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Aggregation of recombinant hepatitis B surface antigen in *Pichia pastoris*

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Abstract

The combination of immunoaffinity and size-exclusion chromatography (SEC) is a powerful tool to analyze multiprotein particle assembly. This approach was used to investigate the source of aggregation of recombinant hepatitis B surface antigen (HBsAg) detected in purified material. As HBsAg aggregation does not originate in the stresses, such as the concentration of HBsAg solutions, temperature and chaotropic agents, it is less probable that the HBsAg aggregate is produced during the process. To test whether aggregation takes place in vivo, crude yeast extract containing the expressed HBsAg was fractioned on a Sephacryl S-400 column just after cell disruption, and each fraction immunopurified individually. As a result, the HBsAg aggregate was isolated from a fraction corresponding to the elution of large particle aggregates only, not native HBsAg particles. It was biologically active, which demonstrates aggregate formation by specific assembly of partially or wholly folded HBsAg intermediates.

Keywords: Hepatitis B surface antigen

1. Introduction

The demonstration in 1982 [1] that yeast-derived hepatitis B surface antigen (HBsAg) could self-assemble into antigenic 20-nm particles spurred the attention towards an important class of proteins: virus-like particles. Nowadays, the use of recombinant virus-like particles as carriers for foreign antigenic determinants has more and more become a matter of interest in vaccine applications [2–6]. To demonstrate the faithfulness of the expression, multiprotein structures are commonly characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, immunoblotting, isopycnic centrifugation in CsCl gradients and electron microscopy; SDS-PAGE and immunoblotting demonstrate purity and identity of monomer subunits expressed, whereas CsCl sedimentation analysis and electron microscopy reveal important characteristics of self-assembled particles, such as their buoyant density, shape and size. Less frequently, size-exclusion chromatography (SEC) is

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used to demonstrate the efficiency of self-assembly. SEC is a powerful technique for the analysis of large protein aggregates [7,8], organic polymers [9], inorganic colloidal particles [10] and another supramolecular assemblies [11,12]. It is currently in use for quality control of HBsAg produced by recombinant DNA technology [13]. Using SEC, the purified HBsAg was shown to be heterogeneous, as evidenced by the presence of a shoulder before the main peak in the chromatogram (Fig. 1), suggesting the presence of soluble aggregates. However, other commonly used methods (SDS-PAGE, immunoblotting and electron microscopy) failed to detect the heterogeneity of HBsAg, even in the SEC-separated aggregate fraction [14]. A similar shoulder in the SEC profile has been reported for plasma-derived HBsAg [15] and HBsAg expressed in cell cultures [16]; in these preparations, the shoulder corresponded to the elution of filamentous particles, as shown by electron microscopy [15,16]. However, no filaments were detected by electron microscopy of the HBsAg aggregate fraction isolated from purified, *Pichia pastoris* expressed HBsAg [14]. The heterogeneity of HBsAg is not detected when the TSK G6000 PW column is used (Fig. 2). Since the absence of aggregates in the final drug is highly recommended [17], the HBsAg is purified by SEC at the last processing step using the TSK G5000 PW column [18].

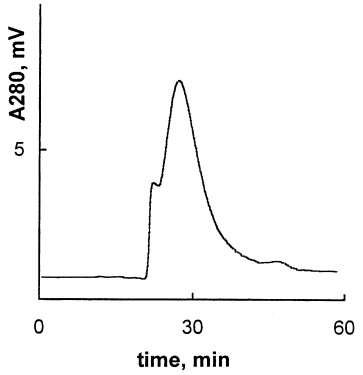

Fig. 1. Chromatogram of HBsAg-containing material before SEC purification. Conditions: TSK G5000 PW (600×7.5 mm I.D.) supplied with a TSK GPW guard column (75×7.5 mm I.D.); eluent, PBS, pH 7.0; flow-rate, 0.5 ml/min; detection, UV at 280 nm; injection volume, 100 μl; sample concentration, 1.35 mg/ml; retention time of HBsAg, 29.63 min.

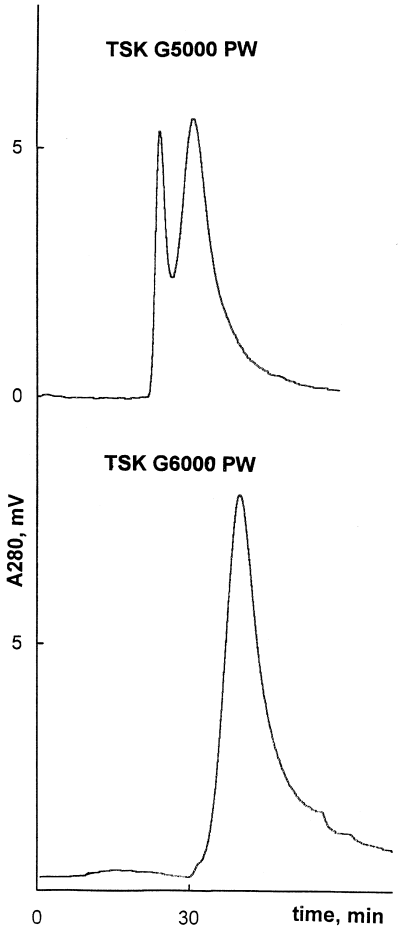

Fig. 2. Chromatographic performance of the mixture containing aggregate and aggregate-free HBsAg controls. Conditions as described in Fig. 1. TSK G6000 PW (600×7.5 mm I.D.) supplied with a TSK GPW guard column (75×7.5 mm I.D.); injection volume, 150 μl; sample concentration, 0.25 mg/ml (aggregate), 0.77 mg/ml (aggregate-free HBsAg).

Without a doubt, aggregate formation affects the recovery of recombinant products. By determining the source and cause of aggregation, it would be possible to limit or prevent it, thereby maximizing the yield of correctly assembled particles. The study reported here was carried out to determine whether HBsAg aggregate is formed during fermentation broth or purification.

2. Experimental

2.1. Materials

All chemicals used were analytical grade purchased from Merck (Darmstadt, Germany). Sephacryl S-400, superfine, was from Pharmacia (Uppsala, Sweden). 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, 250 m*M* NaCl and 0.02% NaN_3, pH 7.0. Purified HBsAg (>95% HPLC, SDS-PAGE) was obtained from yeast fermentation after multistep purification process [18], in the National Center for Bioproducts (Havana, Cuba). It contained 1.55 mg/ml HBsAg and was used for the preparation of aggregate-free HBsAg control and as a standard in SDS-PAGE and protein quantitation. The Sepharose-immobilized CB-Hep.1 mouse monoclonal antibodies were purchased from the Center for Genetic Engineering and Biotechnology (Havana, Cuba).

2.2. Preparation of aggregate and aggregate-free HBsAg controls

The HBsAg aggregate is copurified with the correctly folded HBsAg particles through the whole purification process [16]. It was separated by SEC at the last step of purification: the concentrated, purified HBsAg (200 mg) passed through a TSK G5000 PW column (Tosohaas; 600×55 mm I.D.) fitted with a TSK GPW guard column (Tosohaas; 75×55 mm I.D.). Elution was with PBS at 7 ml/min. Detection was at 280 nm. The fractions (7 ml) containing HBsAg aggregate only (100%, HPLC) were pooled. To prepare aggregate-free HBsAg control, purified HBsAg was rechromatographed on the TSK G5000 PW column as described above, and the fraction corresponding to the maximum of HBsAg peak was collected.

2.3. Preparation of crude yeast extract

Recombinant HBsAg was produced by fermentation of a recombinant strain of *Pichia pastoris* C226 in saline medium supplemented with glycerol, and its expression was induced with methanol. The cells were harvested by centrifugation and disrupted on a bead mill (KDL type, WAB, Switzerland) at 4°C. Disruption buffer contained 20 m*M* Tris–HCl, pH 8.0, 3 m*M* EDTA, 0.3 *M* NaCl, 3 *M* KSCN and 10 g/l sucrose. The homogenate was centrifuged for 30 min at 10 000 *g*. The temperature was fixed at 4°C during centrifugation. The supernatant was carefully separated, passed through 0.8-μm pore size filter and immediately subjected to SEC fractionation.

2.4. SEC fractionation of crude yeast extract

A column XK 50/60 (Pharmacia) was packed with Sephacryl S-400 (785 ml; bed height 40 cm) and equilibrated (flow-rate 1 ml/min) with PBS. The column was operated in conjunction with an FPLC instrument (Pharmacia). Detection was at 280 nm. The conditions of fractionation were adjusted based on the retention volumes of aggregate and aggregate-free HBsAg controls determined previously. Clarified yeast extract was applied on to the column (25 ml) and the eluting material collected into three major fractions: (1) highly turbid, corresponded to the elution of large particle aggregates only; (2) less turbid, in which correctly assembled, nonaggregated HBsAg particles were expected to coelute and (3) transparent, highly dense yellow solution, corresponded to the elution of lower-molecular-mass proteins and yeast components. Each fraction collected was subjected to SEC analysis. Total protein in crude yeast extract and in the fractions separated was determined based on binding to Coomasie blue dye as described by Bradford [19] using purified HBsAg as a reference standard.

2.5. SEC analysis

Analysis was performed on a HPLC system (Pharmacia) which included a 2248 pump, variable-wavelength UV detector operated at 280 nm and a 2221 programmable integrator. The system was fitted with a TSK G5000 PW column (Tosohaas; 600×7.5 mm I.D.) and a TSK GPW guard column (Tosohaas; 75×7.5 mm I.D.). The samples were filtered through 0.45-μm pore filter and fixed volumes (50 μl) injected into a 200-μl loop; elution was with PBS at 0.5 ml/min.

2.6. Immunoaffinity chromatography

A column (XK 16/20 (Pharmacia) was packed with Sepharose-immobilized CB-Hep.1 mouse monoclonal antibodies (15 ml; coupling density, 5.0 mg IgG per ml of gel; bed height, 12 cm). and equilibrated with PBS. All affinity column operations were performed at a flow-rate of 50–100 cm/h and detection at 280 nm. Each SEC fraction of yeast extract was processed individually. Fractions 1 and 2 (25 and 10 ml, respectively) were loaded directly on to the column, whereas fraction 3 (35 ml) was previously dialysed against PBS. In each experiment, the amount of HBsAg applied was 20–25 times smaller than the maximal adsorptive capacity of the column, as assayed by ELISA. After loading, the column was washed with 70 ml of PBS. Bound HBsAg was eluted with PBS containing 3 *M* KSCN, pH 7.0, and assayed by SEC, SDS-PAGE, enzyme-linked immunosorbent assay (ELISA), Lowry method [20] and electron microscopy. The immunoaffinity column was stored in PBS at 4°C when not in use.

2.7. SDS-PAGE

Yeast extract was treated with the same volume of sample buffer containing SDS and 2-mercaptoethanol and 30 μl of the reduced sample were applied on to 12.5% gel according to Laemmli [21]. Purified HBsAg, a standard, was treated and electrophoresed in the same way. The gel was stained with Coomasie blue (Bio-Rad, Richmond, CA, USA). For silver staining [22], fractions 1, 2, 3 and immunoaffinity column washes were diluted five times with water prior to reduction. The immunoeluates of HBsAg were not diluted. The samples were reduced by adding the same volume of sample buffer and 30 μl were applied to the gel.

2.8. ELISA

The ELISA system used lamb polyclonal antibodies against HBsAg for coating plates and a conjugate of antibodies with horseradish peroxidase for detection, both purchased from the Center for Genetic Engineering and Biotechnology. The working standard preparation of HBsAg was calibrated against the Paul Erlich Institute (Frankfurt, Germany) standard.

2.9. Measurement of HBsAg aggregates

Measurement of HBsAg aggregates was carried out using the above-described SEC procedure and programmable integration. The peak areas were previously normalized using detector response factors for aggregate and aggregate-free HBsAg controls. After that, the area percentage of HBsAg aggregate peak was used to calculate the percent aggregate.

To test aggregate formation during concentration, the aggregate-free HBsAg control was concentrated in Centricon tubes. The samples containing 0.5–10 mg/ml HBsAg, as shown by the Lowry test, were analyzed by SEC. To test aggregate formation in 3 *M* KSCN, the aggregate-free HBsAg control was stored at 37°C in PBS containing 3 *M* KSCN buffer, pH 7.0 and daily assayed by SEC. To test aggregate formation by preformed aggregates, the aggregate HBsAg control (50 μg) was added to the aggregate-free HBsAg control containing 150 or 350 μg of HBsAg. The experiment was carried out in PBS. The mixture (400 μl) was tested by SEC just after mixing and then after storage for 2, 6, 24, 48 h and 1 week at 37°C.

2.10. Electron microscopy

Two drops of sample were placed on to a 400-mesh copper grid coated with formvar-carbon film. After 1 min contact time, excess sample was blotted off. Grids were stained with 2% aqueous solution of uranyl acetate and examined in a Jeol-JEM 2000EX transmission electron microscope, acceleration voltage 80 kV and magnification 50 000× (Fig. 10A) or 40 000× (Fig. 10B, C and D).

3. Results and discussion

3.1. Resistance of HBsAg to aggregation

Recombinant proteins experience stress conditions throughout a wide range of processing environments: high and low temperature, extremes of pH, high

concentrations of chaotropic agents, inadvertent crossings of solubility thresholds in purification and exposure to solid and air interfaces during concentration, centrifugation, filtration and pumping. These stresses can induce structural changes in the protein leading to aggregation [23–25]. To determine if the HBsAg could be altered by the process, we carried out various analyses using aggregate-free HBsAg control. Its SEC-profile is shown in Fig. 3A. Under stress conditions, appearance of a 23.23-min peak corresponding to the elution of HBsAg aggregate control would indicate aggregate formation. The stress conditions tested were the concentration process and presence of 3 *M* KSCN, due to their relevance in HBsAg purification. After concentration, SEC showed no significant amount of soluble HBsAg aggregates, even after prolonged aging of concentrated solutions (Table 1). No aggregates were detected after storage of aggregate-free HBsAg control for 2 days at 60°C (data not shown), or for 1 week at 37°C in PBS buffer containing 3 *M* KSCN, pH 7.0 (Fig. 3B). On the other hand, under these conditions, the HBsAg aggregate failed to undergo degradation, as detected by SEC (data not shown). In general, the resistance of a given protein to the stresses is related to the stability of its native conformation. The conformational stability of HBsAg monomers in the assembled particles should be high, due to a rigid particle structure: HBsAg contains a well-developed hydrophobic core [26] and other tertiary interactions in the helices are stabilized mainly by lipid–protein interactions. Hence, it is less probable that HBsAg aggregate is produced as a result of alterations in the HBsAg structure by the process.

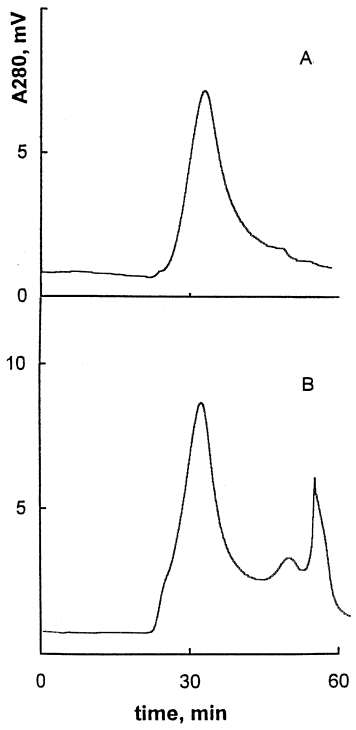

Fig. 3. Chromatogram of aggregate-free HBsAg control in PBS (A) and in PBS containing 3 *M* KSCN, after storage for 1 week at 37°C (B). Conditions as in Fig. 1. Injection volume, 100 μl; sample concentration, 1.55 mg/ml; retention time, 30.25 min.

Table 1
Formation of HBsAg aggregates after concentration and storage of the aggregate-free HBsAg solution, as determined by SEC

HBsAg (mg/ml)	Storage time (months)	HBsAg aggregate (%)
0.5	0	0
0.5	6	0.4
2.5	0	0
2.5	6	0.3
5.0	0	0.4
5.0	6	0.8
10.0	0	1.3
10.0	6	3.5

Incubation conditions, PBS, pH 7.0; 37°C; chromatographic conditions as described in Fig. 1; injection, 50 μg.

To examine whether HBsAg aggregation can be seeded by preformed aggregates, different amounts of aggregate control were added to the aggregate-free HBsAg solution, and the sample mixtures assayed by SEC. As a result, the HBsAg aggregate was capable of aggregating a small portion of native HBsAg just after mixing (Fig. 4, Table 2). No further aggregation occurred during incubation for 1 week at 37°C. The HBsAg aggregate probably contains few active sites on its surface available to accommodate HBsAg particles. As the saturation of these sites is reached, the aggregation stops. Hence, the seeding of HBsAg aggregation by preformed aggregates is impossible. A similar result was obtained for ß-amyloid peptide, prion protein, transthyretin, islet amyloid polypeptide, P22 tailspike and P22 coat protein [27–31], whose aggregation was shown to proceed via specific association of partially folded intermediates. Hence,

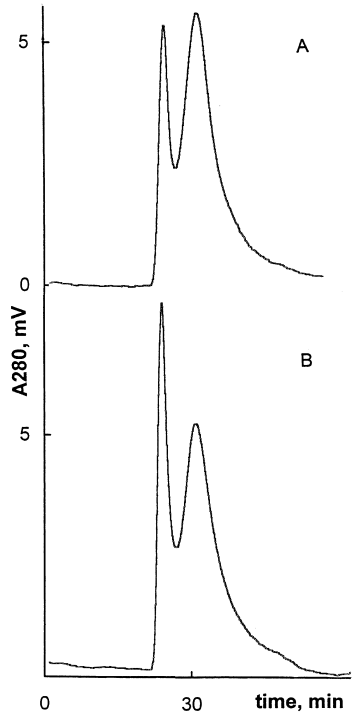

Fig. 4. Chromatogram of the mixture containing aggregated and nonaggregated forms of HBsAg just after mixing (A) and after incubation of the mixture for 2 h at 37°C (B). Conditions as in Fig. 1. Injection volume, 100 μl. HBsAg aggregate: concentration, 0.125 mg/ml; retention time, 23.23 min. Nonaggregated HBsAg: concentration, 0.375 mg/ml; retention time, 30.25 min.

the HBsAg aggregation is not related to seeding by the HBsAg aggregate.

3.2. Isolation of HBsAg aggregate from crude yeast extract

If HBsAg aggregation takes place during the fermentation process, the HBsAg aggregates should be present in crude yeast extract just after cell disruption. To isolate HBsAg from cell cultures, isopycnic centrifugation in CsCl gradients is commonly used. However, this method was reported to alter the particle composition of HBsAg, in particular its lipid content [32]. Hence, in the present work, the HBsAg species were isolated from yeast extract by combination of SEC with immunoaffinity chromatography. The immunoaffinity column was packed with the Sepharose-bound CB-Hep.1 mouse monoclonal antibodies. These antibodies recognize conformation-non-dependent epitopes on the HBsAg particle surface and retain and concentrate all the HBsAg monomer-containing species formed independently on folding and aggregation [33]. By using SEC, the possibility of structural alteration of HBsAg is completely excluded [13,14]. To prove this with respect to immunoaffinity chromatography, the column was saturated with the aggregate-free HBsAg control preparation and the bound material eluted after 2, 12 and 24 h on the column. No

Table 2
Induction of aggregation by addition of HBsAg aggregate to the aggregate-free HBsAg solution, as determined by SEC

Composition of mixture	Incubation time (h)	HBsAg aggregate (%)
50 μg HBsAg aggregate	0	23.68
+150 μg aggregate-free HBsAg	2	30.39
	6	30.39
	24	36.43
	48	33.51
	168	37.29
50 μg HBsAg aggregate	0	7.77
+350 μg aggregate-free HBsAg	2	10.11
	6	11.54
	24	10.48
	48	11.55
	168	13.54

Incubation conditions, PBS, pH 7.0; 37°C; chromatographic conditions as described in Fig. 3.

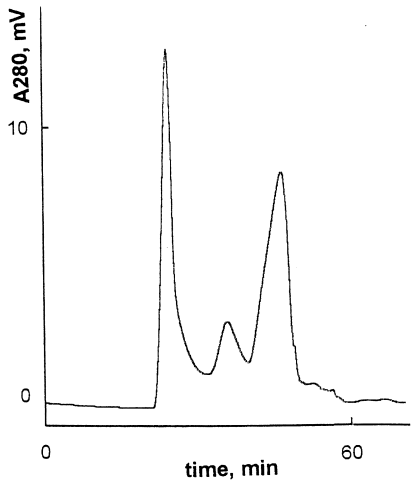

Fig. 5. SEC profile of crude yeast extract containing expressed HBsAg. Conditions as in Fig. 1. Injection volume, 25 μl; total protein concentration, 7.5 mg/ml; HBsAg concentration, 0.15 mg/ml.

HBsAg aggregation was detected by SEC in the eluate.

The SEC profile of yeast extract is shown in Fig. 5. The HBsAg aggregate, if present, should coelute in the first peak. Hence, the strategy used was: (1) to separate a fraction of yeast extract corresponding to the elution of large particle aggregates only, not smaller HBsAg particles; (2) separate the HBsAg species eluting in this fraction by immunoaffinity chromatography and (3) obtain the SEC profile of immunopurified material. Five repetitive processes including fermentation broth, cell disrupture, SEC fractionation of yeast extracts and immunopurification were carried out to demonstrate consistency of the results. The three fractions separated by SEC were: (1) corresponding to the elution of HBsAg aggregate only; (2) in which native HBsAg particles are expected to elute and (3) corresponding to the elution of lower-order protein oligomers (Fig. 6). These fractions, assayed by SDS-PAGE under reducing conditions, showed the presence of 24 000 HBsAg monomer band (Fig. 7B). Since this band is stained weakly with Coomasie blue (Fig. 7A) and fairly well with silver nitrate (Fig. 7B), the latter method was used for detection. By using silver staining, the concentration of immunopurified samples prior to electrophoresis was avoided. As determined by ELISA, the distribution of antigen expressed between fractions 1, 2 and 3 was 42±3, 33±2 and 25±5%, respectively. Surprisingly, a large portion of active material eluted in the HBsAg-aggregate-containing fraction. To separate HBsAg-containing material from yeast contaminants, each fraction was passed through the immunoaffinity column. The binding was efficient, because the column washes did not contain HBsAg, as established by ELISA and SDS-PAGE (Fig. 7B, lane 2). Bound material was eluted by PBS in 3 *M* KSCN and immediately assayed by SEC and SDS-PAGE.

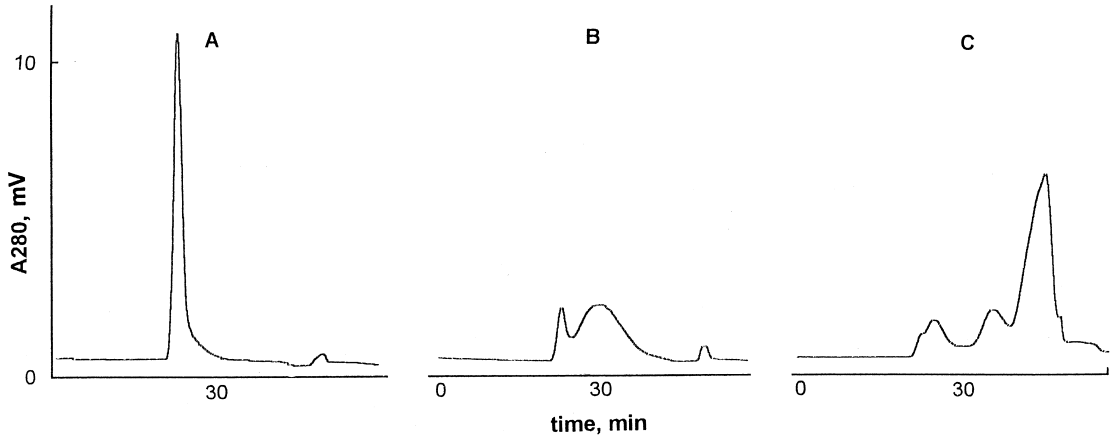

Fig. 6. SEC profile of fraction 1 (A), fraction 2 (B) and fraction 3 (C) separated from crude yeast extract on XK-60 column packed with Sephacryl S-400. Conditions as described in Fig. 1. Injection volume, 50 μl.

The immunoeluate from fraction 1 gave a SEC-peak coinciding with the HBsAg aggregate control (Fig. 8A); in SDS-PAGE under reducing conditions, it migrated as the 24 000 monomer of HBsAg (Fig. 7B, lane 3). The immunoeluate from fraction 2 produced two peaks in SEC corresponding to aggregated and correctly folded HBsAg (Fig. 8B). The active material from fraction 3 was represented mainly by the lower-order oligomer of HBsAg eluting in the 46.4 min peak (Fig. 9). This structure was described in our previous work [34]. When the 46.4-min peak of HBsAg was collected and reassayed by SEC, the 26.45 and 34.25 min peaks

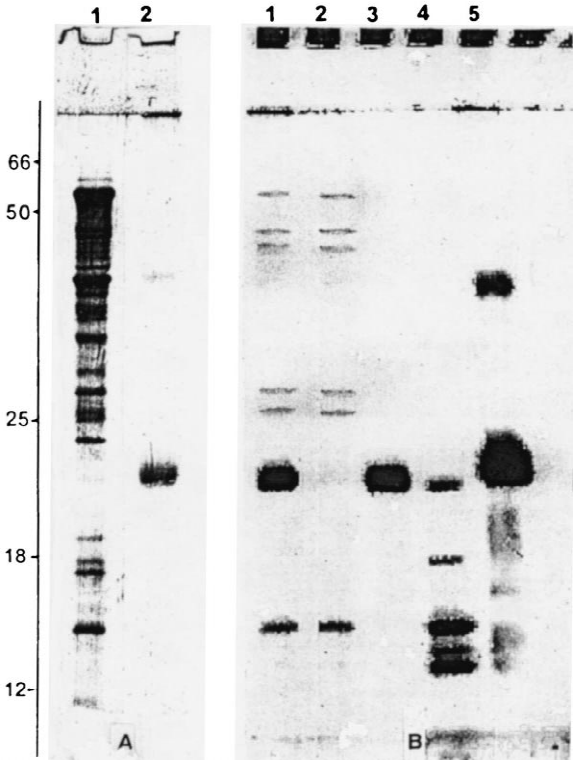

Fig. 7. SDS-PAGE followed by Coomasie staining of crude yeast extract (A, lane 1) and HBsAg control (A, lane 2). SDS-PAGE followed by silver staining of fraction 1 (B, lane 1), nonretaining by immunoaffinity material from fraction 1 (B, lane 2), immunoeluate from fraction 1 (B, lane 3 and 5) and fraction 3 (B, lane 4). Conditions: gel, 12.5%; the sample buffer, 0.06 *M* Tris–HCl, 2% (w/v) SDS, 5% (v/v) mercaptoethanol and 10% (w/v) glycerol; application volume, 30 μl; Amount of protein applied, 200 μg (A, lane 1), 25 μg (A, lane 2), 20 μg (B, lane 1, 2, 4), 0.7 μg (B, lane 3) and 3.5 μg (B, lane 5).

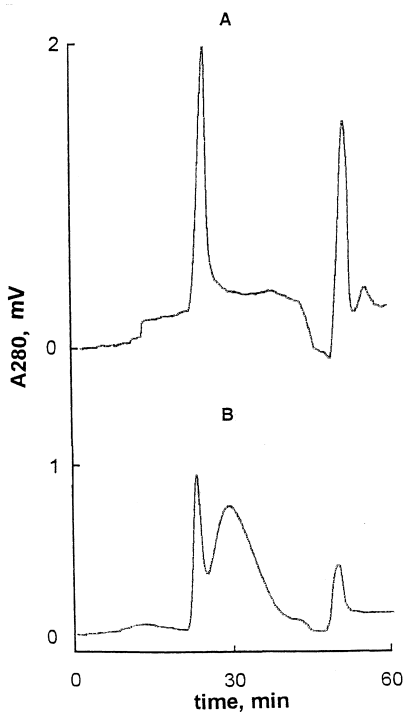

Fig. 8. SEC profile of fraction 1 (A) and fraction 2 (B) from crude yeast extract cleaned up using immunoaffinity chromatography. Conditions as described in Fig. 1. Injection volume, 200 μl; retention time of HBsAg, 23.23 min (A), 23.23 min and 29.85 min (B).

appeared in the chromatogram (Fig. 9B). Hence, this oligomer may be a precursor in self-assembly of a determinant population of HBsAg particles. The presence of HBsAg aggregate in fraction 1 in which only large particle aggregates are eluted, is evidence that the HBsAg aggregate was already formed in vivo. On the other hand, this fact excludes once more a possibility of aggregate formation after immunopurification, as a result of alterations in the HBsAg assembly induced by drastic elution conditions.

The freshly isolated HBsAg aggregate was biologically active ($[HBsAg]_{ELISA}/[HBsAg]_{Lowry}=$ 1.05) and represented about 40% of total HBsAg expressed. The high antigenicity of HBsAg aggregate is in contrast with our previous results that the $[HBsAg]_{ELISA}/[HBsAg]_{Lowry}$ ratio in purified aggregate control is 0.20. The HBsAg aggregate probably looses antigenicity with aging.

The denatured and reduced HBsAg aggregate

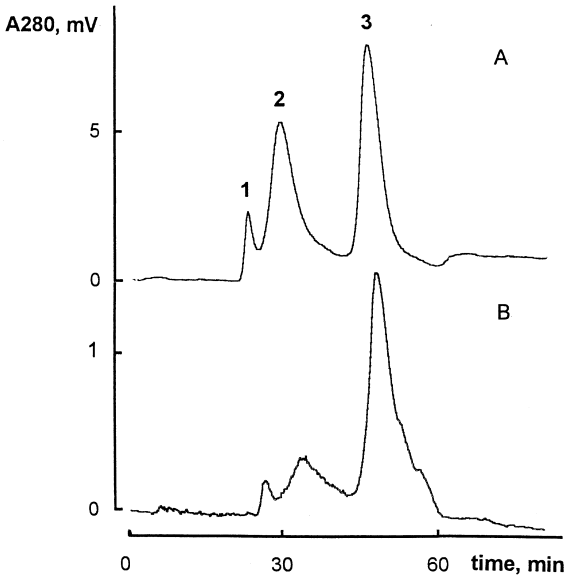

Fig. 9. SEC profile of fraction 3 from crude yeast extract cleaned up using immunoaffinity chromatography (A). Conditions as described in Fig. 1. Injection volume, 200 μl; retention time of HBsAg, 23.23 min (1), 30.25 min (2) and 46.4 min (3). The maximum of peak 3 was collected, concentrated and rechromatographed (B). Injection volume, 200 μl.

showed the presence of dimers in SDS-PAGE when considerably large amounts were loaded onto the gel (Fig. 7B, lane 5). Dimerization is the first step in self-assembly of HBsAg [35]. In general, dimer formation of membrane proteins requires previous extensive folding of interacting structures that may be whole monomer subunits or their transmembrane domains [36,37]. Hence, the presence of dimers in the HBsAg aggregate provides evidence that the precursor of aggregation is assembled from folded monomers and dimers. The detection of HBsAg dimers by SDS-PAGE under reducing conditions is not surprising because the native helical structure of membrane proteins is frequently preserved after SDS denaturation [38,39] and reduction [40].

Photomicrographs of the HBsAg aggregate-free and aggregate controls, as well as the HBsAg aggregate isolated directly from crude yeast extract by SEC–immunoaffinity chromatography are shown in Fig. 10. All these samples are represented by 22-nm spherical antigen particles; apparently, no alterations in the morphology of HBsAg particles occur in the aggregate samples. The HBsAg aggregate isolated directly from yeast extract by SEC–immunoaffinity chromatography seems to contain noneliminated components of yeast cells (Fig. 10D). These impurities are not present in the HBsAg aggregate control purified by the procedure used for production of recombinant HBsAg (Fig. 10B and C). Unlike the HBsAg aggregate-free control (Fig. 10A), the HBsAg particles in aggregate samples are fixed by adhesion (Fig. 10B, C and D). Since this pattern was observed for a sample isolated from yeast extract just after cell disruption, it is highly likely that this homotypic adhesion had occurred in yeast cells. The extent of aggregation increases with aging (Fig. 10C). As mentioned above, the HBsAg aggregate was stable during storage in the presence of 3 *M* KSCN. Hence, the particles should be bound each other by nonionic interactions.

4. Conclusions

When all the supporting evidence is considered (resistance of HBsAg to aggregate formation and detection of HBsAg aggregate in the yeast extract), there is no doubt that HBsAg aggregation takes place in yeast. The HBsAg aggregate is assembled in 22-nm particles; it migrates as monomers and dimers in SDS-PAGE, like correctly folded HBsAg; it is biologically active, as shown by ELISA, although inactivation seems to occur with storage. Being strongly antigenic, it may be more immunogenic, than nonaggregated HBsAg fraction, and, thus, useful in vaccine applications. The results obtained here refute our initial hypothesis that HBsAg aggregate formation is due to inefficient folding. From electron microscopic measurements, the HBsAg aggregate is produced by particle adhesion. This raises an important question concerning the origin of HBsAg particle adhesion and point us towards several areas of HBsAg chemistry to explore.

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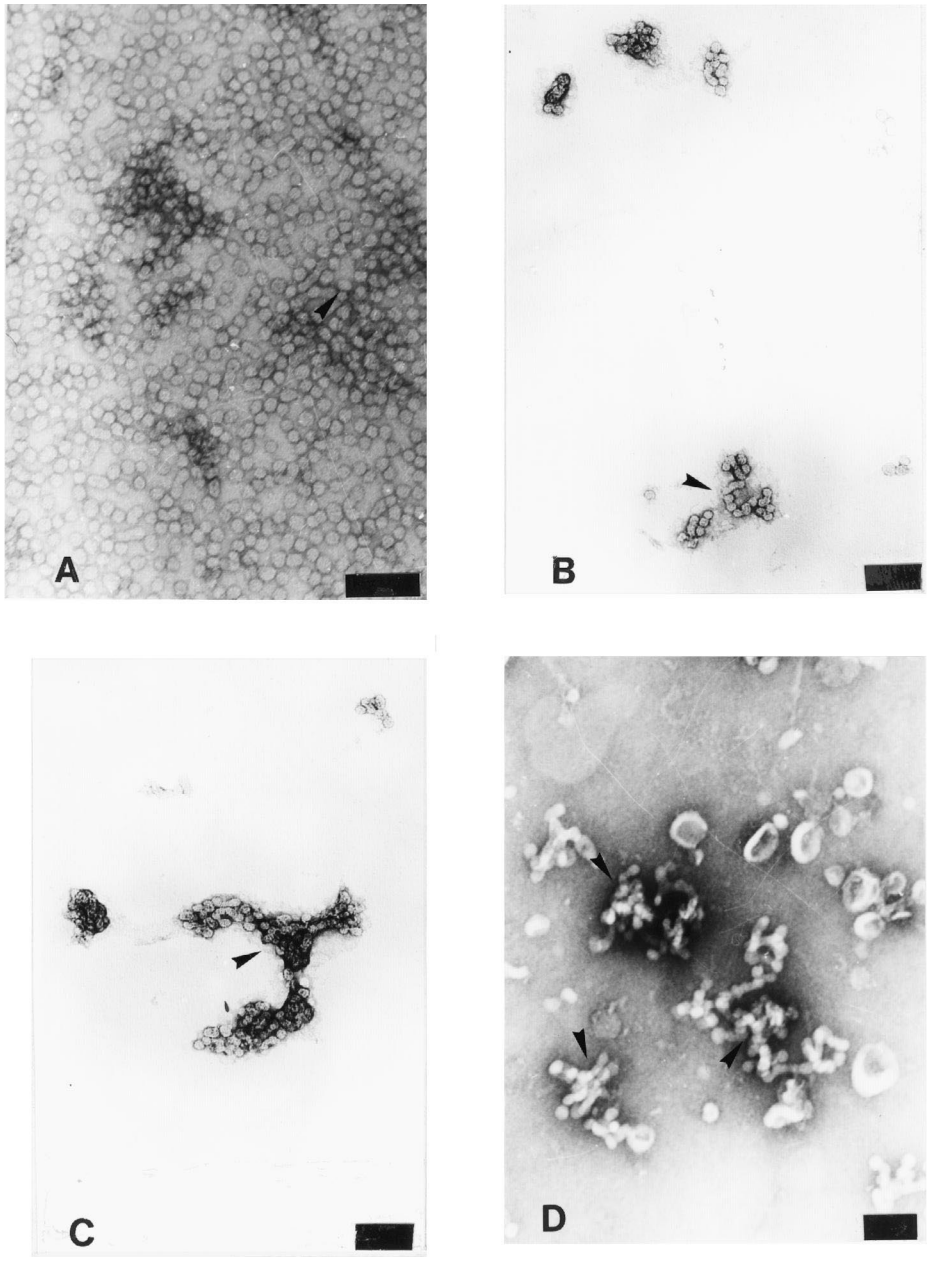

Fig. 10. Electromicrographs of HBsAg aggregate-free control (A), HBsAg aggregate control (B), HBsAg aggregate control stored at 4°C for 12 months (C) and the HBsAg aggregate isolated from crude yeast extract as described (D). Experimental conditions as in text. Scale bar is 100 nm.

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