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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR ANTIBODIES, GLYCOSIDASES AND MEMBRANE PROTEINS

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SUMMARY

The broad range of applications of high-performance liquid chromatography (HPLC) in biochemistry and cell biology is demonstrated by the purification of antibodies, separation of glycosidases and isolation of a liver membrane protein with a molecular weight of 65 000–67 000 daltons. The advantage of HPLC over classical chromatographic methods is shown by the purification of the glycosidases from *Streptococcus pneumoniae*. These enzymes can be purified to a degree similar to what can be achieved by "classical" ion exchange, combined with affinity chromatography, but the time needed for the HPLC experiment is much shorter and the yield at least three to five times higher. Particular attention is directed to sample preparation before HPLC separation. For the best results, a combination of HPLC with other biochemical and immunochemical methods is necessary, as is also demonstrated.

INTRODUCTION

Significant progress has recently been made in the high-performance liquid chromatography (HPLC) of macromolecules, such as polysaccharides, nucleic acids and proteins. Even the separation of hydrophobic proteins has been carried out by means of HPLC. The newly developed stationary phases for the separation of macromolecules by size-exclusion, ion-exchange, reversed-phase and hydrophobic interaction HPLC have provided solutions to many separation problems that can arise in the laboratory[1–3]. However, as a rule, it is impossible to apply crude samples to a column. In the present paper we describe an approach to this problem, in which extensive use of highly specific HPLC techniques has been combined with more classical methods of protein separation during sample preparation. The goal has been the

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design of protocols capable of achieving maximum resolution, recovery and, if possible, retention of biological activity of macromolecules.

MATERIALS AND METHODS

Antibodies

Rabbit antiserum against human transferrin or mouse ascites fluid, containing monoclonal anti-transferrin receptor antibody OKT 9, was used for antibody purification. The antigen-binding fragment (Fab) from monoclonal anti-transferrin receptor antibody OKT 9 was used for size-exclusion HPLC purification. In order to remove albumin, rabbit serum was first purified by chromatography on an Affi-Gel Blue column (Bio-Rad, Richmond, VA, U.S.A.).

Glycosidases

A glycosidase mixture from *Streptococcus pneumoniae* was a gift from Dr. G. Ashwell, National Institutes of Health, Bethesda, MD, U.S.A. It contained neuraminidase, endo-β-galactosidase, β-galactosidase, *endo*-α-N-acetylglucosaminidase, β-N-acetylglucosaminidase and *endo*-β-N-acetylglucosaminidase. No protease activity could be detected. Only neuraminidase, β-galactosidase and β-N-acetylglucosaminidase were assayed, as described by Glasgow *et al.*[4].

Membrane proteins

Membrane protein samples were obtained from plasma membranes of rat liver or Morris hepatoma 9121 or 7777. The plasma membranes were isolated and selectively extracted as described[5,6].

High-performance liquid chromatography

The HPLC system consisted of two pumps, a gradient mixer, a controller, filter photometer, spectrophotometer and a RH 7125 loop injection valve (all from LKB, Bromma, Sweden). For size-exclusion HPLC the TSK-3000 and TSK-4000 SW columns (600 × 7.5 mm, 10 μm; LKB) or a Superose 12 column (300 × 8.0 mm; Pharmacia, Uppsala, Sweden) were used. A Mono Q column (50 × 5.0 mm, 10 μm; Pharmacia) or a TSK DEAE-5 PW column (75 × 7.5 mm, 10 μm; LKB) were used for ion-exchange HPLC. Hydrophobic interaction HPLC was carried out with a Spherogel-TSK phenyl PW column (75 × 7.5 mm, 10 μm; Altex-Beckmann Instruments, Palo Alto, CA, U.S.A.) or a SynChropak propyl column (75 × 4.0 mm, 10 μm; Bischoff Analysentechnik, Leonberg, F.R.G.). All chemicals used in the mobile phase were HPLC grade. The solutions and gradients used are stated in the figure legends. Fractions were collected with a Super-Rac fraction collector (LKB).

RESULTS AND DISCUSSION

Purification of antibodies

Fig. 1 shows the purification of polyclonal anti-transferrin antibodies from rabbit serum. As in Fig. 1b, the serum albumin can be almost completely removed by preliminary purification on an Affi-Gel Blue column. This considerably increased the column capacity for subsequent HPLC separations. The subsequent purity con-

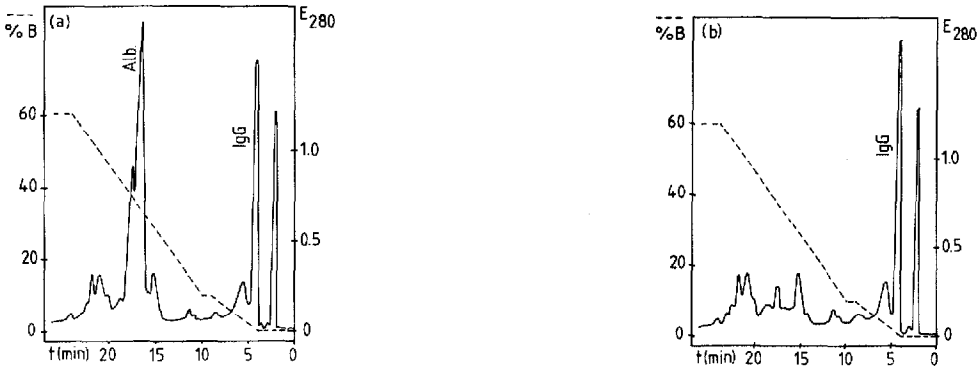

Fig. 1. Ion-exchange HPLC of rabbit anti-transferrin antiserum before purification on an Affi-Gel Blue column (a) and after removal of albumin (A1b.) on an Affi-Gel Blue column (b). Conditions: column, Mono Q, 50 × 5 mm (Pharmacia); buffer A, 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 6.05); buffer B, 1 *M* sodium chloride in buffer A; the gradient is as indicated; flow-rate, 1 ml/min; pressure, 20 bar; room temperature.

trol by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) showed that the immunoglobulin-fraction was homogeneous. Similar results were obtained for the purification of other monoclonal antibodies from mouse ascites fluid (not shown). The possibly differing retention times for the immunoglobulin subclasses have to be taken into account (see also ref. 7).

For the separation of the Fab fragments from the remaining uncleaved immunoglobulin, the application of size-exclusion HPLC is recommended. The fragment with M_r of about 50 000 daltons could be fully separated from the immunoglobulin, as shown in Fig. 2. The TSK-3000 and the Superose 12 columns yielded similar results.

Purification of glycosidases

Fig. 3 shows the separation of glycosidases from *Streptococcus pneumoniae*. Ion-exchange (Fig. 3a) or hydrophobic interaction HPLC (Fig. 3b) was actually the

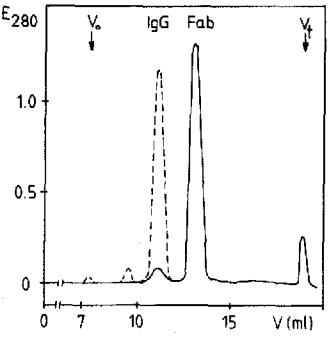

Fig. 2. Separation of Fab fragment from immunoglobulin (IgG) by size-exclusion HPLC on a TSK-3000SW column (600 × 7.5 mm). – – –, IgG (purified monoclonal antibody OKT 9); ———, Fab fragment after purification on protein A-Sepharose (remaining IgG appears in the first peak). Conditions: buffer, Tris buffered saline (TBS); flow-rate, 0.5 ml/min; pressure, 10 bar; room temperature.

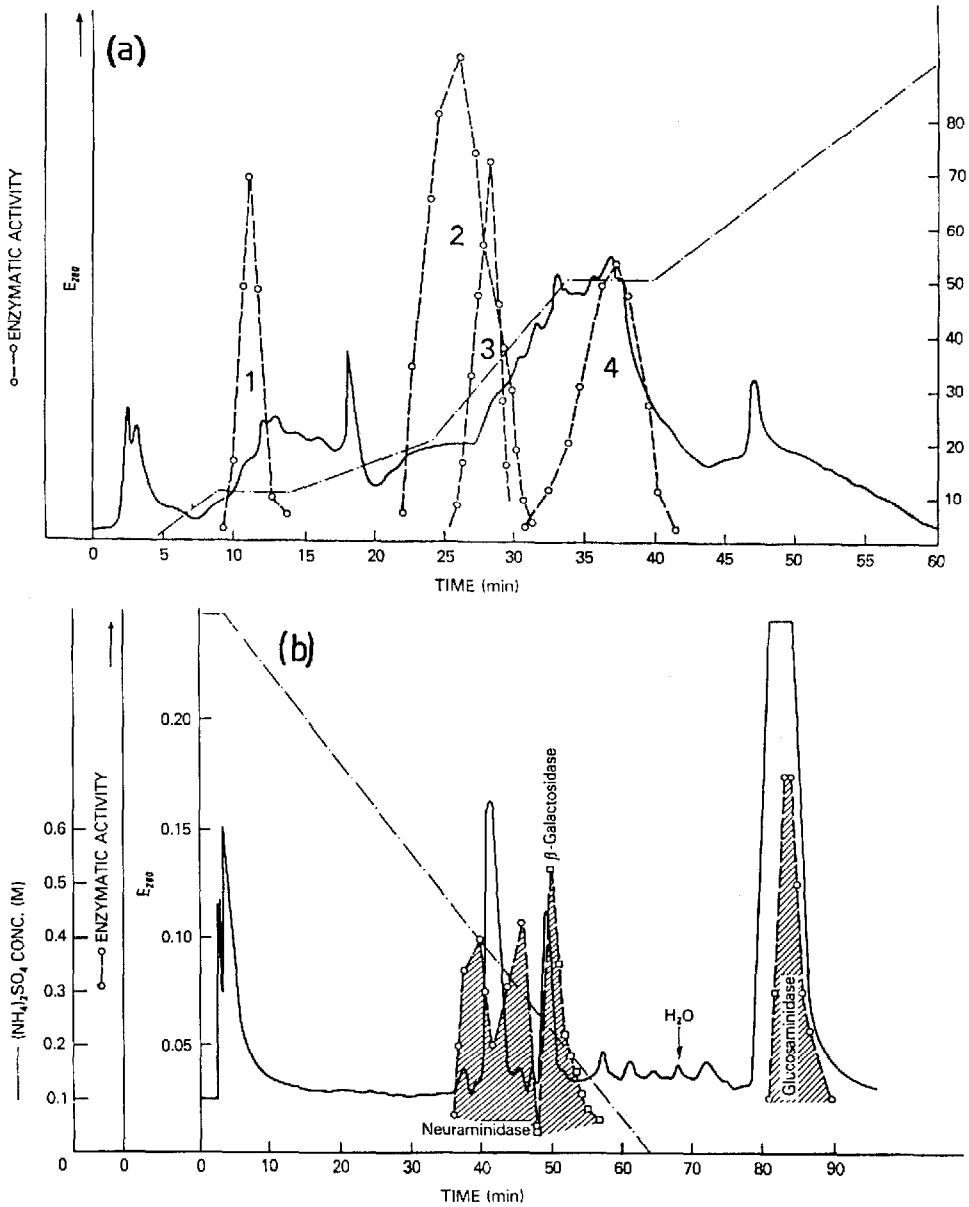

Fig. 3. Separation of glycosidases from *Steptococcus pneumoniae*. (a) Ion-exchange HPLC on a TSK DEAE-5 PW column (75 × 7.5 mm): 1 ml of a glycosidases mixture was applied after purification by ammonium sulphate precipitation, and the enzymatic activity was determined. Peaks: 1 and 2 = neuraminidase; 3 = β-galactosidase; 4 = β-N-acetylglucosaminidase. Conditions: buffer A, 10 m*M* HEPES (pH 6.05); buffer B, 1 *M* sodium chloride in A; flow-rate, 1 ml/min; pressure, 16 bar; room temperature. (b) Same mixture as in (a), applied to an hydrophobic interaction HPLC column, TSK phenyl PW (75 × 7.5 mm). Activity applied: neuraminidase, 2.1 units; β-galactosidase, 2.14 units; β-N-acetylglucosaminidase, 8.4 units. The activity recovered was between 70% (β-N-acetylglucosaminidase) and 92% (β-galactosidase). Conditions: buffer A, 1 *M* ammonium sulphate in 0.1 *M* potassium phosphate (pH 7.0); buffer B, 0.1 *M* potassium phosphate (pH 7.0), gradient as indicated; after 68 min (arrow) the column was washed with water for 20 min; flow-rate, 1 ml/min; pressure, 14 bar; room temperature.

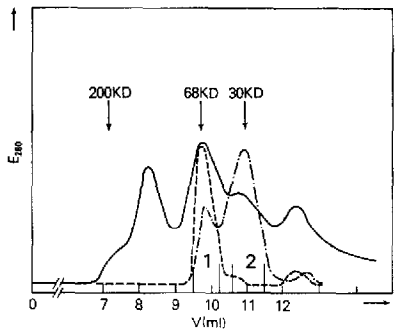

Fig. 4. Size-exclusion HPLC of CHAPS/EDTA extract from plasma membranes of liver. ———, First separation. The fractions were collected and rechromatographed; the resulting separations are – – – (fraction 1) and — · — (fraction 2). The protein with the apparent M_r of 65 000–67 000 daltons could be isolated in fraction 1 after rechromatography. Fraction 2 contains some of this protein and contaminating proteins with an apparent molecular weight around 30 000 daltons. Conditions: column, Superose 12 (300 × 8.0 mm); buffer, TBS with 0.1% (w/v) SDS and 0.5% (v/v) mercaptoethanol: flow-rate, 0.5 ml/min; pressure, 8 bar; room temperature.

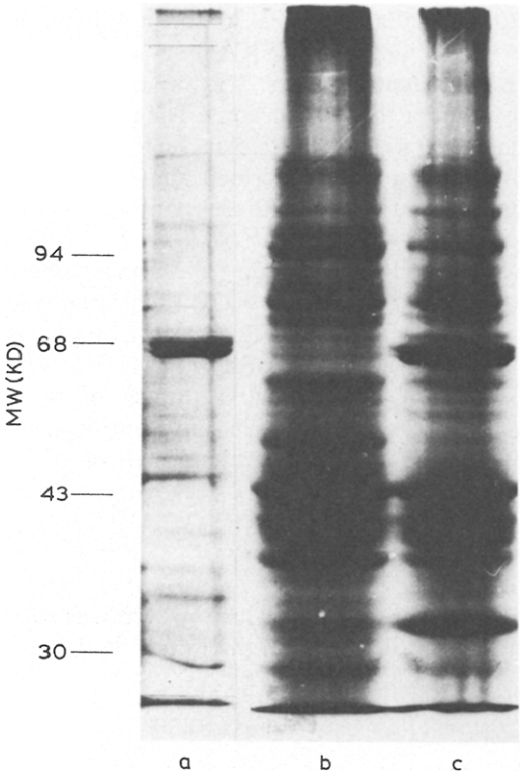

Fig. 5. SDS–PAGE of different membrane extracts. (a) Extract with 1% CHAPS and 20 mM EDTA. The membranes were first extracted with sodium hydroxide at pH 11 and subsequently with 1% Nonidet P-40 in TBS. (b) The membranes were first extracted with only sodium hydroxide at pH 11, washed and subsequently extracted for 6 h with 1% CHAPS in TBS without EDTA. (c) The membrane extraction was carried out as in (b) but 20 mM EDTA was added to the extraction buffer. By this means the additional extraction of polypeptides with apparent M of 67 000, 65 000 and about 30 000 daltons was achieved.

last step in the isolation process. After removing the bacterial biomass, the supernatant was purified by precipitating it twice with ammonium sulphate (for the complete purification protocol see ref. 4). Then size-exclusion HPLC on a TSK-4000 column was performed. All enzymes investigated had M_r between 100 000 and 300 000 daltons. This step results in a good preliminary purification, but is not absolutely necessary. As Fig. 3b shows, a much better separation can be achieved by hydrophobic interaction HPLC. Neuraminidase shows heterogeneity in both hydrophobic interaction and ion-exchange HPLC separations, as evidence by the two peaks with neuraminidase activity (see Fig. 3).

Purification of membrane proteins

Fig. 4 shows the size-exclusion HPLC purification of a membrane protein with a M_r of 65 000–67 000 daltons in SDS–PAGE. This protein was first purified by stepwise extraction of plasma membranes from rat liver. A fraction which contained more than 60% of these polypeptides was obtained by extraction with the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS), combined with ethylenediaminotetraacetic acid[6].

As Fig. 4 shows, these polypeptides could be fully separated from the other polypeptides in this fraction with M_r of 30 000–45 000 daltons. As the mobile phase contains 0.1% SDS and 0.1% mercaptoethanol, the use of TSK columns is not recommended, because they are sensitive toward reducing agents. The Superose 12 column proved to be more chemically resistant under these conditions. The importance of the method of sample preparation is demonstrated, in particular, by the isolation of this protein. Fig. 5a shows the last extraction step, as described above, after the removal of the extrinsic membrane proteins (by extraction with the non-ionic detergent Nonidet P-40)[6].

Fig. 5b and c show an extract of unpurified liver plasma membranes, where CHAPS was used. For the preparation in Fig. 5b the calcium was not chelated (the solubilization buffer did not contain EDTA), and for that in Fig. 5c the calcium was chelated by adding EDTA. As Fig. 5c shows, the polypeptide with M_r 65 000–67 000 daltons could be extracted by CHAPS and EDTA. However, a number of other membrane proteins now appear as well, in the absence of preliminary purification. These are then very difficult to remove by HPLC. This problem has been solved by selective preliminary purification, as demonstrated in Fig. 5a.

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