminary results demonstrate that 2-DE is a suitable and powerful method superior to SDS–PAGE for screening and identifying vesicular and micellar protein patterns of human gallbladder bile. Clearly, further studies are needed to characterize the complex protein patterns of mixed micelles or vesicles in gallbladder bile. These investigations will give more insight in the physiologically important

role of lipid–protein interactions in bile and their relevance for the development of cholesterol gallstones.

4. Conclusions

We presented 2-DE protein patterns of lipid vesicles and mixed micelles of human gallbladder bile. 2-DE may be a powerful technique providing insight into the molecular mechanisms of lipid transport and of vesicle-mediated protein transport between organelles and organs.

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