JOURNAL OF CLINICAL MICROBIOLOGY, June 1976, p. 626-631

Vol. 3, No. 6
Printed in U.S.A.

Large-Scale Purification of Hepatitis B Surface Antigen

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Received for publication 10 March 1976

Hepatitis B surface antigen was concentrated and purified from plasma by two simple steps of purification. In the first step the antigen was purified 24-fold by polyethylene glycol precipitation. An additional 10-fold purification was achieved by batchwise adsorption to hydroxylapatite and subsequent elution with 0.02 M sodium phosphate buffer.

As it appears probable that inactivated preparations of hepatitis B surface antigen (HBsAg) particles may be useful in active immunization of humans against hepatitis B virus infections, a need exists for methods of purifying these particles suitable for use on a large scale. A variety of methods for purification of HBsAg has been reported. These include methods based on pelleting and treatment with enzymes (11) or low pH (4), methods based on zonal ultracentrifugation (1, 6), immunoadsorption (7, 8) and concanavalin A affinity chromatography (12), adsorption to and elution from Aerosil (5), and others.

We report herein a modification of the polyethylene glycol (PEG) method originally reported by DeRizzo et al. (3) which, when followed by a batchwise adsorption with hydroxylapatite, results in a 250-fold purification of HBsAg with a 40% recovery. These procedures permit final steps of purification involving density gradient centrifugation to be carried out with 1% of the original sample volume.

MATERIALS AND METHODS

Source of HBsAg. Whole plasma units containing HBsAg of both adw and ayw subtypes were obtained from apparently healthy blood donors. The plasma was stored frozen at −70 C until used for purification.

Fractionation by PEG 6000. The following modification of the method described by DeRizzo et al. (3) was used. (i) The pH of the plasma was adjusted to 4.6 at room temperature by addition of concentrated HCl and the precipitate formed was removed by centrifugation at 1,500 × *g* for 20 min. (ii) To the supernatant a 30% (wt/vol) solution of PEG 6000 in distilled water was added dropwise until a 2% concentration was obtained, and the resulting suspension was left overnight in the refrigerator. (iii) After centrifugation at 1,500 × *g* for 20 min, the precipitate from step (ii) was discarded, the PEG concentration of the supernatant fluid was adjusted to within the range of 4.0 to 4.5% (wt/vol) and it was stored overnight in the refrigerator. (iv) The precipitate from step (iii) was collected by centrifugation, and the pellet was resuspended in distilled water to approximately the original volume of the plasma. (v) The bulk of the PEG and a small amount of protein were removed from the suspension from step (iv) by centrifugation after adjusting the pH with 1 N NaOH to 5.0. (vi) The clear supernatant fluid from step (v) was readjusted to pH 4.6, PEG was added to a final concentration of 4.0 to 4.5% (wt/vol), and the material was again stored overnight in the refrigerator and centrifuged. (vii) The precipitate from step (vi), containing the antigen, was resuspended in distilled water to approximately one-half of the original volume of plasma.

Batchwise treatment with hydroxylapatite. The resuspended PEG precipitate of HBsAg was adjusted to pH 6.8 by adding 10 ml of 0.5 M sodium phosphate buffer. The sample was stirred with 150 ml of packed hydroxylapatite (Bio-Rad Laboratories) for 2 h at room temperature. The slurry was equally divided into two 200-ml centrifuge tubes and centrifuged for 5 min at 1,500 × *g*, and the supernatant fluid was recovered. The sediment was washed twice by stirring for 30 min with 100 ml of 0.02 M sodium phosphate buffer (pH 6.8) and centrifuged. The supernatant and the washings were pooled and concentrated by ultrafiltration in an Amicon concentrator using a PM-30 membrane, washed with 0.02 M sodium phosphate buffer (containing 0.02% NaN_3), and adjusted to a final volume of 12 ml with the same buffer.

The washed sediment of hydroxylapatite was eluted twice with 100 ml of 0.1 M sodium phosphate buffer, pH 6.8, and twice with 100 ml of 0.2 M buffer by stirring for 30 min each at room temperature. The eluates were pooled and concentrated by ultrafiltration. The sample was washed twice with 0.02 M sodium phosphate buffer containing 0.02% NaN_3 and adjusted to final volume of 12 ml with the same buffer.

Isopycnic banding. The concentrated sample after hydroxylapatite treatment was applied onto a continuous CsCl gradient (in 0.02 M sodium phosphate buffer containing 0.02% NaN_3) of density ranging between 1.05 and 1.35 g/ml and centrifuged for 24 h at 35,000 rpm in a Spinco SW65 rotor. Fractions of 10 drops were collected from the bottom of the tubes. The samples were analyzed for proteins

and HBsAg after dilution to 0.5 ml with 0.02 M sodium phosphate buffer (containing 0.02% NaN_3).

Rate zonal centrifugation. After isopycnic centrifugation, the dialyzed and concentrated sample of HBsAg was applied onto a linear 10 to 25% sucrose gradient buffered to pH 6.8 with 0.02 M sodium phosphate buffer and centrifuged for 16 h at 23,000 rpm in a Spinco SW25.1 rotor. Fractions of 0.5 ml were collected and analyzed for proteins and HBsAg.

Serological techniques. The antigenic activity at individual steps of purification of HBsAg was monitored by counterelectrophoresis (13) and solid-phase radioimmunoassay (Ausria-125, Abbott Laboratories). The counterelectrophoresis titer of an HBsAg preparation corresponded to the reciprocal of its highest dilution at which the antigen was still detectable. All materials tested by radioimmunoassay were diluted at least 1:10 in normal serum (free of HBsAg detectable by radioimmunoassay and anti-HBs detectable by passive hemagglutination [14]).

Protein determination. The protein contents were monitored by measuring the optical density at 280 nm. The protein concentration was calculated arbitrarily using $E^{0.1\%}_{1\,cm} = 1.42$ for partially purified samples and $E^{0.1\%}_{1\,cm} = 3.73$ for final purification products of HBsAg (15). The protein concentration in the original plasma and in the PEG precipitate of HBsAg was determined by the micro-Kjeldahl technique (10).

Cellulose acetate electrophoresis. Samples after each step of purification were analyzed by cellulose acetate electrophoresis under the conditions described for the Beckman microzone apparatus. The strips were stained with Ponceau red and scanned in a Beckman densitometer.

Immunoelectrophoresis. Each preparation of purified HBsAg was examined for the presence of normal serum proteins by immunoelectrophoresis in the Gelman-LKB equipment. The antisera used were prepared by immunization of sheep with HBsAg-free human plasma.

Electron microscopy. Specimens were deposited on carbon-coated grids and stained with 2% phosphotungstate (pH 6.8 to 7.0). Pictures were taken in a JEM-100B electron microscope at a ×40,000 magnification.

RESULTS

Purification of HBsAg by PEG 6000 precipitation. Fractionation of HBsAg by PEG precipitation can be followed in Fig. 1, which represents the results of analyses by cellulose acetate electrophoresis at individual steps of purification. Line 1 shows a typical plasma protein distribution pattern of the original HBsAg-containing plasma. Line 2 represents the supernatant of plasma after adjustment to pH 4.6. It shows losses in the fibrinogen and in the $\alpha 1$ protein region. Line 3 illustrates that a further lowering of contaminants (especially fibrinogen) was achieved by precipitation at 2% PEG. Line 4 shows that a large amount of proteins were removed in the first 4.0 to 4.5% PEG supernatant. From line 5 it is apparent that the resuspended HBsAg precipitate still contained a high amount of protein contaminants. Line 6 shows that the protein distribution pattern was not changed substantially after precipitating the bulk of PEG at a pH of 5.0. The second precipitation (line 7), at 4.0 to 4.5% PEG con-

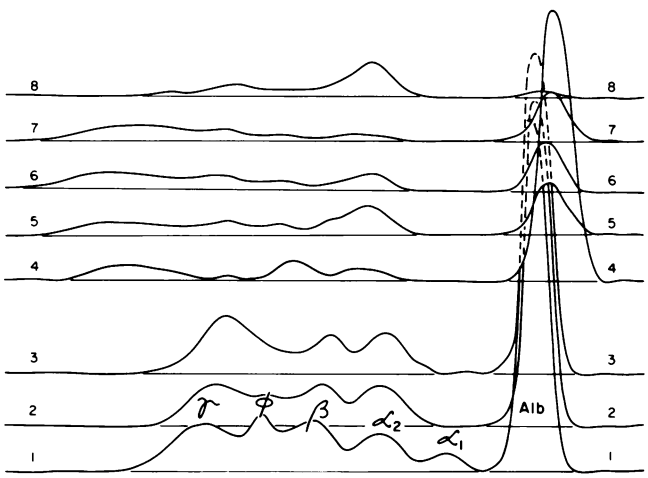

FIG. 1. *Cellulose acetate electrophoresis of samples from PEG fractionation of HBsAg. (1) Original HBsAg-containing plasma; (2) supernatant of plasma after adjustment to pH 4.6; (3) supernatant after 2% PEG precipitation; (4) supernatant after 4.0 to 4.5% PEG precipitation of HBsAg; (5) resuspended first 4.0 to 4.5% PEG precipitate of HBsAg; (6) pH 5.0 supernatant of HBsAg from step vi; (7) supernatant from the second 4.0 to 4.5% PEG precipitation of HBsAg; (8) resuspended second 4.0 to 4.5% PEG precipitate of HBsAg.*

centration, removed substantial amounts of contaminants in the supernatant. Line 8 shows that the final precipitate of HBsAg (at twofold concentration, relative to the original volume of plasma) contained a major peak only in the $\alpha 2$ region and has two minor peaks in the albumin and fibrinogen regions.

The results of purification by PEG precipitation are summarized in Table 1. It is evident that in the prepurification steps (steps 2 and 3) 16% of contaminating proteins were removed without loss of HBsAg. The first 4.0 to 4.5% precipitation (step 6) gave fourfold purification, and the second 4.0 to 4.5% PEG precipitation (step 8) yielded a total of 24-fold purification of the HBsAg.

Purification of the PEG precipitate of HBsAg on hydroxylapatite. The resuspended PEG precipitate of HBsAg was further purified by batchwise adsorption to hydroxylapatite and subsequent elution with sodium phosphate buffers at pH 6.8. From the sample adsorbed to hydroxylapatite, about 50% of antigenic activity, with less than 5% proteins, was recovered in the 0.02 M buffer eluate. This represents a total of approximately 250-fold purification of HBsAg (Table 2). The residual HBsAg was eluted at 0.1 to 0.2 M buffer concentrations. Since this material showed substantial plasma protein contamination it was not further purified. However, a sample was pelleted and examined electron microscopically. This revealed large filaments and Dane particles as well as the spherical forms of HBsAg.

Density gradient centrifugation of HBsAg eluted from hydroxylapatite. The 0.02 M eluate of HBsAg concentrated to a 12.0-ml volume by ultrafiltration (Table 2, eluate A) was further purified by isopycnic banding in a CsCl gradient. The results presented in Fig. 2 show only a minor peak of high-density protein contaminants (peak A) distinctly separated from

TABLE 1. *Purification of HBsAg with PEG 6000*

Step	Material	Vol (ml)	Protein[a] (g)	% Original protein	1/HBsAg Titer (CEP)[b]
i	Original plasma	240	14.2	100	256
ii	Plasma, pH 4.6	240	13.2	93	256
iii	2% PEG supernatant	250	12.0	84	256
iv	1st 4.0 to 4.5% PEG supernatant	270	8.2	58	0
v	1st 4.0 to 4.5% PEG precipitate	200	3.6	25	256
vi	1st 4.0 to 4.5% PEG precipitate pH 5.0 supernatant	200	3.4	24	256
vii	2nd 4.0 to 4.5% PEG supernatant	230	2.4	18	2
viii	2nd 4.0 to 4.5% PEG precipitate	100	0.5	3.6	512

[a] Kjeldahl method.
[b] CEP, Counterelectrophoresis.

TABLE 2. *Results of purification*

Sample	Vol (ml)	1/HBsAg titer (CEP)[a]	Total protein (mg)	Sp act[b]	Fold purification[c]	% Yield of HBsAg[d]
Original plasma	240.0	256	14,200.0[e]	4.3		100.0
Resuspended 2nd PEG precipitate	100.0	512	500.0[e]	102.4	23.7	83.3
0.02 M PO_4^- OHap. eluate (A)	12.0	2,048	22.8[f]	1,077.0	249.5	40.0
0.1 + 0.2 M PO_4^- OHap. eluate (B)	12.0	2,048	240.0[g]	102.4	23.7	40.0
Isopycnic banding of (A)	4.0	6,400	16.3[f]	1,570.5	363.5	41.7
Rate zonal centrifugation						
Pool I	3.0	640	1.06[f]	1,811.3	419.3	3.1
Pool II	2.5	1,280	1.93[f]	1,658.0	383.8	5.2
Pool III	3.0	6,400	6.84[f]	2,807.0	649.8	31.2
Pool IV	3.0	800	0.45[f]	5,333.3	1,234.5	3.9
Pools I–IV	11.5	2,323	10.28[f]	2,599.2	601.6	43.5

[a] CPE, Counterelectrophoresis.
[b] ([1/HBsAg] × volume)/total protein (milligrams).
[c] (Specific activity − sample)/(specific activity − plasma).
[d] ([1/HBsAg] − sample × volume)/ ([1/HBsAg] − plasma × volume) × 100.
[e] Kjeldahl method.
[f] Optical density at 280 nm, $E^{0.1}_{1cm}$ = 3.73.
[g] Optical density at 280 nm, $E^{0.1}_{1cm}$ = 1.42.

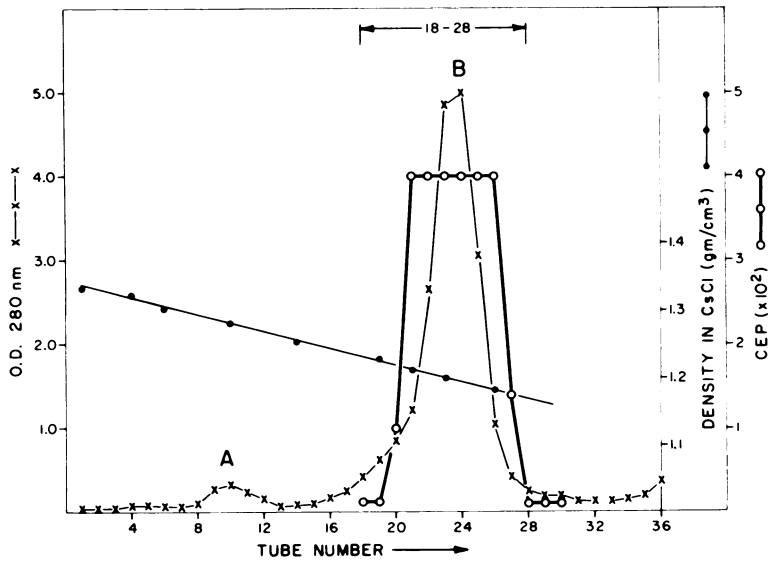

FIG. 2. *Isopycnic banding of HBsAg eluted from hydroxylapatite with a 0.02 M phosphate buffer, run on a linear CsCl gradient for 24 h at 35,000 rpm in a Spinco SW65 rotor.*

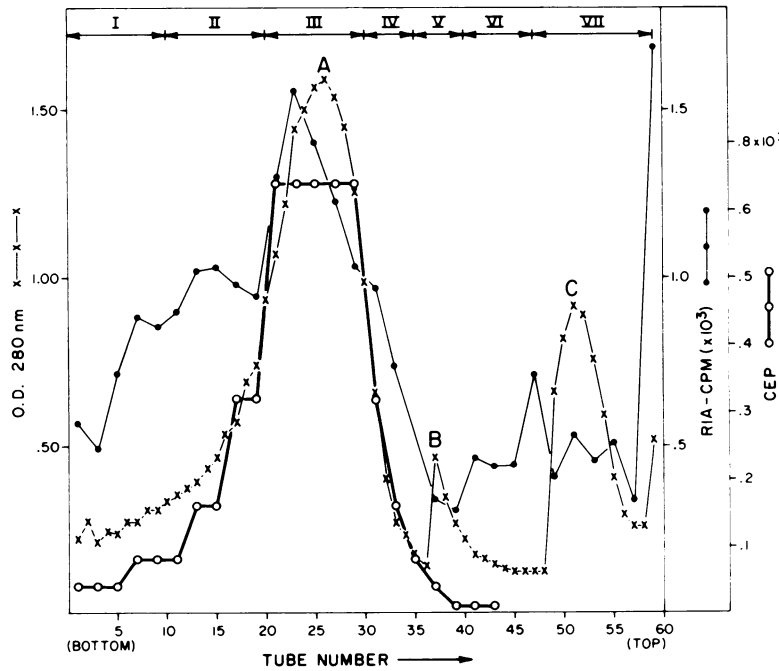

FIG. 3. *Rate zonal centrifugation of HBsAg from CsCl gradient, run on a linear sucrose gradient for 16 h at 23,000 rpm in a Spinco SW25.1 rotor.*

the major HBsAg-containing peak (peak B). The results summarized in Table 2 show more than 360-fold purification of HBsAg.

The pooled and concentrated eluate of HBsAg from the CsCl gradient was submitted to rate zonal centrifugation in a linear sucrose gradient. The results illustrated in Fig. 3 show only two minor peaks of low-molecular-weight protein contaminants (peaks B and C). Fractions from the peak region of antigenic activity (Fig.

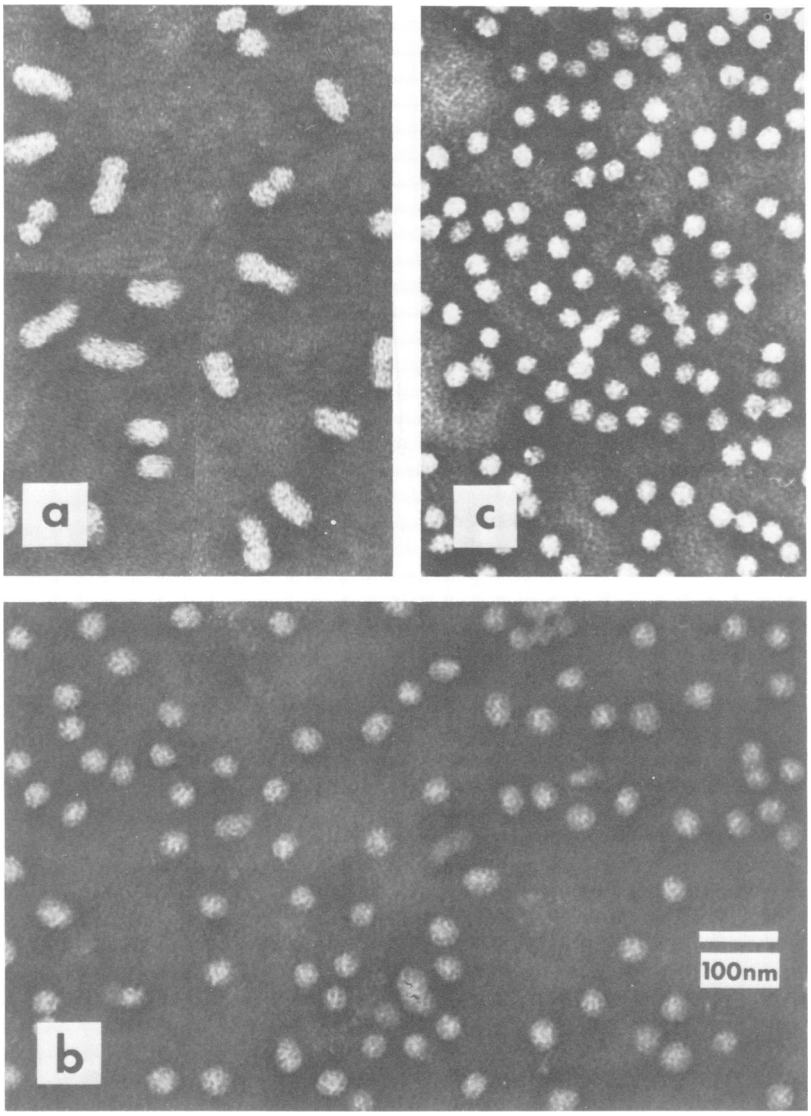

FIG. 4. *Electron microscopy of HBsAg separated by rate zonal centrifugation. Pictures were taken after phosphotungstate staining, at a ×40,000 magnification (60 KV). (a) Fraction I, a composite of two fields is shown; (b) fraction II; (c) fraction III.*

3, peak A) were distributed into four pools which were dialyzed and concentrated by ultrafiltration. Analyses for protein content and antigenic activity (Table 2) show an average of 600-fold purification of HBsAg. Immunoelectrophoresis of samples revealed no detectable contaminants with sheep antiserum against normal human plasma. Electron microscopy showed short filaments and spherical particles of 22 to 27 nm in diameter in fraction I (Fig. 4a); fraction II was composed mostly of 22- to 27-nm spherical particles (Fig. 4b), fraction III contained spherical particles of 20 to 22 nm in diameter (Fig. 4c), and fraction IV consisted mostly of small 16- to 22-nm spherical particles (not shown).

DISCUSSION

DeRizzo et al. reported eightfold purification of HBsAg from plasma by using PEG precipitation (3). However, using the same procedure, only twofold purification had been achieved in our laboratory. Later modification of the DeRizzo procedure resulted in a relatively low degree of purification or low yields of recovery of HBsAg (2, 9). In a further modification of the

DeRizzo method, we removed 96% of plasma proteins by PEG precipitation of HBsAg. The factors that contributed to the improved results were: (i) utilizing two prepurification steps to remove interfering plasma protein contaminants; (ii) using concentrated solutions instead of solid PEG to better control purification conditions; and (iii) lowering the temperature at each purification step to lower the PEG concentration required for selective and quantitative precipitation of HBsAg.

The resuspended PEG precipitate of HBsAg was further purified on hydroxylapatite. Since column chromatography on hydroxylapatite gave very slow flow rates, a batchwise procedure was used. The plasma protein contaminants were almost quantitatively removed by adsorption to hydroxylapatite and subsequent elution of approximately 50% of HbsAg with a 0.02 M phosphate buffer of pH 6.8. The residual impurities were removed in the final steps of purification by isopycnic banding and rate zonal centrifugation. Studies by electron microscopy revealed populations of HBsAg particles composed mostly of 20- to 28-nm spherical particles and a few short filaments.

If the methods described were to be applied to vaccine preparation, it would be necessary to replace sodium azide with a nontoxic preservative such as merthiolate.

ACKNOWLEDGMENTS

We wish to thank Mary Chen for her excellent electron microscopy work and M. Stryker for help with protein analyses.

This investigation was supported by Public Health Service research contract 70-2236 from the National Heart and Lung Institute.

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