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High-performance size-exclusion chromatography and molar mass measurement by low-angle laser light scattering of recombinant yeast-derived human hepatitis B virus surface antigen vaccine particles

YOSHIKO SATO
Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)
NOBUYOSHI ISHIKAWA
Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa 768 (Japan)
and
TOSHIO TAKAGI*
Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)

SUMMARY

Two kinds of recombinant yeast-derived human hepatitis B virus surface antigens (HBsAg) for use as vaccines were analysed by high-performance size-exclusion chromatography using a TSK-GEL G6000PWXL column. One consisted of the surface antigen, a glycosylated polypeptide with of about 230 amino acid residues identical with that of human hepatitis B virus in addition to lipids derived from the yeast cell. The other differed in that it consisted of a polypeptide with an additional peptide of nine amino acid residues corresponding to the pre-S2 region. Elution from the column was monitored with a low-angle laser light-scattering detector and also UV and refractive index detectors. The results obtained indicate that both of the antigen particles gave a single symmetrical peak in the elution curves, the molar masses of the particles were 5040 and 4640 kg/mol for the former and the latter, respectively, and the particle size was homogeneous for both of the HBsAg particles. The present approach seems suitable for the characterization of such antigen particles, which will be widely used as vaccines.

INTRODUCTION

Hepatitis type B virus is a widespread disease affecting an estimated 200 million persons throughout the world. An antigenic particle discovered in a human serum in the 1960s was named Australian antigen, and was eventually shown to be hepatitis B virus surface antigen (HBsAg). The antigen turned out to be a particle with a complex composition consisting of glycosylated protein polypeptides and lipids with

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a diameter of about 22 nm as determined by electron microscopy. The HBsAg was purified from sera of carriers of hepatitis B virus, and treated with heat and formaldehyde to produce a hepatitis B vaccine. Limitation of supply and danger of infection were the major demerits of the use of the natural HBsAg particle as a vaccine. Development of the gene engineering technique triggered efforts to develop of a vaccine free from such disadvantages. The production of HBsAg in the form of a particle similar to the natural one was reported by Miyanohara *et al.*[1] using yeast as the host. Recently the vaccine thus prepared became commercially available.

One of our research groups has also succeeded in the production of HBsAg using yeast as the host[2]. Two kinds of the antigens were produced. One containing a protein polypeptide with an amino acid sequence identical with that of the natural HBsAg and the other having a different protein polypeptide in that it had an elongation on the N-terminus consisting of nine additional amino acid residues corresponding to the pre-S2 region. The engineered HBsAg particles can be intensively studied without fear of the infection pertinent to the natural counterpart. The detailed characterization of the yeast-derived HBsAg will also be useful as a test of studies that should be carried out for any such antigen particles produced for various virus diseases. This paper reports the results obtained by characterization of the engineered HBsAg particle by high-performance size-exclusion chromatography (HPSEC) and monitoring of its elution by the low-angle laser light-scattering (LALLS) technique. This study forms part a series of efforts to apply the light-scattering technique to the physico-chemical characterization of biological macromolecules and particles[3].

EXPERIMENTAL

Yeast cells in which HBsAg genome was cloned were cultured in Burkholder medium and were broken by high-pressure homogeneizer. The extract was purified by adsorption–desorption and centrifugation. Further purification was carried out by two subsequent density-gradient centrifugations using sucrose in the first and potassium bromide in the second gradient former. Fractions active with respect to the HBsAg activity were collected and dialysed against 5 m*M* sodium phosphate buffer (pH 7.6) containing 0.02% gelatin. The final concentration was estimated to be 94.4 μg/ml according to the Lowry method using bovine serum albumin as the standard.

For a single high-performance liquid chromatographic (HPLC) analysis according to our procedure, 100 μl of solution containing 30 μg of the vaccine were required. This solution was concentrated prior to HPLC by ultrafiltration using a Centricon-30 tube (Amicon). The gelatin of low molecular weight added for stabilization during storage could be removed by filtration.

The columns were equilibrated and eluted with 0.1 *M* sodium phosphate buffer (pH 6.9) containing 0.02% sodium azide at 25°C. HPLC analysis was carried out at a flow-rate of 0.30 ml/min at room temperature (25°C). In the initial phase of the study, a tandem arrangement of TSK-GEL G6000PWXL and TSK-GEL G4000SW columns in addition to a guard column (Tosoh) was used. The SW column was specially selected for lipoprotein analysis. Most of the later experiments were carried out without the G4000SW column. For application of standard proteins, the column(s) was replaced with a TSK-GEL G3000SWXL column.

Elution was monitored with three detectors connected in series in the following

sequence: a UV absorption photometer (UV detector), a low-angle laser light-scattering photometer (LS detector) and a precision differential refractometer (RI detector). The outputs of these three detectors were used to calculate the molar masses of the HBsAg particles.

RESULTS

Fig. 1 is a schematic diagram of the measuring system used. Fig. 2 shows a typical example of triple sets of elution curves obtained using this measuring system for the two kinds of engineered HBsAg particle. The retention time corresponding to the void volume of the series of columns was 26.5 min as determined by the frontal elution of dextran T500 (Pharmacia). It should be noted that the scattering detector, which is extremely sensitive to the presence of large particles, detected nothing at the retention time corresponding to the void volume. The particle was eluted as a single symmetrical but broad peak at 35 min for both types of HBsAg particles. No protein was eluted behind the peak of the HBsAg particles, despite the deliberate use of the special G4000SW column with a low affinity for lipoproteins in the initial phase of the experiments.

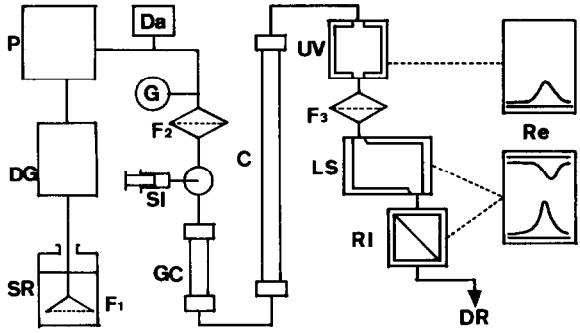

Fig. 1. Schematic diagram of instrumentation. SR = Solvent reservoir; F_1 = sintered stainless-steel filter (Umetani Seiki, Model SFY); DG = degasser (Erma Optical Works, Model ERC-3310); P = dual pump (Tosoh, CCPD); Da = bellows-type damper (Umetani Seiki, Model S-100) with a helically coiled stainless-steel tube (2 m × 0.1 mm I.D.) on the downstream side; G = pressure gauge with safety device to shut down the pump when the pressure exceeds a predetermined value (Umetani Seiki); F_2 = sintered stainless-steel filter (Umetai Seiki, Model SLF); SI = sample injector with a sample loop of 100-μl internal capacity; GC = guard column (Tosoh, TSK-GEL GPWXL, 4.0 cm × 6.0 mm I.D.); C = column (Tosoh, TSK-GEL G6000PWXL, 30 cm × 7.8 mm I.D.); UV = UV detector (Tosoh UV-8000); F_3 = ultrafilter with pore size 0.5 μm (Millipore, Type FHLP 01300); LS = low-angle laser light-scattering photometer (Tosoh, LS-8000); RI = RI detector (Tosoh, RI-8011); Re = two double-pen recorders.

In Fig. 2, retention time was that recorded by the scattering detector. Traces from the other two detectors were corrected for displacements due to the difference in pen positions and the location of the detectors along the flow-line. The displacements were calculated using proteins such as bovine serum albumin and *E. coli* β-galactosidase. The peak positions were identical, irrespective of the method of detection, as expected for a sample without size distribution.

The three detectors give outputs, hereafter denoted by (UV), (LS) and (RI),

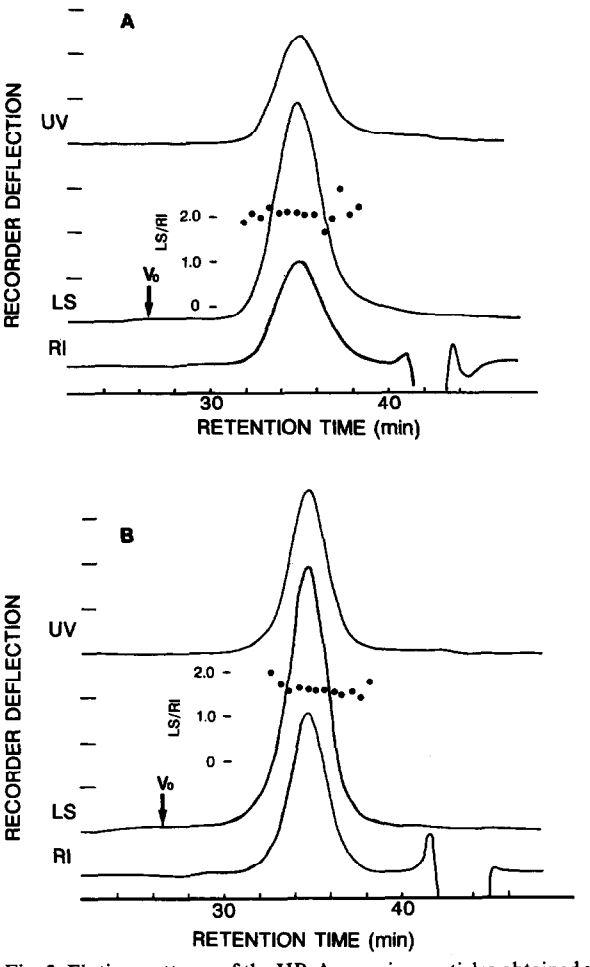

Fig. 2. Elution patterns of the HBsAg vaccine particles obtained using the measuring system shown in Fig. 1 (A) Pre S(+); (B) Pre S(−). The amounts of samples were 39 and 42 μg for A and B, respectively. UV, LS and RI indicate traces given by the respective detectors. The sensitivity setting for these detectors were 0.32, 4 and 2, respectively, for both A and B. As the RI detector is set at the next to the highest sensitivity, the room temperature must be well thermostated in order to obtain a flat baseline. V_0 indicates the retention time corresponding to the void volume detected by the LS detector. ● = (LS)/(RI) ratio.

reflecting changes in UV absorption, light scattering and refractive index. The molar mass (M_r) of a particle the size of which is small enough compared with the wavelength of the light (633 nm) can be determined according to the following equations[3],

$$M_r = K_1 \frac{(\mathrm{LS})}{(\mathrm{d}n/\mathrm{d}c)\ (\mathrm{RI})} \tag{1}$$

or

$$M_r = K_2 \frac{(\mathrm{UV})\ (\mathrm{LS})}{E_1^{1\%}{}_{\mathrm{cm}}\ (\mathrm{RI})^2} \tag{2}$$

where (dn/dc) is the specific refractive index increment and $E_1^{1\%}{}_{cm}$ is the extinction coefficient.

Fig. 2 includes a plot of the (LS)/(RI) ratio that is flat over most of the peak. The (RI)/(UV) ratio also gave a similar flat plot for each of the types of the vaccine particles. This constancy of the (RI)/(UV) ratio strongly suggests that the composition of the HBsAg particle with respect to protein, lipid and carbohydrate is homogeneous. The result does not exclude the possibility that the sample is heterogeneous with respect to composition but homogeneous with respect to size. However, this possibility can be excluded, because the particle was shown to give a narrow band in density gradient centrifugation. If the content of the two major components, protein and lipid, which have markedly different densities were present in different proportions, the sample should not behave in this manner. The sample can be therefore concluded to be homogeneous with respect to the value of the specific refractive index, and hence the flat plot of (LS)/(RI) shown in Fig. 2 is a strong indication that the particles are homogeneous with respect to molar mass according to eqn. 1. The plot of $(UV)/(LS)/(RI)^2$ (not shown) was, however, less flat. This is an inevitable consequence of the serial arrangement of the three detectors, which adversely affects the solute distribution and thus the conformity of the three elution curves.

The specific refractive index increment in eqn. 1 was calculated to be 0.164 ml/g for the Pre S(+) vaccine particle from the weighted average [protein, 53%; lipids, 36%; sugars, 11% (w/w)] of the refractive index increments of the components, assumed to be 0.187, 0.134 and 0.148 ml/g, respectively. For the Pre S(−) particle, no data are available for its composition and the same value as that for the Pre S(+) particle was tentatively assumed. This might be allowed as a first approximation, because the (RI)/(UV) ratio for the Pre S(−) particle was the same as that for the Pre S(+) particle.

Using the value of (dn/dc), the molar masses of the Pre S(+) and Pre S(−) HBsAg particles were calculated to be 5040 ± 480 and 4640 ± 240 kg/mol ($n=3$), respectively, according to eqn. 1. The scale of concentration in the value of (dn/dc) is the weight of the total vaccine particle, and the molar mass thus obtained by light-scattering measurements is the total molar mass of the particle.

On the other hand, the molar mass can also be obtained by using eqn. 2. The extinction coefficient was obtained by the procedure proposed by Edelhoch[4], in which the extinction coefficient on the basis of concentration expressed in terms of g/dl can be calculated from the equation

$$E_1^{1\%}{}_{cm} = (10/M_W)(N_{Trp} \cdot 5690 + N_{Tyr} \cdot 1280) \quad (3)$$

where M_W is molar mass of the Pre S(+) surface atigen protein, N_{Trp} and N_{Tyr} are the contents in residue numbers of tryptophan and tyrosine, respectively, in the Pre S(+)surface antigen protein molecule and 5690 and 1280 are the extinction coefficients of tryptophan and tyrosine residues, respectively. Although eqn. 2 is for a denatured protein in 6 *M* guanidine hydrochloride, the same values can be safely assumed for a native protein without correction, as a first approximation. The Pre S(+) surface antigen protein molecule contains thirteen tryptophan and five tyrosine residues. The extinction coefficient, $E_1^{1\%}{}_{cm}$ in eqn. 3, was found to be 30.9 dl/g · cm. The value for the Pre S(−) surface antigen protein was calculated to be 32.1 dl/g · cm, taking the absence of the Pre S peptide into consideration.

Using the values of the extinction coefficients the molar masses of the Pre S(+) and Pre S(−) vaccine particles were calculated to be 3030 ± 380 and 2440 ± 150 kg/mol ($n=3$), respectively, according to eqn. 2. The scale of concentration in the value of (dn/dc) is the weight of the protein moiety of the respective vaccine particle, and the molar mass thus obtained by light-scattering measurements is the molar mass of the protein moiety.

DISCUSSION

In serum from a patient or carrier of human hepatitis B, a small particle with a diameter of about 22 nm can be observed and has been found consist of the surface antigen of hepatitis B virus particle (HBsAg). The HBaAg particle described here is a particle equivalent to the above natural particle, produced by yeast according to the gene engineering technique. It is interesting that the engineered yeast produces a particle similar to that produced by human cell invaded by hepatitis B virus. Physico-chemical characterization of the egineered particle seemed to provide useful information for the utilization of the particle as a vaccine against hepatitis B, and the present study was started with this motivation.

Electron microscopic observations suggested that there is limited distribution in their sizes[2]. The present results however, strongly indicate that the vaccine particles, both Pre S(+) and Pre S(−) types, are homogeneous with respect to their molar masses and sizes. The molar masses of the Pre S(+) and Pre S(−) HBsAg particles were calculated to be 5040 and 4640 kg/mol, respectively and those of the protein moiety 3030 and 2440 kg/mol, respectively. These values indicate that the Pre S(+) and Pre S(−) particles are composed of 116 and 102 copies of the HBsAg proteins, respectively. The Pre S(+) particle was calculated to be 9 and 24% larger in its molar mass than the Pre S(−) type particle with respect to the total particle and protein moiety, respectively. These figures should not show such a difference if their compositions with respect to molar ratios were the same. Both are, however, significantly larger than the increase expected solely from the presence of the additional nonapeptides. This point needs further careful study and is under further investigation using the dynamic light-scattering technique. The effect of the presence of such an additional peptide or even protein on the nature of such a protein–lipid vaccine particle is worthy of further detailed investigation, because such particles seem to be excellent vehicles for covalently bound biologically active components.

As shown in Fig. 2, the ratios of the outputs of the detectors, such as (LS)/(RI), were constant in the peak region except at the edges. The peaks were unexpectedly broad, however, for the HBsAg particles giving a broad peak. Rechromatography gave a single peak at the same position as in the initial run whether the sample was collected in the front or in the rear region. Application of a sample such as dextran T500 with a skewed molar mass distribution gave an asymmetric peak.

The present results show that particles of large size without a molar mass distribution give a wide peak in the elution curve. It is not yet clear whether a pure molecular sieve effect of the column is responsible for this broadening, or other effects due to the large size of the particle involved. We are currently comparing the elution behavior of other particles of comparable molecular size with that of the vaccine particles.

This study has shown that the engineered vaccine of particle nature against human hepatitis B can be successfully applied to the HPSEC columns and the eluate monitored by a measuring system provided with detectors for molar mass determination. Based on these results, further physico-chemical characterization of the HBsAg particles is now in progress. These studies should be of use in providing examples for the physico-chemical characterization of future vaccine particles for application against various viral diseases.

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