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The Isolation and Purification of Pre-S2 Containing Hepatitis B Virus Surface Antigen by Chemical Affinity Chromatography

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ABSTRACT

A simple, rapid, and efficient method was developed to isolate and purify pre-S2 containing HBsAgs from the plasma of a single chronic carrier of HBsAg (adw) by ammonium sulfate fractionation, hydroxyapatite column chromatography, and polymerized human serum albumin-affinity column chromatography. About 500 μg of pre-S2 containing HBsAg was obtained from 140 mL of plasma containing 4,200 μg of HBsAg. Two purified pre-S2 containing HBsAgs were analyzed by SDS-polyacrylamide gel electrophoresis and their molecular weights were determined to be 31,000 and 68,000 respectively. No significant amount of HBsAg or its derivative was detected in the final product.

Index Entries: Pre-S2; HBsAg; pHSA affinity chromatography.

INTRODUCTION

Chinese people are suffering the highest incidence of hepatocellular carcinoma among the world's population. Hepatitis B virus (HBV) causes the acute and chronic hepatitis, and it is most likely an important agent to cause hepatocellular carcinoma (*11*). HBV envelope contains hepatitis B virus surface antigen (HBsAg) carried by the major polypeptides with a mol wt 26,000, its glycosylated derivative with a mol wt of 30,000 and two

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minor polypeptides with a mol wt of 31,000 and 68,000, respectively. The minor polypeptides are carrying the receptor activity for polymerized human serum albumin (pHSA) (*7,12–15*).

Present investigation is to develop a fast, simple, and efficient method for the isolation and purification of p31 and p68 of HBsAgs that could be produced by recombinant DNA techniques in many laboratories. The pHSA-Sepharose 4B affinity column chromatography technique was employed as a major and important step for the purification of pre-S2 containing HBsAg.

MATERIALS AND METHODS

Human serum albumin and glutaraldehyde (99% purity) were purchased from Sigma Chemical Co. Sepharose 4B was the product of Pharmacia Co. Hydroxyapatite was purchased from Bio-Rad Laboratories.

Preparation of Polymerized Human Serum Albumin (pHSA)

The preparation of pHSA was carried out according to the method of Lenkei et al. (*1*). Human serum albumin (200 mg) was dissolved in 9 mL of .1*M* phosphate buffer pH 6.8, and 1 mL of 2.5% glutaraldehyde was added. The reaction mixture was kept at room temperature for 2 h and then at 4°C for 12 h, and the reaction was terminated by dialyzing against .01*M* phosphate buffered saline (PBS) pH 6.8 at 4°C for 36 h. The dialyzate was further purified by gel filtration using a Sepharose 4B column (1.6× 90 cm) to remove monomeric and dimeric albumins. The flow rate was 15 mL/h, and each fraction of 4 mL was collected. The fractions containing the polymerized albumin were pooled, concentrated and dialyzed against PBS, ph 7.0 at 4°C for 36 h. This preparation was designated as pHSA.

Preparation of pHSA-Sepharose 4B (pHSA-S-4B) Affinity Column

Sepharose 4B gel was first activated with cyanogen bromide (CNBr) according to the method described by Dean et al. (*2*). 70 gm gel was treated with 1.6 g of CNBr at pH 11.0 for 20 min. The CNBr activated gel was washed with .1*M* sodium bicarbonate buffer pH 9.0 and then it was coupled with pHSA in .05*M* borate buffer pH 10.5 at 4°C for 12 h. In order to react all the activated Sepharose hydroxy groups, the unreacted groups were blocked by reacting the gel with .1*M* glycine in .05*M* borate buffer, pH 10.5 for 6 h at 4°C.

Determination of HBsAg

HBsAg was quantitatively determined by using commercial radioimmunoassay (RIA) kits from Abbott Laboratories. It was carried out by using a standard from NIH in a parallel line assay (*3*).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS—PAGE)

SDS-PAGE of slab gel was carried out according to the method of Laemmli (4). Purified HBsAg was treated with 1% SDS, 5% 2-mercapto-ethanol, and 8*M* urea in .0625*M* Tris-chloride buffer, pH 7.2 for 30 min at 100°C to dissociate HBsAg polypeptides before electrophoresis. After electrophoresis, the gel was stanied for protein by staining with .05% coomassie brilliant blue or .012*M* silver nitrate.

For quantitation of pre-S2 containing HBsAgs, the gel that was stained for protein analysis was scanned at a wavelength of 550 nm with an Auto Ciba Corning 780 Fluorometer/Densitometer (Ciba Corning Diagnostics Co. Medfield, MA).

Purification of pre-S2 Containing HBsAg

All pre-S2 containing HBsAgs purified in the present investigation were obtained from a single chronic carrier of HBsAg (adw).

The plasma was treated with 60°C for 10 h and centrifuged at 10,000 xg for 30 min to remove the insoluble particles present in the plasma. The clear supernatant was subjected to ammonium sulfate fractionation; HBsAgs were precipitated between 15 and 45% saturation of ammonium sulfate. The precipitates were collected by centrifugation at 10,000 xg for 20 min. The precipitates were dissolved in a minimum volume of water, and then dialyzed against .01*M* potassium phosphate buffer, pH 6.8. The dialyzate was applied to a hydroxyapatite column (2.2×40 cm) that was previously equilibrated with .01*M* potassium phosphate buffer pH 6.8. The column was first eluted with .1*M* equilibrium buffer, and then with .5*M* the same buffer. The flow rate was 30 mL/h, and each fraction of 4 mL was collected. Pre-S2 containing HBsAg and HBsAg was detected in the first peak.

The fractions containing HBsAg were pooled and concentrated by ammonium sulfate as described above. The dialyzate was then fractionated by affinity chromatography using a pHSA-Sepharose 4B affinity column. The column (.9×35 cm) was pre-equilibrated with .01*M* PBS, pH 6.8. After the application of the dialyzate to the column, HBsAg without pre-S2 as well as other contaminating proteins were eluted with equilibrium buffer and then pre-S2 containing HBsAg was eluted with .01N HCl. The flow rate was 12 mL/h, and each fraction of 2 mL was collected. The fractions containing pre-S2 containing HBsAgs were neutralized immediately with .01N NaOH and subjected to quantitative analysis with RIA kits and qualtitative analysis by SDS-PAGE.

Western Blot Analysis

SDS-PAGE gel was subjected to Western blot analysis according to the method of Towbin et al. (*15*).

RESULTS

Preparation of pHSA

pHSA prepared by the present method was found by gel filtration to contain 1% of monomeric and 2% dimeric forms, respectively. The fractions of polymers were pooled for the purpose of purification of pre-S2-HBsAg.

Purification of pre-S2 Containing HBsAg

After ammonium sulfate fractionation, the fractions containing HBsAg and pre-S2 containing HBsAg were applied to the hydroxyapatite column. As shown in Fig. 1, the surface antigens of HBV were detected in the first peak were eluted with .1*M* phosphate buffer, pH 6.8, whereas the second peak eluted by .5*M* phosphate buffer was found to be contaminating proteins. The fractions containing the surface antigens of HBV were pooled and concentrated by precipitating with ammonium sulfate. After dialysis, the dialyzate was subjected to further purification by affinity chromatography with a pHSA-Sepharose 4B column, and the results were summarized in Fig. 2. The first peak was eluted with .01*M* phosphate buffered saline, pH 6.8, and was found to be contaminating protein. The second peak was eluted with .01N HCl, and was found to contain two pre-S2 containing HBsAgs, as shown by SDS polyacryamide gel electrophoresis. The acidity of the second peak was neutralized with .1N NaOH right after elution from the column. The yield of pre-S2 containing HBsAg of a typical experiment is about 500 μg from 140 mL plasma of starting material which contained about 4,200 μg surface antigens.

The results of one of the typical experiments used to isolate and purify pre-S2 containing HBsAg are summarized in Table 1. An overall yield of 8.8% of total HBsAg activity is obtained, which was based on commercial RIA kits from Abbott Laboratories. Approximately 500 μg of pre-S2 containing HBsAg is obtained from 140 mL of the high titer plasma.

Table 1
Purification of pre-S Containing HBsAg

Step	Volume	Titer	Total activity	A_{280}	Total A_{280}	Yield %
$HBeAg^+$ plasma	140 ml	30.0 μg/mL	4200 μg	60.4	8456	100
60°C, 10 h	185 ml	12.0 μg/mL	2220 μg	9.3	1721	52
Ammonium sulfate	43 ml	48.8 μg/mL	2098 μg	25.4	1092	50
Hydroxyapatite	250 ml	8.1 μg/mL	2525 μg	1.7	425	48
pHSA-Sepharose 4B	10 ml	50.0 μg/mL	500 μg	.06	.6	8.8

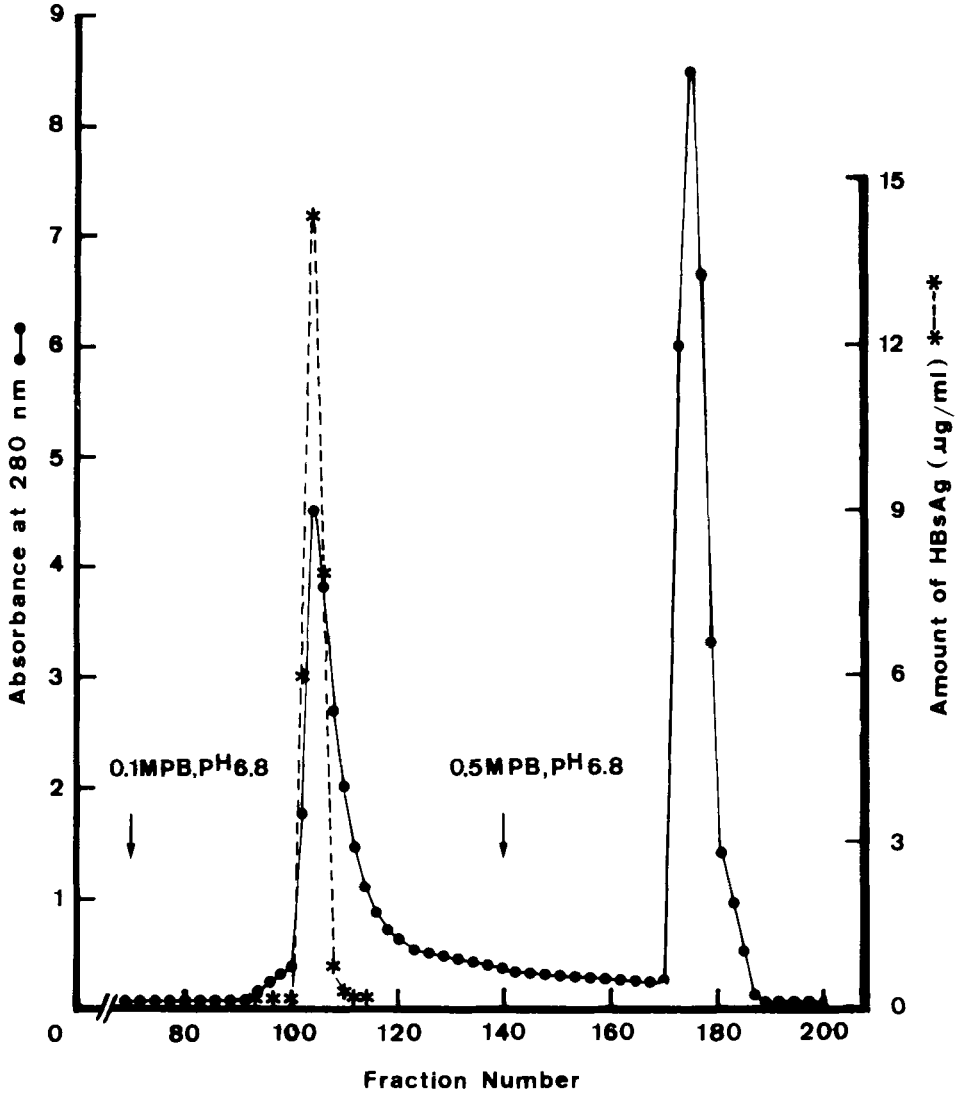

Fig. 1. Fractionation of pre-S2 containing HBsAg and HBsAg by column chromatography with a hydroxyapatite column (2.2×40 cm). The column was first eluted with .1*M* phosphate buffer pH 6.8, and then with .5*M* phosphate buffer pH 6.8. Pre-S2 containing HBsAg and HBsAg was found in the first peak. The flow rate was 30 mL/h, and each fraction of 4 mL was collected.

SDS Polyacrylamide Gel Electrophoresis

The pre-S2 containing HBsAgs isolated and purified by pHSA-Sepharose 4B column chromatography was subjected to SDS-polyacryamide gel electrophoresis analysis. Two pre-S2 containing HBsAgs were detected by silver stain as well as Western blot methods, and the results were sum-

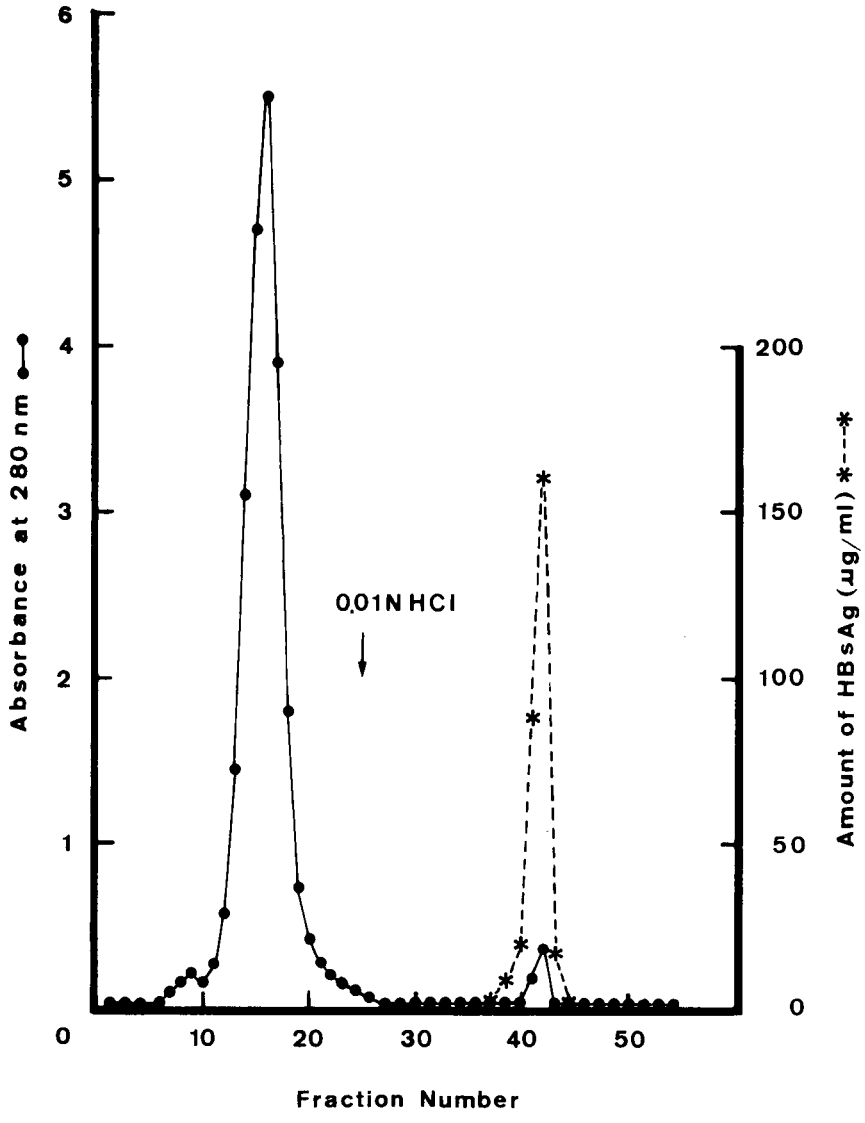

Fig. 2. Purification of pre-S2 containing HBsAg by glutaraldehyde polymerized human serum albumin affinity column chromatography. The column (.9×35 cm) was first eluted with .01*M* phosphate buffer, pH 6.8, and then pre-S2 containing HBsAg was eluted with .01*M* HCl. The flow rate was 12 mL/h and each fraction of 2 mL was collected.

marized in Fig. 3. The apparent mol wt of pre-S2 containing HBsAgs were measured to be 31,000 and 68,000, respectively.

The purity of final purified pre-S2 containing HBsAg was examined quantitatively with a densitometer. On the basis of pre-S2 containing HBsAg polypeptides, the purity was estimated to be 92%.

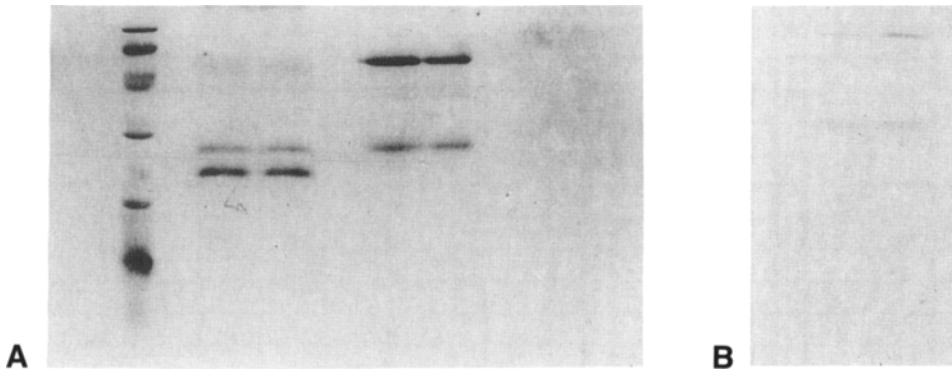

Fig. 3. (A) SDS-polyacrylamide gel electrophoresis of pre-S2 containing HBsAg-detected by Silver staining method. Lane 1: Standard protein: phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, alpha-lactalbumin. Lanes 2 and 3: CsCl density gradient centrifugation purified HBsAg was treated with 5% MSH and 8*M* urea. Lanes 4 and 5: Purified pre-S2-HBsAg was treated with 5% MSH and 8*M* urea. (B) SDS-polyacrylamide gel electrophoresis of pre-S2 containing HBsAg detected by Western blotting method.

DISCUSSION

The purification of HBsAg was reported previously by several other research units, and the methods they employed are similar but not identical (*7,10,16*). The common principle they used for purification of HBsAg was to apply a series of rate zonal centrifugation, first by sucrose density gradient centrifugation, followed by CsCl density gradient centrifugation. However, by those methods, pre-S containing HBsAgs are purified together with HBsAgs without pre-S peptide.

The results of present investigation demonstrated that pHSA bound pre-S2 containing HBsAgs specifically, and no significant amount of HBsAg (MW 26,000) or its glycosylated derivate (MS 30,000) was absorbed by pHSA. These results are in an agreement with those of Yu et al. and Machida et al. (*6,7*). They showed that HBsAg particles were also bound by pHSA, probably because of the presence of pre-S HBsAg in the particles.

Since pHSA binds to pre-S2 containing HBsAg but it does not bind to HBsAg without pre-S2, it strongly suggests that the pre-S2 region has the binding site for pHSA. Neurath et al. reported that the 55 amino acid residues of pre-S2 region contained the epitopes for immunoglobulin of hepatitis B virus and that the synthetic peptide of the first 26 amino acid terminal residues was shown to induce antibodies (*8*). The pre-S2 struc-

ture was suggested to be involved in the attachment of HBV to liver cells. Therefore, to induce the antibodies against the infection by HBV, the pre-S2 containing HBsAg could be a much better antigen than HBsAg for preparing the vaccine against hepatitis B virus infection.

The results of the present investigation can be applied for the recovery of pre-S2 containing HBsAg produced be recombinent DNA techniques such as the expression of pre-S2 containing HBsAg in *Escherichia coli* (*9*) or Chinese hamster ovary cells (*10*). pHSA-Sepharose 4B affinity chromatography has two advantages: It has the specific affinity toward pre-S2 containing HBsAg, and it has another advantage over immunoadsorbant affinity chromatography, because pHSA is the product made from normal human serum, whereas for the immunoadsorbant affinity technique, the materials are obtained from monoclonal antibody of hybridoma of polyclonal antibody of immuned human being, which suffer either from safety or economic problems.

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