# Sodium dodecylsulfate polyacrylamide gel electrophoresis of recombinant hepatitis B surface antigen particles

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## Abstract

To investigate the factors leading to broadening of the recombinant hepatitis B surface antigen (HBsAg) peak in size-exclusion chromatography, the HBsAg particles eluting in different regions of the peak were subjected here to electrophoretic analysis. In nonreduced samples, the 24-kD band corresponding to the $S$ monomer was detected when excessively large amounts of HBsAg were loaded onto the gel. Hence, some monomers are not disulfide-crosslinked in assembled particles. On the other hand, the results of alkylation experiments indicated the presence of free sulfhydryl group(s) in a little portion of freshly-purified HBsAg which was retarded on the size-exclusion chromatographic column and had significant antigenicity. This fraction of HBsAg was shown to be oligomeric and capable of spontaneous assembly into higher-order structures during aging. © 1998 Elsevier Science B.V.

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## 1. Introduction

Hepatitis B surface antigen (HBsAg) is considered as a potential carrier for foreign antigenic determinants to enhance their immunogenicity [1]. In order to rationally design such gene vectors and vaccines, a detailed understanding of the mechanism of HBsAg assembly is essential. This subject is complex because information about the chemical structure of HBsAg at a molecular level can only be provided by physicochemical studies of HBsAg crystals that are not yet in sight. Nonetheless, with this reservation in mind, some features of particle assembly have been elucidated from the intensive experimental work done in the field of physicochemical characterization [2–7], kinetic studies of in vitro expression [8,9], mutational [10–14] and pulse-

chase [15] analysis of HBsAg. A short review on this theme is presented in [1].

It is highly likely that the molecular events resulting in particle formation may be responsible for some peculiar physicochemical properties of HBsAg, such as its variability in size [2–5] and lipid content [3,6,7], as well peak broadening in size-exclusion high-performance liquid chromatography (SEC-HPLC) [5,16]. In our recent work [5], we found that antigen fractions which were widely separated by SEC-HPLC showed differences not only in particle size distribution but also in antigenicity expressed by the $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio. It is known that antigenicity of HBsAg strongly depends on the integrity of disulfide bonds within the particle [2,17]. HBsAg contains 14 cysteine residues in each copy of the $S$ monomer, all of which have been shown to be

involved in extensive intra- and intermolecular disulfide crosslinking [18]. Other reports, however, contradict this [13,14]. Since breaking of disulfide bonds can increase the side chain volume of a protein [19], the supposition has been made that variability in the extent of disulfide crosslinking between particles might be responsible for the variable size and antigenicity of the SEC-separated HBsAg fractions. In the present paper, this hypothesis is further supported by the data from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of HBsAg.

## 2. Experimental

### 2.1. Materials

Tris(hydroxymethyl)aminomethane, dithiotreitol, sodium dodecyl sulfate and mercaptoethanol were obtained from Merck (Darmstadt, Germany). The remaining electrophoresis reagents were purchased from Pharmacia (Uppsala, Sweden). All solutions were made in Milli-Q grade water. Recombinant HBsAg (>95% HPLC, SDS–PAGE) was obtained from yeast fermentation after a multistep purification process [20]. It was purchased as a 1.5 mg ml$^{-1}$ solution in phosphate-buffered saline (PBS) (1.7 m$M$ KH$_2$PO$_4$, 7.9 m$M$ Na$_2$HPO$_4$, 2.7 m$M$ KCI and 250 m$M$ NaCl, pH 7.0) from the National Center for Bioproducts (Havana, Cuba).

### 2.2. SEC

The HPLC system included a Pharmacia LKB 2248 pump, Knauer degasser, Pharmacia 2141 variable-wavelength UV detector operated at 280 nm and Pharmacia 2221 programmable integrator. The columns were from Tosohaas (Stuttgart, Germany). For fractionation, about 100 mg of HBsAg were loaded onto a TSK G5000 PW (20 $\mu$m, 600×55 mm I.D.) supplied with a TSK GPW guard column (75×55 mm I.D.). Elution was achieved with PBS at 7 ml min$^{-1}$. Fractions of 7 ml were collected and subjected to SEC analysis by injecting 100 $\mu$l onto a TSK G5000 PW (17 $\mu$m, 600×7.5 mm I.D.) supplied with a TSK GPW guard column (75×7.5 mm I.D.). Elution was with PBS at 0.5 ml min$^{-1}$. The

fractions eluting in the same volume were pooled, rechromatographed and subjected to electrophoresis under nonreducing and reducing conditions.

### 2.3. SDS–PAGE

Electrophoresis was run in 12.5% gels according to Laemmli [21] followed by Coomasie staining (Bio-Rad, Richmond, CA). Prior to analysis, the fractions containing HBsAg at concentrations less than 1 mg ml$^{-1}$, as assayed by Lowry method [22], were concentrated in Centricon-3 tubes (Amicon Inc, Beverly, USA). Aliquots of concentrated samples (75 $\mu$l) were incubated in a boiling water bath for 10 min with 25 $\mu$l of sample buffer (0.3 $M$ Tris–HCI, 10% (w/v) SDS and 50% (w/v) glycerol). Aliquots of 50 $\mu$l were applied onto the gel.

Fully-reduced samples were prepared by diluting the bulk HBsAg and fraction 3 with an equal volume of sample buffer (0.06 $M$ Tris–HCI, 2% (w/v) SDS, 5% (v/v) mercaptoethanol and 10% (w/v) glycerol) and then boiling for 10 min at 100°C. Subsequently, carboxamidomethylated HBsAg was prepared by adding the same volume of freshly-prepared iodoacetamide (400 m$M$ in water) to the reduced samples. The mixture was allowed to stand for 30 min at room temperature in the dark.

### 2.4. Western blot

After electrophoresis, the gel was incubated with monoclonal antibodies raised against HBsAg polypeptide and developed with protein A conjugated to aminobenzidine [23].

## 3. Results and discussion

It is well-known that recombinant HBsAg particles vary in size [2–5]. Their mean diameter differs from one report to another depending, probably, on the purification protocol used [2]. In our recent work [5], we investigated if this phenomenon has any relation with broadening of the HBsAg peak in SEC. For this purpose, HBsAg was fractionated on a preparative SEC column and the fractions were subjected to analysis by transmission electron microscopy and SEC. As a result, the 16–32 nm particles were found

in the overall region of the peak. The fractions which were widely-separated by SEC differed both in size distribution and retention time. With increasing fraction number, the HBsAg peak shifted toward higher retention times corresponding to the preferential elution of smaller particles. However, slight changes in size distribution could not alone account for the great differences in elution time between the fractions. Enzyme-linked immunosorbent assay (ELISA) of these samples showed differences in the $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio. Since HBsAg used for fractionation was free from foreign proteins ($>$95% HPLC, SDS–PAGE), total protein content of each fraction determined by the Lowry method corresponds to the total amount of HBsAg monomers whereas ELISA measures the amount of HBsAg monomers in the assembled form only. Hence, the $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio represents the efficiency of the HBsAg-monomer assembly which, as shown in [5], varies from one fraction to another.

In the present work, SEC-separated fractions were analyzed by SDS–PAGE. Unfortunately, SDS–PAGE does not allow us to judge about physical homogeneity and state of aggregation of HBsAg: the particles are disrupted to monomers and dimers by boiling with SDS and 2-mercaptoethanol, whereas under nonreducing conditions, they are retained on top of the stacking gel (Fig. 1B). The analysis of entire HBsAg particles by SEC-HPLC [5] is somewhat questionable because of unexplainable broadening of the HBsAg peak. No superior assay specific for nonreduced HBsAg has been developed that is effective in determining the grade of oligomerization of HBsAg. In the present work, HBsAg has been tested simultaneously by SDS–PAGE and SEC-HPLC. The results obtained here are internally consistent with the recent studies [13,14,24] which provide critical insight for the trend in HBsAg-particle assembly.

Under nonreducing conditions, a weak 24 kD band was appreciated when excessively large amounts of HBsAg (40 $\mu$g) were loaded on the gel (Fig. 1). This band was recognizable in Western blot (data not shown) proving its correspondence to the *S* monomer. It was present in HBsAg samples from fractions 2 and 3, but not in that from fraction 1, suggesting physical heterogeneity between HBsAg particles eluting in different regions of the peak. In HBsAg

from fraction 1, the protein bands were mainly retained at the site of application and a little fraction migrated up to top of the stacking gel. The elution volume of fraction 1 is close to the exclusion volume of TSK G5000 PW column suggesting particle aggregation [5]. Since no free monomers were detected by elution of fractions 2 and 3 from the TSK G3000 PW column (data not shown), the monomers detected by SDS–PAGE under nonreducing conditions should be bound to the assembled HBsAg. Since this binding is disrupted by SDS treatment, it should have noncovalent character. The fact that purified HBsAg contains trace amounts of *S* monomers which do not participate in disulfide crosslinking, is in agreement with the mechanism proposed for HBsAg assembly in yeast [25]: (1) HBsAg is formed presumably as large particles in which monomeric subunits are lipid-associated and held together by noncovalent interactions and (2) disulfide-linked dimers and higher order oligomers are formed in vitro during purification.

Another result of the present study comprised the finding that a nonreduced monomer from fraction 3 migrated with a slightly higher mobility compared to that from fraction 2 (Fig. 1). To reproduce this result, fractionation of HBsAg was repeated using different lots of freshly-purified material. It is interesting that in the chromatogram of freshly-obtained HBsAg (Fig. 2A), there was a little peak at 46.4 min, eluting after the main HBsAg peak (29.9 min), which showed significant antigenicity in ELISA ($[HBsAg]_{ELISA}/[HBsAg]_{Lowry}=1$) and had electrophoretic behavior similar to fraction 3. As has been shown for fraction 3 (Fig. 1), a nonreduced sample from the 46.4-min peak was presumably retained at the top of the stacking gel suggesting high disulfide crosslinking. However, a tiny amount of the *S* monomer entered the gel and migrated with unusually higher mobility compared to that of the corresponding band from the bulk HBsAg (Fig. 3). The peculiar migration of the 24-kD band from fraction 3 was preserved after sample reduction with 2-mercaptoethanol and changed after alkylation of the reduced sample with iodoacetamide (Fig. 3). After that, the 24-kD band migrated similarly to the bulk HBsAg. Since iodoacetamide reacts with free SH groups only, the observed effect should be related to the presence of free SH group(s) in the *S* monomer from
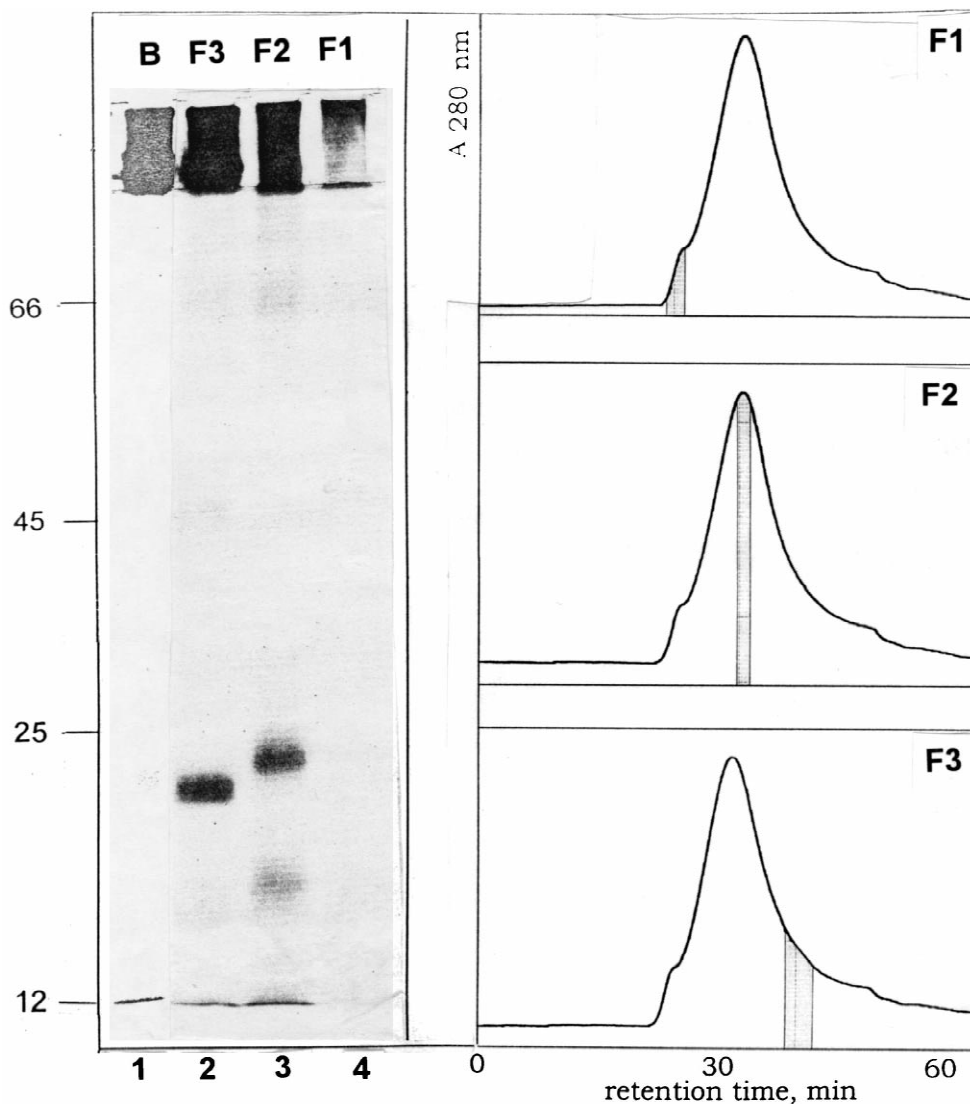
Fig. 1. Electrophoretic analysis of bulk HBsAg (left, lane 1) and SEC-separated fractions of HBsAg (lanes 2–4). Conditions: sample buffer, 0.3 *M* Tris–HCl, 10% (w/v) SDS and 50% (w/v) glycerol; application volume, 50 µl; HBsAg amount, 20 (lane 1) and 40 µg (lanes 2–4). Analyzed fractions indicated on chromatographic profile of HBsAg in SEC-HPLC (right). Conditions: TSK G5000 PW (600×7.5 mm I.D.); eluent, PBS, pH 7.0; flow-rate, 0.5 ml min$^{-1}$; detection, UV at 280 nm; HBsAg amount, 50 µg.

fraction 3. A similar retardation in electrophoretic mobility of the 24-kD band upon alkylation has been reported for the wild-type and mutant S proteins expressed in liver cells [13]. It was shown that alkylation did not affect migration of the sextuple mutant in which the cysteines 48, 65, 69, 76, 90 and 221 were replaced by either alanine or phenylala-nine. Hence, some of these residues, being free from disulfide bond formation in freshly-obtained HBsAg particles, may be responsible for the observed phe-nomena. All the cysteines mentioned above are found outside the major hydrophilic region and were shown to be dispensable for antigenicity of HBsAg [13]. It was shown that the 46.4-min peak is pre-
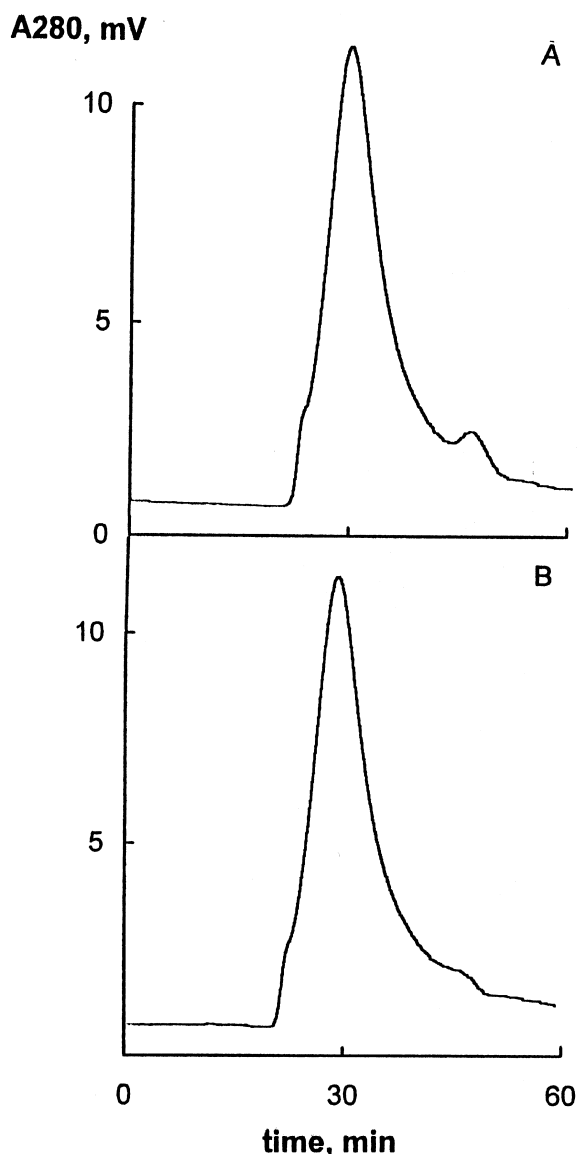
## A280, mV



Fig. 2. Chromatogram of HBsAg. Conditions: TSK G5000 PW (600×7.5 mm I.D.) supplied with a TSK GPW guard column (75×7.5 mm I.D.); eluent, PBS, pH 7.0; flow-rate, 0.5 ml min$^{-1}$; detection, UV at 280 nm; injection volume, 40 µl; HBsAg concentration, 1.35 mg ml$^{-1}$; the freshly-purified sample, (A) and the same sample stored at 4°C for a month after purification (B).

region seem to be crosslinked in the corresponding particles although assigning free SH group(s) to specific cysteine residue(s) is impossible due to the high number of cysteines in HBsAg.

There are several reports indicating that free SH group(s) may alter mobility of protein bands in SDS–PAGE. For example, a shift in migration of the 26-kD protein, apparently of the order of 1 kD, was observed after a unique Arg/Cys mutation [26]. Two species of *Aspergillus uryzae* α-amylase differing only in the location of one disulfide bond and one sulfhydryl group have different mobilities on SDS–polyacrylamide gel [27]. Probably, the presence of free cysteine(s) in the secondary structure of a protein induces conformational changes compared to the protein structure in which all cysteines are disulfide bonded. Both conformations may be stabilized in HBsAg through lipid association and differ from each other in the extent of detergent binding. As a result, the SDS–protein micelles formed may slightly differ in size and electrophoretic mobility.

As shown in Fig. 2, the 46.4-min peak in the chromatogram of HBsAg was diminished when freshly-purified HBsAg was stored at 4°C during a month. Moreover, in contrast to the freshly-separated sample from the 46.4-min peak, the stored aliquot showed the same migration of the 24-kD band as the bulk material (data not shown). These observations suggest that structural transformation of HBsAg from the 46.4-min peak into larger particles may occur during storage. This is in agreement with the existing hypothesis that higher crosslinking is a spontaneous event during aging of subviral particles [1]. Further structuration of HBsAg during storage may explain why no difference in migration of the 24 kD-band between early- and lately-eluted HBsAg was detected in our previous work where this aspect was not taken into account [5].

Hence, it was found that freshly-purified HBsAg contains a little fraction characterized by (1) retarded elution in SEC, (2) presence of free SH-groups and (3) significant antigenicity. Since no adsorption of HBsAg onto the TSK G5000 PW column has been previously demonstrated [5], the observed SEC-retardation is apparently size-related. Thorough electron-microscopic analysis of this sample is currently in progress. One can speculate that HBsAg eluting in the 46.4-min peak is composed presumably of lower-

sumably retained at the top of the stacking gel under nonreducing conditions. On the other hand, it has significant antigenicity in ELISA [5]. All this strongly suggests that eight cysteines of the major antigenic
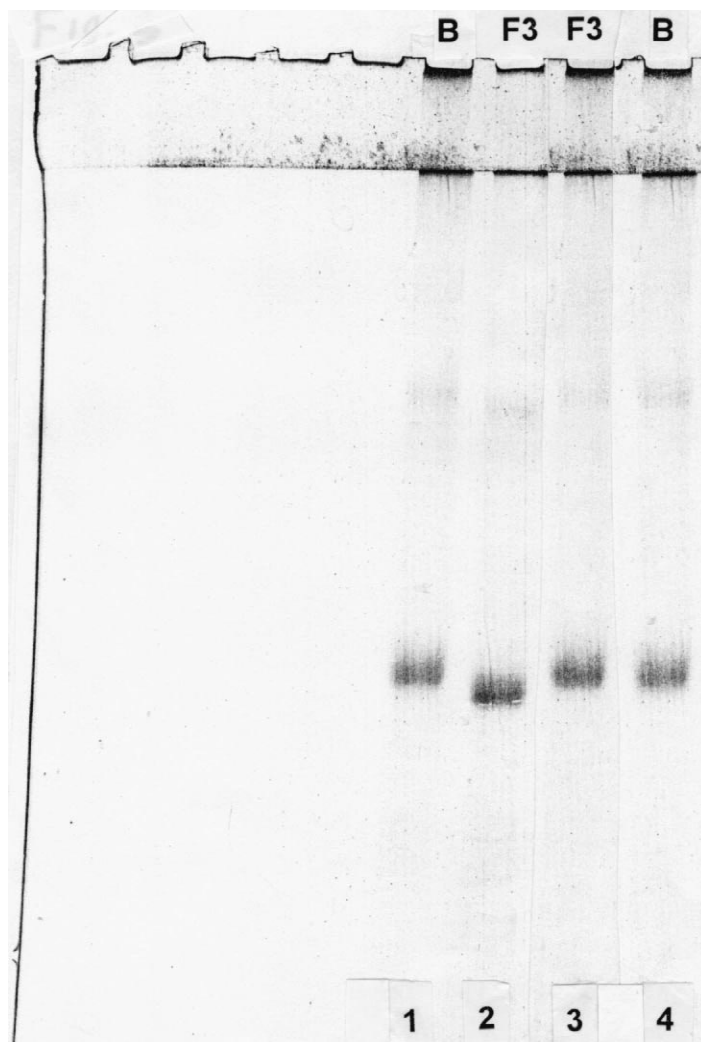
Fig. 3. Electrophoretic analysis of bulk HBsAg (lanes 1 and 4) and fraction 3 corresponding to the peak at 46.4 min in the chromatogram from Fig. 2 (lanes 2 and 3). Conditions: the sample buffer, 0.06 $M$ Tris–HCl, 2% (w/v) SDS, 5% (v/v) mercaptoethanol and 10% (w/v) glycerol; application volume, 15 µl; HBsAg amount, 20 µg. Reduced HBsAg was subsequently alkylated with iodoacetamide (lanes 3 and 4).

order oligomers in which disulfide bonds indispensable for antigenicity have been already formed while some SH-group(s), probably locating outside the antigenic region, remain free. These oligomers are capable of spontaneously assemble into higher-order structures through further disulfide bond formation during aging. On reducing polyacrylamide gels, the 24-kD band is unusually broad. As shown in the present work, this may be related to heterogeneity in the disulfide-crosslinking pattern within HBsAg.

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