Hydrophobic Chromatography of Hepatitis B Surface Antigen on 1,9-Diaminononane or 1,10-Diaminodecane Linked to Agarose

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SUMMARY

A series of ω-aminoalkyl-agaroses differing in the number of carbon atoms in the alkyl chains was prepared and used for chromatography of hepatitis B surface antigen (HBsAg). HBsAg was separated from the major part of serum proteins by adsorption to columns of 1,9-diaminononane or 1,10-diaminodecane linked to agarose beads (Sepharose 4B or 2B) followed by elution with 4 M-NaSCN.

Immunosorbents represent the most specific tools for the isolation and purification of antigens. However, they may be less suitable for the large scale purification of antigens if sufficient quantities of monospecific antibodies are not available. Consequently, chromatographic techniques based on less specific interactions than those involved in immunological recognition may be advantageous for the separation of virus antigens from contaminating proteins. The reaction between HBsAg and the lectin concanavalin A has been utilized for the partial purification of HBsAg from human serum (Neurath, Prince & Lippin, 1973; Neurath et al. 1974). Further purification of HBsAg was achieved with molecular exclusion chromatography and high speed centrifuging techniques. In a search for alternative techniques more amenable to an unlimited increase in scale, we turned our attention to hydrophobic chromatography—a method for the separation of proteins differing in the frequency and size of exposed hydrophobic regions, on columns of alkyl- or ω-aminoalkyl-agaroses (Shaltiel & Er-El, 1973). The results presented here demonstrate that hydrophobic chromatography combined with the use of chaotropic agents (Hatefi & Hanstein, 1969) as eluants represents an effective step for the purification of HBsAg.

ω-Aminoalkyl-Sepharose 4B was prepared by mixing 40 ml of 0.25 M solutions of ω,ω-diaminoalkanes (NH₂(CH₂)n-NH₂), dissolved in N,N-dimethylformamide – 0.1 M-NaHCO₃ (1:1) and adjusted to pH 11.0 by addition of concentrated HCl, with 3 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at 23 °C overnight. The aminoalkylated derivatives were washed on a filter with 50 ml of distilled water followed by three alternate washes each with 0.1 M-borate–1 M-NaCl, pH 8.0, and 0.1 M-acetate–1 M-NaCl, pH 4.0. Part of the 1,10-diaminodecane usually precipitated during the reaction with CNBr-activated Sepharose. In that case, N₂,N-dimethylformamide–0.1 M-NaHCO₃ was used for the first wash to dissolve the precipitated diamine.

The separation of the spherical HBsAg particles from the filamentous forms and from the Dane particles by precipitation with polyethylene glycol was carried out as described earlier (Neurath et al. 1973). HBsAg was determined using the radioimmunoassay developed by Ling & Overby (1972). The amount of protein in the chromatography fractions was determined spectrophotometrically at 280 nm. Thiocyanate was determined in the form of its complex with Fe⁺⁺. One ml of 400-fold diluted samples was mixed with 1 ml of acetone and with 1 ml of a solution containing 1 mg of Fe(NO₃)₃ in 1 ml of 0.5 N-HCl. Optical density
Fig. 1. Distribution of protein (shaded columns) and HBsAg (black columns) in fractions after stepwise elution of ω-aminoalkyl-agarose columns (0.9 x 3.5 cm) to which partially purified HBsAg (1 ml) has been applied. The number of carbon atoms in the ω-aminoalkyl groups linked to Sepharose 4B is indicated in insets. Solutions (6 ml each) containing increasing quantities of NaCl in TB were used for elution; the 1 M-NaCl was in 0.1 M-phosphate, pH 10.85.

was measured at 480 nm. The number of aminoalkyl groups linked to agarose was determined spectrophotometrically with picrylsulphonic acid (Falilla & Santi, 1973).

In preliminary experiments, HBsAg partially purified by chromatography on concanavalin A-Sepharose (Neurath et al. 1973) and suspended in 0.01 m-tris, pH 7.2 (TB) was applied to columns of various ω-aminoalkyl-agaroses and eluted with solutions containing increasing quantities of NaCl in TB. These agarose derivatives differed in the number of carbon atoms (C) in their hydrocarbon side chains but contained approx. the same number of ligands bound per ml of agarose gel (range 7.5 to 10.3 μmol). The results in Fig. 1 show that the elution of both serum proteins and HBsAg was shifted to higher concentrations of NaCl with increasing carbon chain length. Whereas the recovery of protein was about 100% for C = 5 to 8 and 90% for C = 9 or 10, the elution of HBsAg decreased drastically with increasing C and dropped to 9 and 3% for C = 9 and C = 10, respectively. Attempts to elute HBsAg with buffers of various pH (2.2 and 10.85 respectively) or solutions containing organic solvents (1 to 25% (v/v) ethanol, 10 to 40% (v/v) dimethylformamide), detergents (0.1% sodium deoxycholate, 0.1% Tween 80) or urea (1 to 7 M), were all unsuccessful.
Finally, we used solutions of chaotropic salts, known to impart lipophilic properties to water (Hatefi & Hanstein, 1969). Complete elution of HBsAg was achieved with 4 M-NaI or NaSCN. In subsequent experiments 4 M-NaSCN was used for elution of HBsAg to avoid potential difficulties due to the presence of free iodine in solutions of NaI.

HBsAg was applied to 0.9 x 10.5 cm columns of ω-aminodecyl-Sepharose 4B. The columns were washed with 30 ml of 0.8 M-NaCl followed by 4 M-NaSCN. Fractions of 2.5 ml each were collected and analysed for protein, HBsAg and NaSCN. The following preparations of HBsAg were chromatographed (the capacity of the column corresponding to the maximum volume of each preparation which can be applied to the column, expressed as the percentage of the column volume, and the degree of purification achieved are given in that order in parentheses): partially purified HBsAg eluted from concanavalin A-Sepharose (70%, 13-fold = 130-fold, compared with original serum); serum containing HBsAg from which Dane particles and filaments had been removed by precipitation with polyethylene glycol (25%, 18- to 66-fold, Fig. 2) and HBsAg-positive serum (10%, 20-fold). These results were obtained with an adsorbent containing 10.9 μmol/ml of aminoalkyl groups. Similar results were obtained with ω-aminononyl-Sepharose which contained 6.1 μmol/ml of ligand and had a proportionately lower capacity to bind HBsAg.

Attempts to replace CNBr-activated Sepharose obtained from Pharmacia with derivatives prepared in our laboratory by activating Sepharose 4B with CNBr at pH 11.0 were unsuccessful. The resulting ω-aminoalkyl-agarose compounds adsorbed both HBsAg and a portion of serum proteins irreversibly. The insolubility of these derivatives in 50% (v/v) acetic acid

![Fig. 2. Separation of HBsAg from the major portion of serum proteins by chromatography on ω-aminodecyl-Sepharose. O---O, extinction (E_{280}); •---•, HBsAg. The arrow indicates start of elution with 4 M-NaSCN. The E_{280} values plotted on the ordinate represent experimentally determined values from which the E_{280} corresponding to the concentration of NaSCN in each fraction was subtracted.](image)
Short communications

at 75 °C - conditions appropriate for melting of agarose for the determination of bound ligands (Failla & Santi, 1973) - suggested a higher degree of cross-linking compared with that of substituted agaroses prepared from commercial CNBr-activated Sepharose which dissolved under these conditions. About 35% of HBsAg was recovered after chromatography on a column of ω-aminodecyl-agarose obtained from Miles-Yeda, Rehovot, Israel. This compound was partly soluble in 50% acetic acid at 75 °C. Activated agarose having the proper characteristic was prepared by mixing appropriate volumes of a 50% (v/v) slurry of Sepharose 2B in 0.1 M-NaHPO₄ and of a freshly prepared solution of CNBr in acetonitrile (300 mg/ml). The pH was maintained at 9.8 to 10.5 for 10 min by addition of 2 N-NaOH. The gel was washed with 0.1 M-NaHCO₃ and added to the solution of the appropriate diamine.

The HBsAg particles eluted from the aminoalkyl-agarose columns had a buoyant density of 1.21 g/cm³ in CsCl and appeared intact under the electron microscope. The partially purified antigen was concentrated by ultrafiltration without prior removal of NaSCN, and the purification was completed by density gradient sedimentation (Gerin, Holland & Purcell, 1971).

Chromatography on aminoalkyl-agaroses containing 4 to 6 carbon atoms in the alkyl chain was successfully used for the purification of some enzymes (Shaltiel & Er-El, 1973; Henderson, Shaltiel & Snell, 1974). The results presented here suggest that chromatography on derivatives having a higher number of carbon atoms in the alkyl chain combined with the use of chaotropic ions as eluants may become a useful tool for the purification and separation of hydrophobic structural proteins of virus envelopes and cell membranes.

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