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Size-exclusion chromatographic study of the reduction of recombinant hepatitis B surface antigen

Dina Tleugabulova

Quality Control Department, National Center for Bioproducts, P.O. Box 6048, Havana 6, Cuba

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Abstract

The reduction of the *P. pastoris*-derived hepatitis B surface antigen (HBsAg) has been investigated by size exclusion chromatography performed in a detergent solution containing 0.3% sodium dodecyl sulfate (SDS) and 0.1 *M* Tris–HCl, pH 7.0. The HBsAg, reduced under different conditions and passed through the TSK G4000 SW column (600×7.5 mm I.D.) at 0.9 ml min^{-1}, was resolved into two peaks corresponding to the reduced, monomeric, and non-reduced forms, respectively. Under these conditions, the antigen fraction corresponding to the HBsAg dimer can be separated and completely reduced to monomers by repeated reductive treatment with simultaneous lipid removal. The efficiency of reduction was maximal after sample treatment with an equal volume of a solution containing 417 m*M* dithiothreitol, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol. In conclusion, complete reduction of recombinant HBsAg to monomer subunits is possible and depends on the efficiency of lipid removal during the reductive treatment.

Keywords: Hepatitis B surface antigen

1. Introduction

Despite the progress made in vaccine development in the last few years, hepatitis B remains a significant health care problem in the world, affecting millions of patients. Infected people develop antibodies to hepatitis B surface antigen (HBsAg), formed by the S surface protein, the predominant constituent of the hepatitis B virus envelope. This protein is used as the active ingredient of several commercially available recombinant hepatitis B vaccines, offering a good opportunity for routine immunization programs to eradicate the disease. The expressed protein is self-assembled with host-derived lipids into 22 nm subviral particles, which can be used not only as vaccines, but also as carriers for foreign antigenic determinants to enhance their immunogenicity. In order to rationally design such gene vectors and vaccines, a detailed understanding of the morphogenesis of viral particles is essential. However, several important aspects of this phenomenon are still unclear. One of the reasons for the difficulty to progress in this field is that there is no convenient assay to monitor oligomerization of the S protein in cell cultures at a molecular level. Attempts have been made to study HBsAg particle formation in yeast by simultaneously testing samples by enzyme-linked immunosorbent assay (ELISA) and Western blot [1], or by radioimmunoassay and reversed-phase high-performance liquid chromatography (RP-HPLC) [2], respectively. In this approach, the concentration of assembled particles has been assessed

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by ELISA [1] or radioimmunoassay [2], whereas determination of the total concentration of the S monomer expressed was performed by Western blot [1] or RP-HPLC [2] after sample reduction. However, the results obtained are somewhat ambiguous and depend on the sample processing and assay (ELISA, radioimmunoassay) variability [2]. In addition, the accuracy of the quantitation of the S monomer strongly depends on the extent of HBsAg reduction, which may be different between nascent, mature and highly aggregated particles.

It has been shown previously that complete reduction of HBsAg to monomer subunits is not an easy task [3]. O'Keefe and Paiva [3] used the RP-HPLC method to monitor the extent of HBsAg reduction as the increase in height of the S monomer peak. The maximum peak height was achieved after treatment of HBsAg with enormously large concentrations of both reducing agents [1.3 *M* dithiothreitol (DTT), 4% (w/v) sodium dodecyl sulfate (SDS) and 65% (v/v) 2-mercaptoethanol]. However, even under these harsh conditions, HBsAg is still not identified in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as a single band (data not shown). The objective of the present study was to investigate whether incomplete reduction is due to the conditions used, i.e. insufficient detergent present, or due to the monomers being so tightly bound in dimers that their dissociation is impossible. For this purpose, the reduction of HBsAg was monitored by size exclusion chromatography (SEC) performed in SDS-containing buffer. In previous reports [4–8], this approach has been very effective for the separation of detergent-solubilized monomeric subunits from protein aggregates. Using this method, the reduced HBsAg was fully resolved into two peaks corresponding to monomeric and non-reduced forms, respectively. It was shown, by monitoring the peak areas under different reduction conditions, that a similar extent of HBsAg reduction can be achieved by treatment with considerably lower concentrations of reducing agents [417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol] than those proposed in the previous study [3]. Under these conditions, complete reduction of HBsAg to monomer subunits was achieved after repeated reductive treatment with simultaneous lipid removal.

2. Experimental

Tris(hydroxymethyl)aminomethane (Tris), dithiotreitol, SDS and mercaptoethanol were obtained from Merck (Darmstadt, Germany). Sephadex G-10 and the other electrophoresis reagents were purchased from Pharmacia (Uppsala, Sweden). All solutions were made in Milli-Q grade water. Recombinant HBsAg, cloned and expressed in the yeast *P. pastoris*, was provided as a purified material (>95% HPLC, SDS–PAGE) after a multistep purification process [9]. It was purchased as a 1.5-mg ml^{-1} solution in phosphate-buffered salane (PBS) (1.7 m*M* KH_2PO_4, 7.9 m*M* Na_2HPO_4, 2.7 m*M* KCl and 250 m*M* NaCl, pH 7.0) from the National Center for Bioproducts (Havana, Cuba).

The SEC system included a Pharmacia LKB 2248 pump, a Knauer degasser, a Pharmacia 2141 variable-wavelength UV detector operated at 280 nm and a Pharmacia 2221 programmable integrator. The column used was a TSK G4000 SW (600×7.5 mm I.D.) supplied with a TSK GSW guard column (75×7.5 mm I.D.), both purchased from Tosohaas (Stuttgart, Germany). Elution was achieved with 0.1 *M* Tris–HCl in 0.3% SDS, pH 7.0, at a flow-rate of 0.9 ml min^{-1}. A larger volume of the reduced sample (200 μl) was injected to recollect and analyze the peaks separated by SEC and SDS–PAGE under non-reducing and reducing conditions. Before analysis, the fractions were concentrated in Centricon tubes (Amicon, Beverly, USA).

Electrophoresis was run in 15% gels according to Laemmli [10] followed by Coomassie- (Bio-Rad, Richmond, CA, USA) or silver staining [11].

For HBsAg reduction, different sample buffers were used: (1) 0.3 *M* Tris–HCl, 10% (w/v) SDS and 25% (v/v) mercaptoethanol, (2) 1.3 *M* DTT, 4% (w/v) SDS, 65% (v/v) 2-mercaptoethanol or (3) 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol. The last one was prepared by mixing 1 *M* DTT in 1% SDS (500 μl), 20% SDS (500 μl) and 2-mercaptoethanol (200 μl). An aliquot (500 μl) of the HBsAg solution was mixed with 100 μl of sample buffer; the mixture was incubated in a boiling water bath for 10 min and then cooled to room temperature. For the SEC analysis, excess reducing agents were eliminated by filtering the sample

through a 7-cm layer of Sephadex G-10 loaded into a 1-ml hypodermic syringe. The matrix had been boiled previously in distilled water for 30 min. For the SDS–PAGE analysis, a small amount of glycerol was added to the reduced samples before the run.

After electrophoresis, the SDS-gel was incubated with monoclonal antibodies raised against the HBsAg polypeptide and was developed with protein A conjugated to aminobenzidine [12].

3. Results and discussion

In previous studies [13], it has been impossible to obtain free, soluble S monomer, due to protein aggregation with the formation of amorphous precipitates. The tendency to aggregate is presumably due to the hydrophobic domains on the S protein, which become uncovered in the disrupted and delipidated HBsAg. The use of denaturing SEC–HPLC brings the possibility of separating and analyzing the reduced S monomers as soluble, protein–SDS complexes. As shown in Fig. 1a, the HBsAg sample reduced with 2-mercaptoethanol and passed through the TSK G4000 SW column gave three well-resolved peaks with retention times of 12.5, 23.5 and 31.0 min, respectively. Each of these peaks, following collection and rechromatography, eluted in the same retention volume. In SDS–PAGE under non-reducing conditions, the corresponding band from peak 1 (retention time, 12.5 min) did not enter the gel (Fig. 2, lane 4), whereas that from peak 2 (retention time is 23.5 min) migrated as a diffuse band corresponding to the 24 kDa monomer (Fig. 2, lane 5). Both protein bands were recognized by monoclonal antibodies raised against the S protein (data not shown). Hence, peaks 1 and 2 correspond to the non-reduced and monomeric forms of HBsAg, respectively. The area of peak 2/area of peak 1 ratio has been used here as a measure of the efficiency of HBsAg reduction. Peak 3 (retention time, 31.0 min) failed to give a band in SDS–PAGE and eluted in the inclusion volume of the TSK column used. It is highly likely that it corresponds to the migration of low molecular mass compounds present in the sample. After HBsAg detergent solubilization, lipid components of HBsAg are released and migrate as

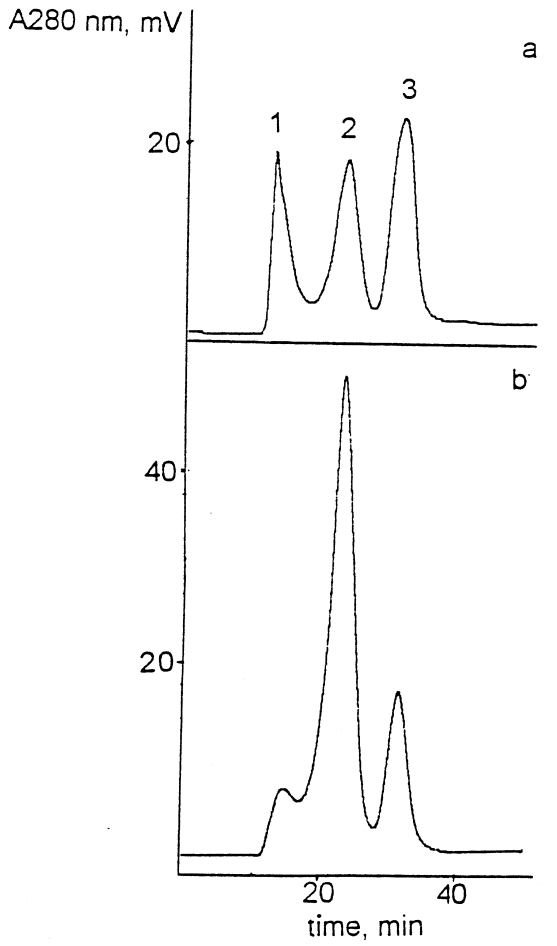

Fig. 1. Chromatogram of reduced HBsAg. Conditions: TSK G4000 SW (600×7.5 mm I.D.); eluent, 0.3% SDS, 0.1 *M* Tris–HCl, pH 7.0; flow-rate, 0.9 ml min^{-1}; detection, UV at 280 nm; injection volume, 75 μl; sample buffer, 0.3 *M* Tris–HCl, 10% (w/v) SDS and 25% (v/v) 2-mercaptoethanol (a), 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol (b). Peaks: 1=HBsAg oligomer, 2=HBsAg monomer and 3=low molecular mass compounds.

lipid–detergent complexes close to the dye front in SDS gels [14]. Besides lipids, intact and oxidized forms of DTT and 2-mercaptoethanol should also co-elute in peak 3.

Peak 2 showed a slight forward tailing (Fig. 1), suggesting the presence of low-order oligomers. After injection of 300 μg of HBsAg, this zone of peak 2 was separated, fivefold concentrated and analyzed by SEC and SDS–PAGE. It migrated as the

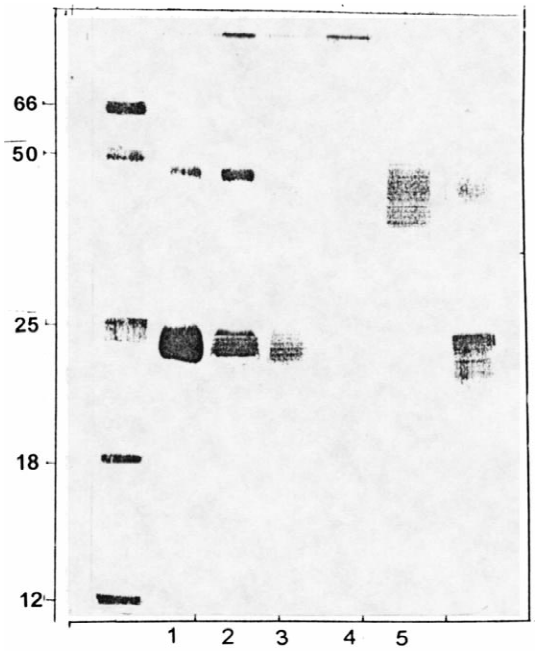

Fig. 2. Electrophoretic analysis of HBsAg reduced with 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol (lane 1), 0.3 *M* Tris–HCl, 10% (w/v) SDS, 25% (v/v) mercaptoethanol (lane 2), 1.3 *M* DTT, 4% (w/v) SDS and 65% (v/v) 2-mercaptoethanol (lane 3); tenfold-concentrated fractions obtained by SEC fractionation of 300 μg of HBsAg reduced with 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol: 12.5 min peak (lane 4), 19.5 min peak (lane 5) and 23.5 min peak (lane 6). Volume applied, 15 μl.

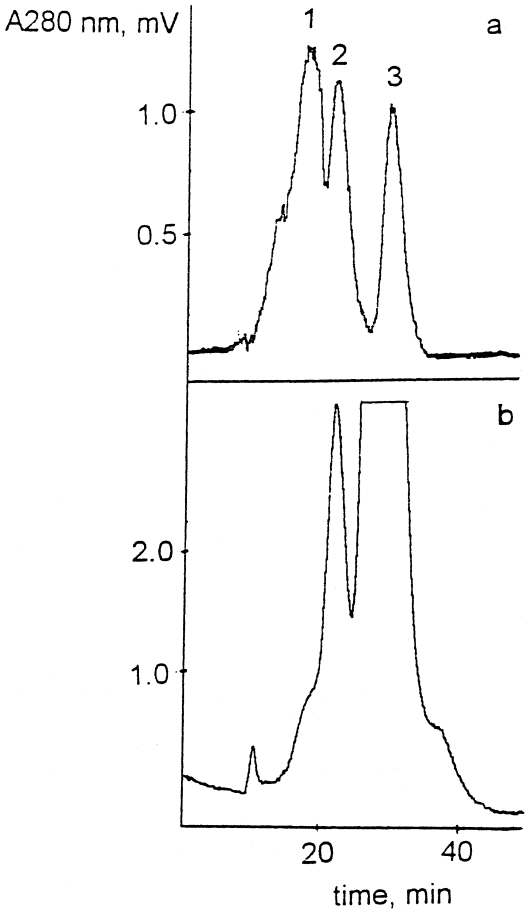

Fig. 3. Chromatogram of the 18–20 min fraction of peak 2. Conditions as in Fig. 3. Injection volume, 100 μl (a), 125 μl (b); sample buffer, 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol (b). Peaks: 1=HBsAg dimer and 2=HBsAg monomer.

19.5 min peak in SEC (Fig. 3a) and as a diffuse 46 kDa band in SDS–PAGE under non-reducing conditions (Fig. 2, lane 5). This pattern was drastically changed after reduction: the sample migrated as a 24 kDa protein; in SEC, the 19.5 min peak was converted into peak 2 (Fig. 3b). The results obtained provide evidence that the 19.5 min peak corresponds to the elution of S dimers. The observed broadening of the corresponding band on SDS–PAGE (Fig. 2, lane 5) is probably related to heterogeneity in the disulfide crosslinking pattern. Hence, the method described is capable of separating the S protein dimers from other reduced forms of HBsAg, allowing for further analysis.

To avoid injecting large amounts of reducing agents onto the column, the reduced samples were passed through a 7-cm layer of Sephadex G-10 gel, loaded into a 1-ml hypodermic syringe. The filtered fraction was collected while that retained by the matrix was discarded. Since low-molecular-mass compounds present in the sample were preferentially retained on the column, their concentration in the reduced sample was essentially diminished by filtration (Fig. 4). Protein recovery was not critical for further analysis. It was more important that the area of peak 1/area of peak 2 ratio was the same before and after filtration, even for samples with different degrees of reduction (data not shown).

In SDS gels, the HBsAg reduced with 2-mercaptoethanol or DTT, usually gives three bands: Two corresponding to monomer and dimer, respectively,

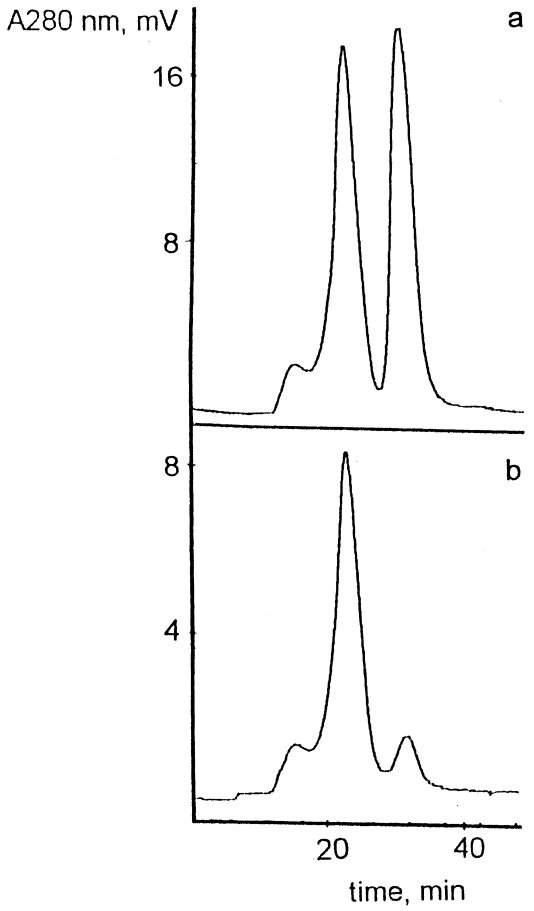

Fig. 4. Chromatogram of reduced HBsAg before (a) and after (b) filtration. Conditions as in Fig. 1. Injection volume, 50 μl; sample buffer, 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol. Reduction procedure as described in Section 2.

and the another one being retained at the top of the stacking gel (Fig. 2, lane 2). From practical experience, the last band does not disappear when higher concentrations of reducing agent are used for HBsAg sample treatment (data not shown). The same observation was reported in the RP-HPLC study of HBsAg reduction [3]. The monitoring of HBsAg reduction by denaturing SEC showed that the height of the S monomer peak is gradually increased with increasing concentrations of reducing agent (up to 25% for 2-mercaptoethanol and 250 m*M* for DTT, respectively; Table 1). No increase in the height of the S monomer peak was detected after increasing the concentrations of reducing agent and/or SDS in

Table 1
Efficiency of the HBsAg reduction, determined as the area of peak 2/area of peak 1 ratio, by SEC–HPLC

Reducing agent	Area of peak 2/area of peak 1
2-Mercaptoethanol	
10%	0.8
20%	1.2
30%	1.3
DTT	
100 m*M*	1.1
200 m*M*	1.3
300 m*M*	1.3
2-Mercaptoethanol+DTT	
10%+100 m*M*	7.5
16%+417 m*M*	10.0
65%+1300 m*M*	10.0

Conditions as in Fig. 1. Reduction procedure as described in Section 2. Sample buffer contained 4% SDS and varying concentrations of reducing agents.

sample buffer. A greater degree of HBsAg reduction can be achieved by using both reducing agents in the sample buffer [3]. As shown in Table 1, reduction with 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol led to a maximal increase in the height of peak 2. No precipitation of insoluble aggregates was detected after centrifugation of reduced samples for 5 min at 10 000 *g*. The notable increase in the intensity of peak 2 after the reductive treatment of HBsAg with combined DTT–mercaptoethanol sample buffer (Fig. 1b) may be related to the drastic increase in the concentration of free monomers, which exposed previously buried tryptophan residues to the aqueous environment after particle reduction [15]. No further increase in the height of peak 2 was observed with increasing concentrations of both reducing agents in the sample buffer, up to 1.3 *M* DTT and 65% 2-mercaptoethanol (Table 1). However, even under these harsh conditions, peak 1 still remained, as shown in the chromatogram on Fig. 1b. This ''unreducible'' fraction of peak 1 was collected, concentrated and subjected to SEC. Surprisingly, in the chromatogram (Fig. 5), a large peak at 31 min, instead of the expected peak at 12.5 min, was observed. Since this fraction was retained at the top of the stacking gel following SDS–PAGE and was not recognizable following Coomassie staining and Western blotting (data not shown), it should correspond to the elution

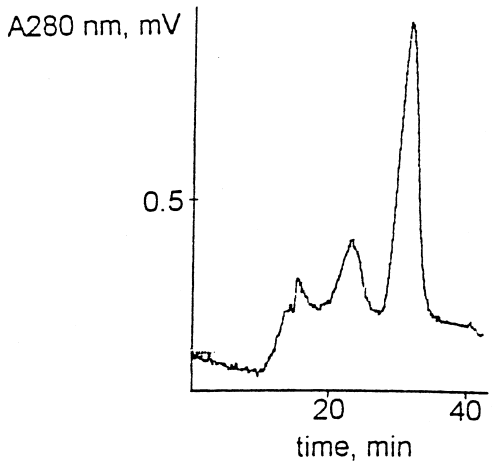

Fig. 5. Chromatogram of fivefold-concentrated fraction corresponding to peak 1 remaining after reduction with 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol. Conditions as in Fig. 1. Injection volume, 50 μl.

of non-protein aggregates. These may be the mixed micelles of SDS with phospholipids, which are known to be large structures (Stokes radius >3.5 nm) [16]. The size of lipid–SDS micelles is known to spontaneously decrease until the smallest attainable size is reached [16], which explains the observed changes in the chromatographic profile of the fraction collected after concentration (Fig. 5). To conclude, the reductive treatment of HBsAg described in the present work leads to the formation of highly uniform monomeric entities, represented by S monomer–SDS complexes. These complexes can be separated by denaturing SEC from non-reduced dimers and mixed lipid–SDS micelles.

One of the typical characteristics of HBsAg is that it is developed as a very intensive black band after silver staining. Hence, it was surprising that HBsAg appeared as a weak band with an unusual yellow shade after reduction with 1.3 *M* DTT, 4% SDS and 65% 2-mercaptoethanol (Fig. 2). Under the same conditions of analysis, the intensity of silver-stained protein bands depends on the content of basic and S-containing amino acids in the polypeptide chain and on their close proximity to each other in the SDS-denatured monomer as well [17]. It is probable that the treatment with 1.3 *M* DTT, 4% (w/v) SDS and 65% (v/v) 2-mercaptoethanol induced strong conformational changes in the S monomer, resulting in dissuading cysteine- and/or basic amino acids-reach regions. In contrast, the intensive silver-staining of the HBsAg protein band observed after treatment with 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol suggests that the S monomer is less conformationally changed.

The difficulty of reducing the HBsAg particle to a single band can be explained in part by the presence of an unusually large number of cysteine residues in the S monomer, all of which were shown to participate in extensive crosslinking via both intra- and intermolecular disulfide bridges [18]. Disulfide bonds, being exposed to the solvent environment or buried in the interior of the particle, differ in their accessibility to reducing agent. On the other hand, protein–protein binding in the assembled HBsAg is strongly stabilized, not only by disulfide bonds but also by hydrophobic interactions and by the association of monomers and dimers with a lipid bilayer [14,15]. Hence, to dissociate HBsAg into monomers, an efficient procedure for the removal of lipid is of primary importance. It is probable that the use of both reducing agents has an additional effect on the delipidation of HBsAg, besides that of reduction. Delipidation can be understood as involving progressive removal of lipids from lipoprotein–detergent complexes that were formed as intermediates during membrane disruption (for review, see Ref. [19]). During delipidation, the lipid contact with protein is exchanged for detergent and transferred into mixed lipid–detergent micelles, resulting in total separation of the lipid from the protein. It was found that SDS may display lipid selectivity; for example, in the treatment of plasma low density lipoprotein, it solubilized phospholipids and free cholesterol more easily than cholesterol esters and triglycerides [16]. The lipid composition of HBsAg is represented by a wide variety of lipids [14,15]. Hence, it cannot be excluded that SDS solubilizes some types of HBsAg-associated lipids more rapidly than others. In partially delipidated HBsAg, some disulfide bonds may be buried in the remaining lipid layer and be inaccessible to reductors. Hence, these structures should be more resistant to reduction than completely delipidated antigen. It is probable that peak 1 corresponds to the elution of partially delipidated HBsAg. The SEC method described here, in combination with lipid determination, provides a means to corroborate

this. It is known that dissociation of the last lipids bound to the protein takes place only when the mixed lipid–detergent micelles are efficiently separated from protein–detergent complexes [19]. Hence, complete disruption of the S dimers into monomers (Fig. 3) was possible due to complete delipidation provided by the strategy used: The S dimers were reduced and gradually delipidated by repeated reductive treatment accompanied by simultaneous removal of lipid–detergent micelles by desalting and SEC. In conclusion, complete reduction of HBsAg to monomer subunits is possible and depends on the efficiency of lipid removal during the reductive treatment.

Establishing the sequence fidelity of recombinant proteins intended for human therapeutic use is essential for demonstrating the faithfulness of expression by the recombinant host and the absence of modifications that could result in alterations of the biological properties of the product. Numerous difficulties have been encountered in attempts to provide direct structural confirmation of the sequence of HBsAg, such as resistance to reduction and tryptic digestion, insolubility in aqueous media and partial blockage of the NH_2 terminus [13]. All of this prevented the straightforward application of standard sequencing strategies involving proteolytic degradation and Edman-based sequence analysis. The structural characterization of recombinant HBsAg was thus achieved by mass spectrometric peptide mapping after nonspecific proteolytic degradation [13]. Since the problems encountered during attempts to sequence the particle-associated S protein are common to structural studies of other integral proteins [20], the efficient reductive procedure described here, combined with simultaneous lipid removal, can be used to improve existing protocols for the structural characterization of membrane proteins.

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