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Short communication

Sodium dodecyl sulphate gel electrophoretic preparation of protein standard human apolipoprotein B-48☆

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Abstract

Quantitation of plasma apo B-48 is currently performed by densitometric analysis of SDS–PAGE zones stained with Coomassie Brilliant Blue, using standard solutions of purified apo B-48. Here, preparative gel electrophoresis with a continuous elution system was used for purifying apo B-48. A chylomicron fraction was isolated by 107 000 *g* ultracentrifugation of a chylous ascite. The proteins were delipidated and precipitated in ethanol–diethyl ether (3:1, v/v), subjected to preparative electrophoresis in a 5% polyacrylamide gel and eluted in 0.1% SDS. The peak containing apo B-48 was eluted at a retention time of 445–480 min. The purity of apo B-48 in this fraction was assessed by the detection of a single band (M_r 260 000) after silver staining and Coomassie staining of 4–15% gradient SDS–PAGE. It was confirmed by the absence of apo B-100 contaminant in Western blot of the purified protein preparation. A linear relationship was observed between the densitometric analysis of SDS–PAGE bands and the apo B-48 in a protein range of 0–3 μg. In conclusion, preparative gel electrophoresis was used in a single step purification of apo B-48 that was adapted to the preparation of a standard solution.

Keywords: Apolipoprotein B-48

1. Introduction

Apolipoprotein-B consists of species with different molecular masses at 550 kDa for apolipoprotein B-100 (apo B-100) and 240 kDa for apolipoprotein B-48 (apo B-48) [1]. The physiologically most important forms are apo B-100 and apo B-48, produced by the liver and the intestine, respectively. Apo B-48 is produced only by the intestine in response to dietary lipid and is one of the main proteins in the chylomicrons and chylomicron remnants of humans [2,3]. This glycoprotein has therefore a clinical interest in lipoprotein metabolism as an indicator of intestinal absorption and blood transport of lipids in postprandial hyperlipemia tests [4,5].

Quantitation of plasma apo B-48 is currently performed by densitometric analysis of SDS–PAGE

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zones stained with Coomassie Brilliant Blue. This assay may be calibrated with a standard solution of purified apo B-48, which is not commercially available. Several methods, such as high-performance liquid chromatography [6] and sequential immunoaffinity chromatography [7], have been proposed for the purification of apo B-48. We describe a new single-step method, which is based on a preparative gel electrophoresis with a continuous elution system for preparing such a standard solution.

2. Experimental

2.1. Materials

PhastGel precast polyacrylamide gradient gels, protein markers and nitrocellulose membranes were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Gelatine from porcine skin, bovine serum albumin and anti-rabbit IgG alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP–NBT) were purchased from Sigma (St Louis, MO, USA). Polyclonal antiserum to human-apolipoprotein B was from Dade-Behring (Marburg, Germany).

2.2. Collection of ascites

A chylomicron fraction was prepared from 1 l of chylous ascites collected by abdominal puncture in a 78-year-old patient. This patient had a chylous ascite because of lymphatic drainage obstruction by a retroperitoneal lymphoma. The chylous ascite collection was needed for diagnosis and treatment and sent to our laboratory for routine biochemical analysis. The remaining sample was used, without storage, for the present study.

2.3. Isolation of chylomicrons by ultracentrifugation

First, the chylous ascite was adjusted to a density of 1.10 with solid KBr, and then overlayed by 0.9% sodium chloride (2:1, v/v). After centrifugation for 45 min at 107 000 *g* at 10°C in a SW 41 Ti swinging bucket rotor (Beckman Coulter, Fullerton, CA, USA), the upper layer was carefully collected. Second, this fraction was centrifuged again under the same conditions in order to obtain a pure chylomicron sample, the purity of which was tested using a Lipofilm Kit (Sebia, Issy-les-Moulineaux, France). Lipofilm is a 3% (p/v) polyacrylamide gel stained with black Soudan and routinely used for lipoprotein identification. The chylomicron sample was concentrated to a final volume of 50 ml, and aliquotted. Total protein content of this chylomicron sample was 552 mg/l (Protein Assay, Bio-Rad, Hercules, CA, USA).

2.4. Delipidation and denaturation of proteins

An aliquot of isolated chylomicrons containing about 2 mg total proteins was incubated with 10 volumes of ice-cold ethanol–diethyl ether (3:1, v/v) for 16 h at −20°C. The protein precipitate was centrifuged at 3500 *g* for 20 min and the pellet was washed twice with 10 volumes of cold anhydrous diethyl ether [8]. The fat-free precipitate was then dissolved in sample buffer (50 m*M* Tris–HCl, pH 7.2, containing 3% v/v β-mercaptoethanol, 3% p/v SDS, 0.001% p/v bromophenol blue and 10% v/v glycerol) to a final concentration of 856 mg/l, and denatured for 3 min at 96°C.

2.5. Preparative electrophoresis with continuous elution

Apo B-48 purification was carried out by a one-step preparative electrophoresis using the Bio-Rad Model 491 Prep Cell with a continuous elution system. This device consisted of a glass tube (2.8 cm I.D.), containing the polyacrylamide gel, mounted on an elution chamber made up of a dialysis membrane (6 kDa MWCO) placed between two thin frits. Buffer was pumped through the elution chamber enabling the collection of migrated proteins from the bottom of the gel.

The denatured sample (1.28 mg) in 1.5 ml was loaded onto a 10.0×2.8 cm I.D. polyacrylamide gel column (acrylamide/Bis, 37.5:1, Bio-Rad) consisting of a 5% (p/v) resolving gel topped with a 1 cm high 4% staking gel. The migration was performed at a constant current of 30 mA for 10 h. Starting from the bromophenol blue dye front, 25 m*M* Tris–192 m*M* glycine buffer (pH 8.3) containing 0.1% SDS was

pumped through the elution chamber at a flow-rate of 0.4 ml/min and divided into 2 ml fractions. Protein elution was monitored by measuring the absorbance at 280 nm.

2.6. Analysis of fraction with SDS–PAGE/ Coomassie brilliant blue staining

Fractions corresponding to the peaks eluted in preparative electrophoresis were pooled and concentrated by ultrafiltration through a 10-kDa MWCO membrane, using a Centricon YM-10 (Millipore, Bedford, MA, USA) centrifuged at 5000 *g*. An aliquot of each fraction (10 μl) was diluted two-fold with a denaturing solution containing 62.5 m*M* Tris–HCl (pH 6.8), 2% SDS, 0.01% bromophenol blue, 10% glycerol and 5% β-mercaptoethanol and was heated at 100°C for 3 min before loading. The separation was carried out in a 4–15% precast SDS–polyacrylamide gel using an automated electrophoresis system (PhastSystem, Amersham Pharmacia Biotech). Separated proteins were identified by silver and Coomassie blue stainings of two different analytical series of SDS–PAGE. The peak of apo B-48 was observed in the fractions of preparative electrophoresis eluted at a retention time of 445–480 min.

2.7. Characterization of isolated apo B-48 by Western blot

Purified apo B-48 sample was characterized by Western blotting (see Fig. 2). The sample was run on 4–15% SDS–PAGE as described above. The proteins were transferred onto a nitrocellulose membrane (0.2 μm nitrocellulose) by blotting using the PhastTransfer unit of the PhastSystem (Amersham Pharmacia Biotech). Unoccupied protein-binding sites of the nitrocellulose sheet were blocked by incubating for 1 h with 3% (p/v) gelatine in 20 m*M* Tris–HCl (pH 7.4), containing 0.5 NaCl (TBS). The nitrocellulose was washed three times with 0.05% (v/v) Tween 20 in TBS and incubated for one night with a polyclonal antiserum to human apolipoprotein B, diluted 1:2000 with 0.05% Tween 20 in TBS. The nitrocellulose was then washed in 0.05% Tween 20 in TBS and incubated for 4 h with an anti-rabbit IgG alkaline phosphatase conjugate diluted 1:500 in 0.05% Tween 20 in TBS. Detection (5 min) was achieved using the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate–nitro blue.

2.8. SDS–PAGE calibration curve of apo B-48

After final concentration, the protein content of the apo B-48 fraction was determined with the protein assay method and bovine serum albumin was used as protein standard. The purity of apo B-48 was then examined using 4–15% SDS gradient gels and verifying that no bands other than apo B-48 were visible on Coomassie-stained gels (Fig. 1(top)). Dried gels were scanned with a laser densitometer (GS-690 Imaging Densitometer, Bio-Rad) equipped with a Multi-Analyst/PC software and computer. The chromogenicity (volume units per μg apoprotein) of apo B-48 was evaluated by loading 0.2–3.0 μg onto SDS gels (Fig. 1(bottom)).

3. Results and discussion

Preparative 5% gel electrophoresis gave a clear separation of apo B-48 from other proteins present in the chylomicron fraction. After Coomassie staining no bands other than apo B-48 were visible in the fractions collected at a retention time range of 445–480 min, as illustrated in Fig. 1. The homogeneity of the purified protein was also assessed by the presence of a single band in silver-stained SDS–PAGE gels. Characterization of the pooled protein by Western blotting (Fig. 2) showed a single band recognized by polyclonal antiserum to apo-B, with a M_r of 240 000, indicating the absence of other species of apo B than apo B-48. Several methods such as sequential immunoaffinity chromatography or HPLC have been proposed for the purification of apo B-48. The principal limitation of these methods is either the necessity for a monoclonal antibody or the small amount of protein that can be loaded on the column (<1 g). Here, we have evaluated a method easier to perform, which allows the purification of apo B-48 from a protein amount greater than 2 g. In addition, in our experience, the chromatographic purification of hydrophobic proteins, such as proteins involved in the storage or the transport of lipids, requires the use of detergents in the mobile phase, which produces protein aggregates [9]. Under these

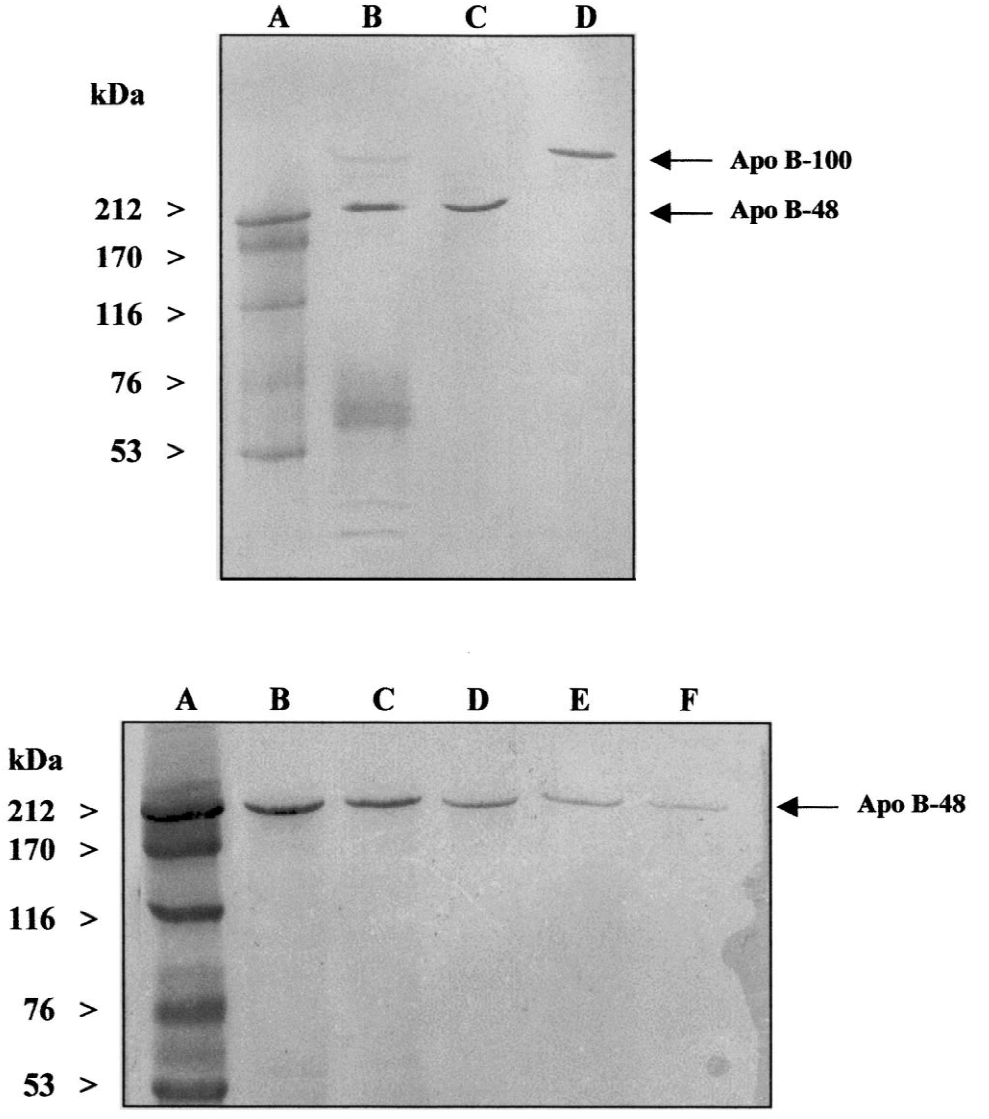

Fig. 1. Top. SDS–PAGE analysis of apo B-48 purified from chylomicrons isolated from chylous ascite. Electrophoresis was run in a 4–15% gradient SDS–PAGE, and protein bands were detected by Coomassie Brilliant Blue staining. Lane A, high-molecular-mass SDS standards; lane B, delipidated chylomicron sample, before preparative electrophoresis; lane C, apo B-48 purified from chylous ascite; lane D, human apo B-100 standard. Bottom. Coomassie-stained 4–15% gradient SDS–PAGE assay of standard solution of apo B-48. Lane A, high-molecular-mass SDS standards; lanes B–F, a reference apo B-48 standard curve (B) 3.00 μg, (C) 1.50 μg, (D) 0.75 μg, (E) 0.38 μg, (F) 0.19 μg.

conditions, the separation of structurally related hydrophobic proteins may be problematic, despite their different molecular size [9,10]. We have recently failed to purify isoforms of peanut oleosins, a lipid storage protein family, by HPLC, whereas the complete separation of isoforms, the molecular size of which differed by only 1500 Da, was achieved by preparative electrophoresis [9]. Finally, this electrophoretic method is inexpensive and produces repeatable results.

In slab gels stained with Coomassie Brilliant Blue, we found that the relation between the densitometric

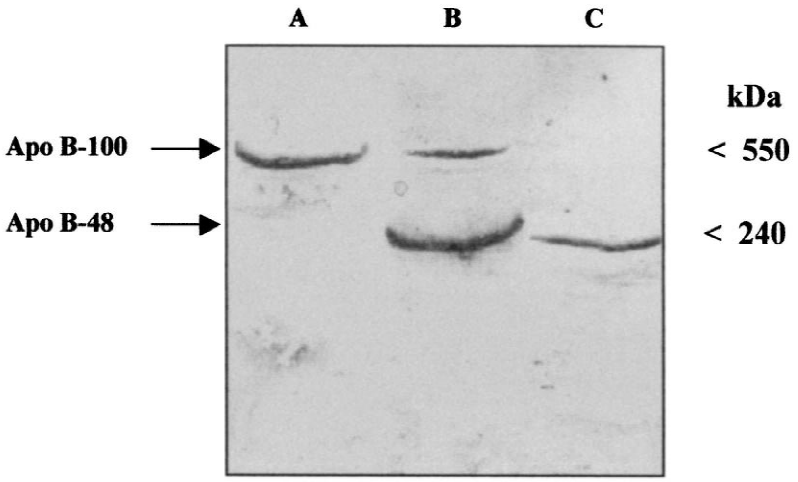

Fig. 2. Western-blot analysis of the pooled fractions corresponding to a retention time of 445–480 min. Samples were run on 4–15% gradient SDS–PAGE, and proteins were transferred onto nitrocellulose. The membrane was then incubated with a polyclonal antiserum to human-apolipoprotein B. Lane A, human standard apo B-100; lane B, chylomicrons isolated from chylous ascite, before preparative electrophoresis; lane C, pooled fractions of apo B-48 from preparative electrophoresis collected at a retention time range of 445–480 min.

analysis of dye uptake and the amount of purified apo B-48 was linear within the 0.2–3.0 μg range (slope 0.145 ± 0.005, $n = 3$, $R^2 = 0.99$). Purified apo B-48 therefore allowed us to obtain a standard linear curve that covered a wide protein range and that could be used for the quantitation of apo B-48 in an automated electrophoresis system. This is advantageous because it is often necessary, especially in postprandial studies, to apply large amounts of chylomicron apo B-48 to SDS–PAGE [5]. These results agree with that of Zilversmit and Shea [11], who found a linear relation between dye uptake and protein amount of rat apo B-48 and human and rat apo B-100. On the other hand, Kotite et al. have found that the relation between dye uptake and the mass of apo B-100 and apo B-48 deviated slightly from linearity and followed a power function over a range of 0–5 μg of apoprotein [7].

The quantitation of apo-proteins by SDS–PAGE requires the preparation of reliable standard solutions of the corresponding purified apo-proteins: quantitation of apo B-48 has been performed with either apo B-100 or with apo B-48 standard solutions [7,11]. In contrast to human apo B-100, purified human apo B-48 is not commercially available. In our opinion, the interest in using apo B-48 rather than apo B-100 is that it serves not only as a calibrator, but also as a control for identifying the protein band which has to be analyzed. In addition, the dye uptake of the standard and assayed proteins is theoretically identical since both proteins have exactly the same amino acid composition.

In conclusion, we have described a new original technique to purify apo B-48 from an apolipoprotein mixture of chylomicrons with good purity and sufficient amount to be used as a standard solution.

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