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Physico-chemical characterization of recombinant hepatitis B surface antigen by a multidimensional approach

Dina Tleugabulova[a,*], Viviana Falcón[b], Eduardo Pentón[c]

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

[b] *Department of Physical Chemistry, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

[c] *Vaccine Division, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

Abstract

Initially, our work was directed to respond to the question: why hepatitis B surface antigen (HBsAg) produces a very broad peak in preparative size-exclusion chromatography (SEC). For this purpose, we used a multidimensional approach based on SEC fractionation of purified HBsAg followed by the individual analysis of SEC fractions by a battery of assays, such as SEC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay and transmission electron microscopy. As a result, HBsAg particles were shown to be heterogeneous in terms of particle assembly. In order to elucidate the origin of HBsAg heterogeneity, we included here the denaturing SEC into a multidimensional approach. The data from denaturing SEC evidenced the fragmentation of protein monomers within the HBsAg particle that, probably, occurs during fermentation broth, rather than during in vitro HBsAg processing. The fractions isolated from widely separated regions of HBsAg peak differed in the extent of protein fragmentation, suggesting that the variable extent of protein degradation within HBsAg particles may be one of the factors responsible for broadening of the HBsAg peak in SEC.

Keywords: Hepatitis B surface antigen; Antigens; Proteins

1. Introduction

Recombinant hepatitis B surface antigen (HBsAg) is produced by expression of the M_r 24 000 polypeptide (HBsAg monomer) in yeast where approximately 100 units of this protein are self-assembled intracellularly into 22 nm lipoprotein particles [1]. Much of the interest in HBsAg arises from its use in vaccines against the hepatitis B virus and as a potential carrier for foreign antigenic determinants to enhance their immunogenicity [2]. Many structural events leading to HBsAg particle assembly remain to be elucidated. In the absence of information on the high-resolution structure of HBsAg, particle folding cannot be assessed directly. For this purpose, alternative approaches based on site-directed mutagenesis [3] and combination of small-angle neutron scattering and contrast variation method [4] have been developed.

In practice, the faithfulness of HBsAg monomer expression in yeast is proved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [5], reversed-phase high-performance liquid chromatography (RP-HPLC) [6] and mass spectrometry [7] after reduction of HBsAg particles by thiol reagents, whereas the assembly of monomeric units into immunogenic particles tested by electron microscopy and enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies [8]. All the mentioned techniques, being efficient in determining

*Corresponding author.

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the general composition of HBsAg expressed, do not discriminate between possible defects in particle folding such as conformational alterations, insufficient disulfide cross-linking [9–11] or particle aggregation [12].

In previous papers, size-exclusion chromatography (SEC) was shown to be a powerful technique to study HBsAg aggregation [12,13]. However, both in preparative and analytical SEC, HBsAg produces an unusually broad peak that limits a more extensive use of this method for HBsAg characterization. Hence, our initial work was directed towards elucidating the factors responsible for broadening of HBsAg peak in SEC [14]. In order to obtain the maximum amount of relevant information consistent with an efficient use of analytical resources, we used a multidimensional approach based on SEC fractionation of purified HBsAg followed by analysis of the isolated fractions by a battery of assays, such as SEC, SDS–PAGE, ELISA and transmission electron microscopy (TEM). As expected, all the fractions gave the same electrophoretic pattern under reducing conditions, suggesting that HBsAg particles are composed by the same monomer independently on their elution in SEC, and were seen in electron microscope as the 16–30 nm particles with minimal differences in size distribution. However, in SEC, reinjection of the isolated fractions demonstrated each peak to have its own, well-defined exclusion volume shifting toward higher values with increasing fraction number. This shift in retention time observed in SEC was accompanied by changes in the efficiency of particle assembly expressed by the $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio ($[HBsAg]_{Lowry}$ is total protein content of each fraction, corresponding roughly to the total amount of HBsAg monomers, and $[HBsAg]_{ELISA}$ is the amount of HBsAg monomers in the assembled form only). The $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio was maximal in the fraction corresponding to the maximum of HBsAg peak. In conclusion, our research on HBsAg peak broadening in SEC resulted in the identification of heterogeneity in the HBsAg assembly pattern [14]. This heterogeneity is expected to be generated by a variety of mechanisms [8,9,12,14].

In order to elucidate the molecular basis of HBsAg heterogeneity, we included here the denaturing SEC into a multidimensional approach. Separation of large proteins under denaturing conditions provides a valuable information on their physical state in solution [15–18]. This approach was recently used to study the reduction of HBsAg by thiol reagents [19]. In the present work, denaturing SEC was applied to the analysis of HBsAg fractions that showed differences in the efficiency of particle assembly.

2. Experimental

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), SDS, 2-mercaptoethanol, sodium chloride, sodium phosphates and other mentioned reagents were analytical grade and obtained from Merck (Darmstadt, Germany). The reagents used in electron microscopy were from Agar Scientific (Essex, UK). All solutions were made in Milli-Q grade water. Phosphate-buffered saline (PBS) contained 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. Recombinant HBsAg (>95% SDS–PAGE), cloned and expressed in yeast *Pichia pastoris*, was provided as a solution in PBS (1.55 mg/ml) from the National Center for Bioproducts (Havana, Cuba).

2.2. Apparatus

2.2.1. SEC

The SEC system included a Pharmacia LKB 2248 pump, Knauer degasser, Pharmacia 2141 variable-wavelength UV detector operated at 280 nm and Pharmacia 2221 programmable integrator. For fractionation, about 200 mg of purified HBsAg (>95% SDS–PAGE) was loaded onto the preparative TSK G5000 PW column (20 μm, 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. Fractions of 7 ml were collected and subjected to SEC analysis by injecting 100 μl onto the analytical TSK G5000 PW column (17 μm, 600×7.5 mm I.D.) supplied with a TSK GPW guard column (75×7.5 mm I.D.). Elution was achieved with PBS at 0.5 ml/min. The fractions eluting in the same volume were pooled and rechromatographed. The three fractions of HBsAg widely separated each

other in SEC were reduced and subjected to denaturing SEC and SDS–PAGE.

The denaturing SEC procedure was similar to that described previously [19]. The TSK G4000 SW column (600×7.5 mm I.D.) was purchased from Tosohaas (Stuttgart, Germany). Elution was achieved with 0.1 *M* Tris–HCl in 0.3% SDS, pH 8.0 at 0.6 ml/min.

2.2.2. SDS–PAGE

Electrophoresis (Hoefer Scientific Instrument) was performed as described by Laemmli [20] on 12.5% gels at 30 mA for 3.5 h at room temperature under reducing conditions. The gels were stained by Coomasie blue dye (Bio-Rad, Richmond, CA, USA) or silver nitrate [21]. For immunoblotting, the gel was incubated with a monoclonal antibody specifically recognized HBsAg monomer and developed with protein A conjugated to aminobenzidine [22].

2.2.3. Transmission electron microscopy

Two drops of HBsAg solution in PBS (0.1 mg/ml) were placed for 5 min onto a 400 mesh copper grid coated with formvar-carbon film. Excess sample was blotted off. Grids were stained with uranyl acetate and examined in a Jeol-JEM 2000EX transmission electron microscope, acceleration voltage 80 kV and magnification 40 000×.

2.3. Reduction of HBsAg particles

Aliquots (200 μl) from SEC-isolated fractions of HBsAg (0.8–1.0 mg/ml HBsAg) were incubated with an aliquot (40 μl) from DTT/M sample buffer [417 m*M* dithiothreitol, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol] for 10 min at 100°C. After reduction, the samples were analyzed by SDS–PAGE/silver staining (1.7 μg per spot) and SEC (75 μg), as described.

2.4. Oxidation of HBsAg

An aliquot (500 μl) of HBsAg fraction separated from the maximum of the SEC peak (0.7 mg/ml, PBS) was incubated for 24 h at 37°C with 1% H_2O_2 (50 μl). After that, the sample (100 μl) was analyzed by denaturing SEC as described.

3. Results and discussion

In the course of HBsAg characterization, we found that there is a discrepancy in the results provided by SEC and electron microscopy. First, HBsAg fractions from the widely separated regions of the SEC peak are apparently composed by the same spherical particles, whereas they essentially differ from each other in retention time [14]. Second, even if SEC analysis shows a considerable extent of HBsAg aggregation in bulk material, no alterations in HBsAg particle size, shape and morphology are detected by electron microscopy (data not shown). To address this question, we fractionated HBsAg by preparative SEC and submitted individual fractions to electron microscopic measurements. Attention has been focused on the fractions from a shoulder before the maximum of peak (fraction 1), maximum of peak (fraction 2) and backward area (fraction 3), respectively (Fig. 1). Indeed, no alterations in particle size, shape and morphology were corroborated. However, a more thorough examination of electronmicrographs has revealed that HBsAg particles from fraction 1 are in aggregated form whereas those from fractions 2 and 3 are not (Fig. 1). The aggregation pattern is somewhat peculiar and consists in adhesion of several, apparently intact, HBsAg particles (Fig. 1). Hence, aggregation leads to a considerable increase in the hydrodynamic diameter of aggregated structures, explaining thus their elution prior to the main HBsAg peak. Besides large inter-particle aggregates containing more than 10 particles, lower-order structures composed of 2–5 particles are also observed, that explains, in part, the observed forward tailing of HBsAg peak in SEC. In contrast, HBsAg particles eluting in fractions 2 and 3 are distributed on the whole grid surface and do not form discrete multiparticle structures (Fig. 1). The fraction 3 was previously shown to contain HBsAg particles with a less efficient disulfide cross-linking compared to those from fraction 2 [11]. However, this information alone is insufficient to explain the difference in the elution volumes between these fractions.

It is thus understandable why we could not detect HBsAg aggregation in bulk material by using electron microscopy: since both aggregated and non-aggregated forms of HBsAg are composed by spherical particles which are indistinguishable by electron

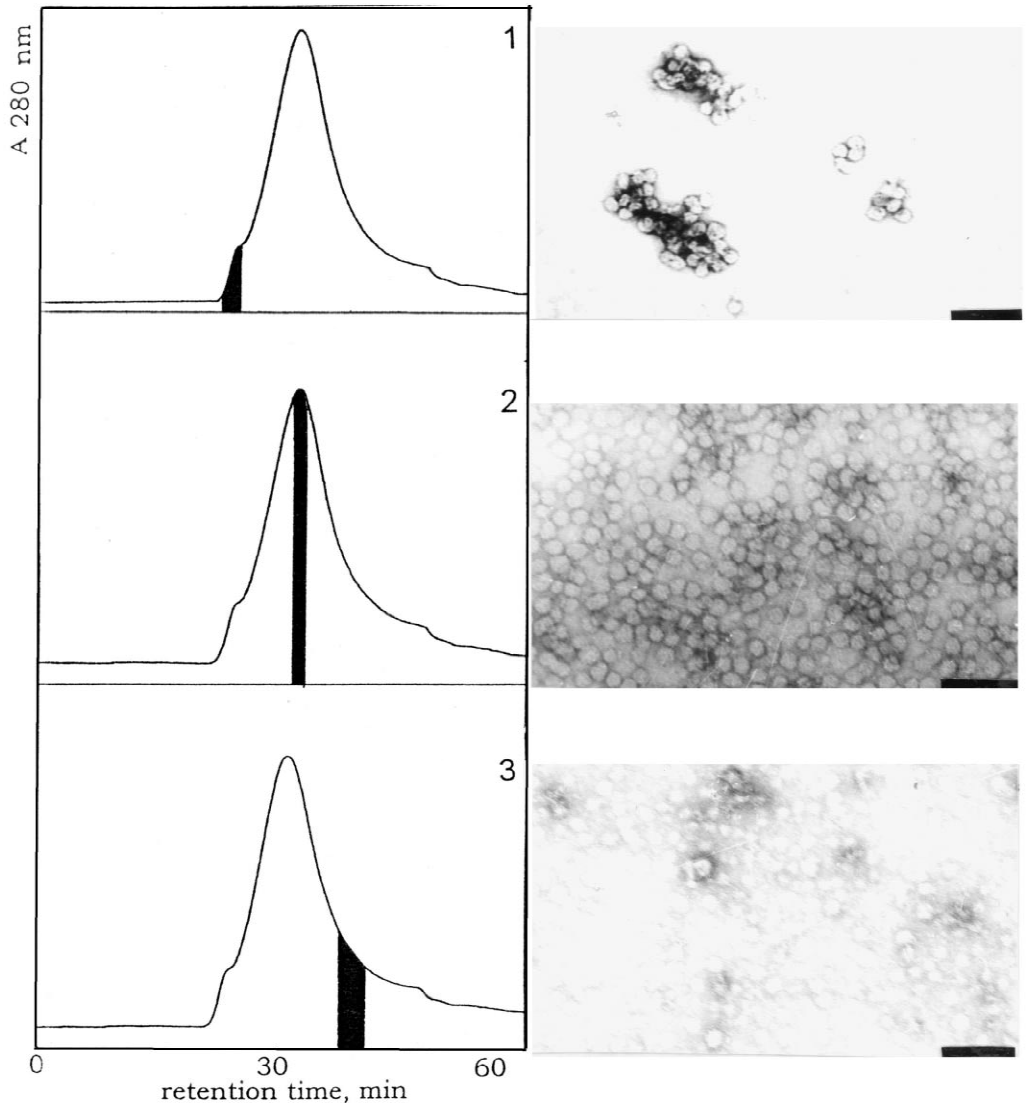

Fig. 1. Preparative SEC chromatogram and electron micrographs of isolated HBsAg fractions. Conditions: TSK G5000 PW (600×55 mm I.D.) supplied with a TSK GPW guard column (75×55 mm I.D.); eluent, PBS, pH 7.0; flow-rate, 7 ml/min; detection, UV at 280 nm; amount of HBsAg loaded, 200 mg. Scale bar is 100 nm.

microscopy, the superposed aggregation pattern is masked.

The denaturing SEC was previously used to demonstrate that an efficient reduction of HBsAg can be achieved by using a mixture of DTT and 2-mercaptoethanol (DTT/M), instead of one of them alone [19]. Probably, thiol reagents do not possess the same reducing power on multiple disulfide bonds within HBsAg particles. The soluble HBsAg–SDS complexes generated after DTT/M reduction are suitable for further analysis by RP-HPLC [6] or denaturing SEC [19].

In denaturing SEC (Fig. 2), the reduced HBsAg is resolved into the three peaks, corresponding to the coelution of nonreduced HBsAg and nonprotein micellar aggregates (peak 1), reduced HBsAg (peak 2) and low-molecular-mass nonprotein compounds (peak 3). It was previously shown that peak 1

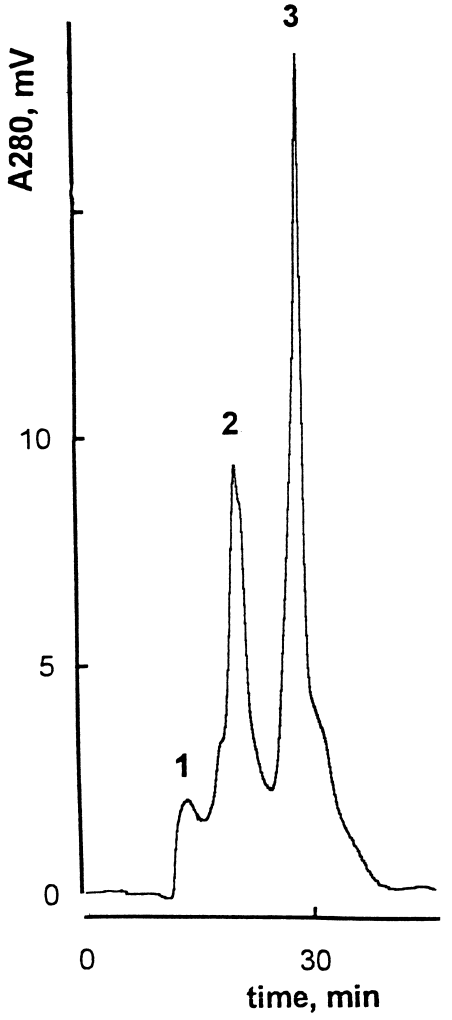

Fig. 2. SEC chromatogram of reduced HBsAg. Conditions: TSK G4000 SW (600×7.5 mm I.D.); eluent, 0.1 *M* Tris–HCl containing 0.3% SDS, pH 8.0; flow-rate, 0.9 ml/min; detection, UV at 280 nm; injection volume, 200 μl; amount of HBsAg, 200 μg; sample buffer, 417 m*M* DTT, 4.2% (w/v) SDS and 16% (v/v) 2-mercaptoethanol. Peaks: 1=lipid–SDS aggregates, 2=reduced HBsAg, 3=low-molecular-mass nonprotein compounds.

remaining after DTT/M reduction is produced by nonprotein aggregates, probably, by lipid–SDS complexes [19]. The presence of shoulders before and after the maximum of peak 2 (Fig. 2) was a hint at the heterogeneity of HBsAg structures formed after particle reduction. To address this question, SEC fractionation of the peak 2 followed by SDS–PAGE analysis of the fractions separated was carried out. As a result, reduced HBsAg was shown to be represented by M_r 46 000 protein dimers, eluting presumably in the forward shoulder of peak 2, M_r 24 000 protein monomers eluting in the maximum of peak 2 and lower-molecular-mass proteins (LMWPs) eluting in the backward shoulder (Fig. 3). These LMWP migrated in SDS–PAGE as multiple bands before the M_r 24 000 monomer (Fig. 3, lane 4). The LMWP bands were appreciated when excessively large amounts of purified HBsAg loaded on to gel (Fig. 3, lane 1), suggesting their presence in minor amounts. However, the LMWP peak in denaturing SEC was high, probably, due to coelution of all LMWPs in the same peak. The detection of LMWPs

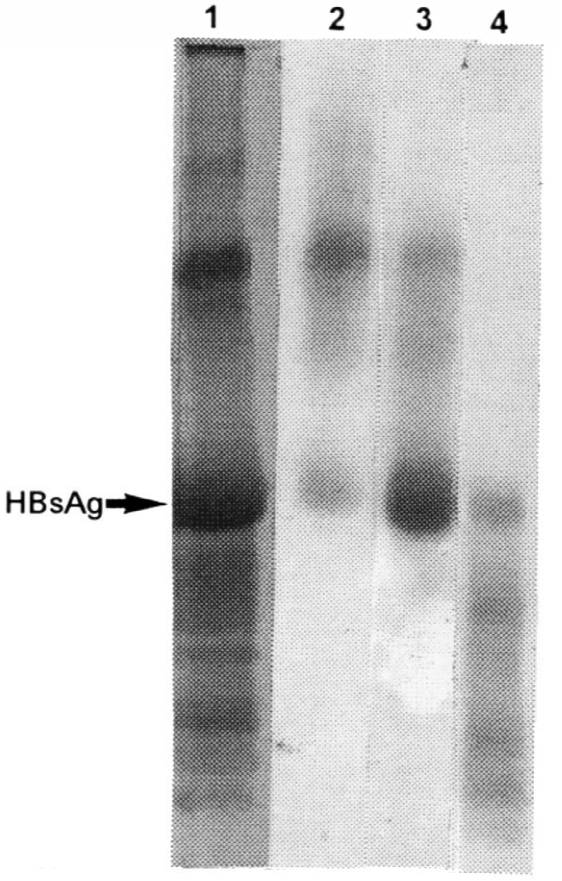

Fig. 3. SDS–PAGE/silver staining of DTT/M-reduced HBsAg (lane 1) subjected to SEC fractionation (lanes 2–4): forward shoulder of peak 2 (lane 2), maximum of peak 2 (lane 3) and backward shoulder of peak 2 (lane 4). Amount, 20 μg (lane 1) and 1.7 μg (lanes 2–4) HBsAg.

by SDS–PAGE is generally less appropriate than by SEC, as small fragments are difficult to retain on gel, and individual LMWP bands may stain differentially depending on their amino acid composition [23]. Hence, estimation of the overall content of LMWPs in the HBsAg sample is more appropriate by SEC than by SDS–PAGE.

The LMWPs were recognized by immunoblotting (data not shown) suggesting their generation from HBsAg monomer. To test whether HBsAg frag-

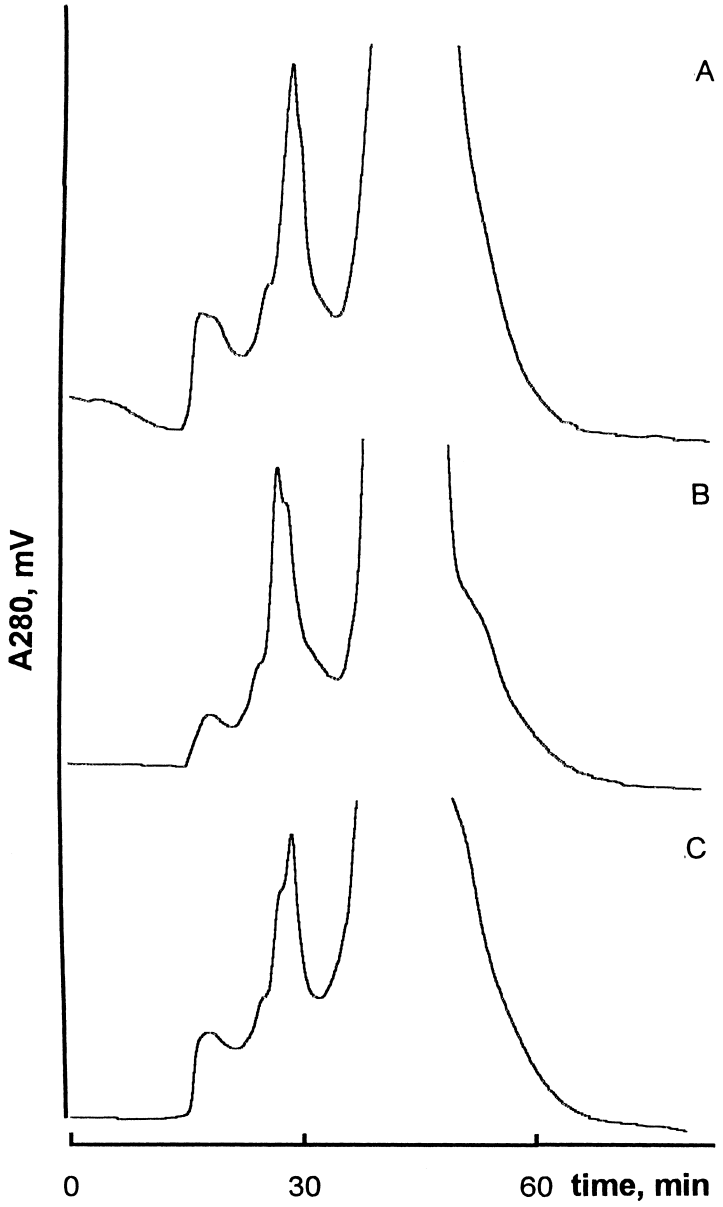

Fig. 4. SEC chromatograms of HBsAg fractions isolated by preparative SEC and reduced with DTT/M sample buffer: fraction 1 (A), fraction 2 (B) and fraction 3 (C). Corresponding fractions indicated on chromatogram in Fig. 1. Preparative chromatographic conditions as in Fig. 1. Analytical chromatographic conditions as in Fig. 2. Flow-rate, 0.6 ml/min; injection volume, 100 μl; amount of HBsAg, 75 μg per injection.

mentation could be produced by drastic reducing conditions used here, the LMWP-free fraction of reduced HBsAg was separated from the maximum of peak 2 (Fig. 3, lane 3) and repeatedly reduced. As a result, no appearance of LMWPs evidenced that these polypeptides are not generated by the reducing procedure.

Under nondenaturing conditions, LMWPs were not detected by SEC, even if large amounts of HBsAg loaded on to the column (data not shown), evidencing their association with the assembled particles. It is in agreement with electron microscopic measurements showing no alterations in the HBsAg assembly pattern, despite the presence of LMWPs in HBsAg particles (Fig. 1).

The presence of LMWPs in HBsAg particles was reported for *S. cerevisiae*- and *H. polymorpha*-expressed HBsAg [24]. Similarly, the corresponding bands were clearly visualized by immunoblotting of crude yeast extracts, suggesting their formation during fermentation broth, rather than during purification.

When HBsAg fractions separated from widely separated regions of SEC peak were reduced and analyzed by denaturing SEC, differences in the chromatographic profile were observed (Fig. 4). Under these conditions, all the fractions showed the presence of LMWPs in the particle composition. However, the LMWP peak was minimal for HBsAg from fraction 1 and maximal for HBsAg from fraction 3, in which it was even higher than the HBsAg monomer-peak. This suggests that HBsAg particles from fraction 3 are mainly composed of LMWPs rather than by intact monomers. Similarly, in SDS–PAGE/silver staining of SEC fractions (Fig. 5), HBsAg from fraction 3 generated the largest number of LMWP bands among the fractions analyzed, although the HBsAg monomer band remained highly intensive, due to a high affinity of HBsAg for silver nitrate [19]. Interestingly, the HBsAg monomer band from fraction 1 migrated slightly larger than the corresponding bands from fractions 2 and 3. In previous work, a similar electrophoretic behavior was described for a small fraction of HBsAg particles retarding on to the SEC column and eluting in SEC after the main peak of HBsAg [11]. This fraction was highly antigenic in ELISA and composed by HBsAg particles similar to those from the maximum of SEC peak, as shown by electron microscopy (data not shown). This fraction was capable of self-assembly into higher-order HBsAg structures, as shown by SEC [11]. However, a more information is necessary to interpret a possible relation between this fraction and HBsAg aggregates.

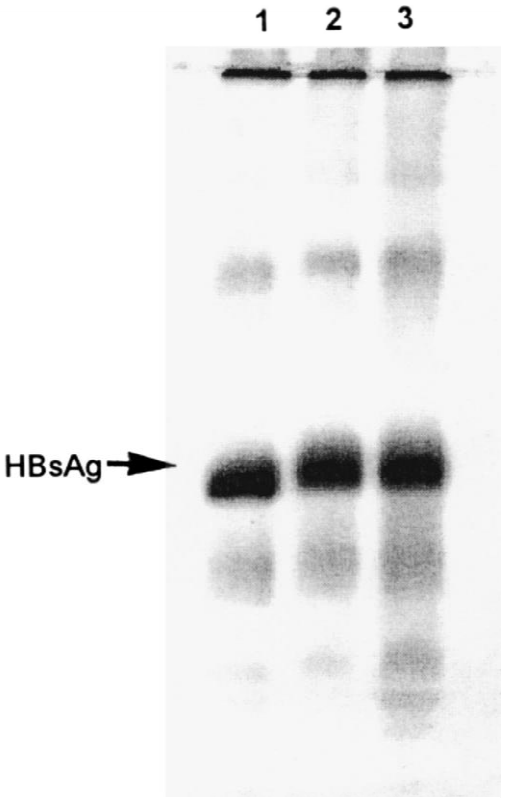

Fig. 5. SDS–PAGE/silver staining of HBsAg fractions isolated by preparative SEC and reduced by DTT/M sample buffer: fraction 1 (lane 1), fraction 2 (lane 2) and fraction 3 (lane 3). Corresponding fractions indicated on chromatogram in Fig. 1. Amount of HBsAg, 1.5 μg per spot.

Since assembled HBsAg particles are known to be resistant to proteases [25], in vivo formation of LMWPs should occur by non-proteolytic cleavage of HBsAg monomers. The HBsAg monomer contains a large number of labile amino acids into surface-exposed hydrophilic domains which are prone to the covalent modifications, such as Ser, Thr beta-elimination and racemization, Asn deamidation, Cys, Trp, Tyr oxidation or the hydrolysis of peptide bonds [26]. In yeast, synthesized HBsAg particles are potentially exposed to the oxidative processes me-

diated by reactive oxygen species. These radicals can lead not only to oxidation of amino acid residue side chains and formation of protein–protein cross-linkages, but also to oxidation of the protein backbone resulting in protein fragmentation [27,28]. Peptide bond cleavage can occur also as a result of radical attack to glutamyl, aspartyl and prolyl side chains [27]. To demonstrate that oxidation of HBsAg can produce protein degradation, HBsAg from fraction 2 was incubated with hydrogen peroxide and the oxidized sample analyzed by denaturing SEC. As expected, a considerable increase in the height of LMWP peak was observed (Fig. 6), suggesting that protein oxidation may be one of the reasons for the appearance of LMWPs in the composition of HBsAg. In accordance to this hypothesis, higher levels of protein degradation detected for HBsAg from fraction 3 may be responsible for its lower antigenicity as compared to HBsAg from fraction 2 [11]. Apparently, HBsAg particles seem to retard on to the SEC column as the extent of protein degradation within HBsAg particle increases. Protein degradation is expected to alter the surface of HBsAg particles inducing large modifications in charge and/or hydrophobicity.

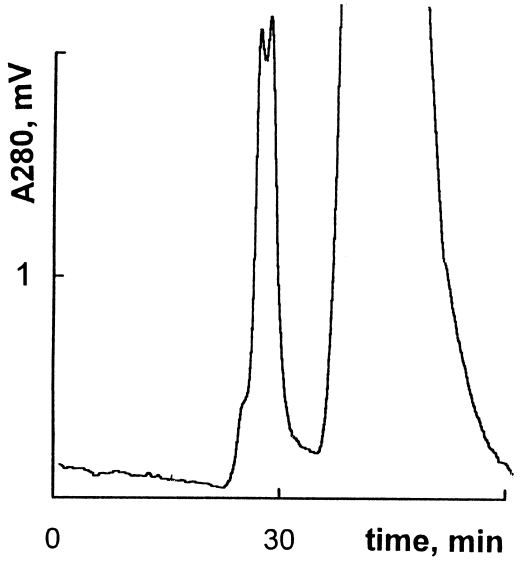

Fig. 6. Chromatogram of HBsAg fraction isolated by preparative SEC from the maximum of HBsAg peak and oxidized with H_2O_2. Preparative chromatographic conditions as in Fig. 1. Analytical chromatographic conditions as in Fig. 2. Injection volume, 100 μl; amount of HBsAg, 150 μg.

Although multidimensional approach to the analysis of HBsAg has provided a considerable amount of valuable information, many issues are still remained unclear: (1) What are the factors originating HBsAg particle heterogeneity? (2) Are the protein or lipid domains on HBsAg particle surface responsible for the heterogeneity, either directly by providing the intermolecular contact sites, or indirectly, via their influence on overall conformation? (3) Has the particle heterogeneity any role in immunological reactivity of HBsAg? Although the information on the high-resolution structure of HBsAg is insufficient to answer these questions, the present report strongly suggests protein degradation (oxidation) within HBsAg particles as a possible source of HBsAg heterogeneity.

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