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ISOLATION AND PURIFICATION OF RECOMBINANT HBsAg OF HUMAN HEPATITIS B VIRUS FROM SILKWORM LARVAE

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Recombinant HBsAg coded by preS1-preS2-S regions of hepatitis B virus was expressed in Bombyx mori *silkworm larvae. Recombinant HBsAg was expressed (30-40 μg/mL, 0.1% of the total amount of extracted protein) by larvae infected with recombinant baculovirus rBmNPV-Hep-preS1-S containing cDNA of HBsAg strictly by the polyhedrin gene promoter. Recombinant HBsAg consisting of a polypeptide of molecular weight ~36 kDa (p36) was purified by gel filtration and affinity chromatography to 92% purity.*

Key words: recombinant HBsAg, baculovirus expression system, affinity chromatography.

Human hepatitis B surface antigen (HBsAg) is one of the principal virus envelope proteins. It exists in mature virions in three different forms: large (LHBsAg), medium (MHBsAg), and small (SHBsAg). All proteins LHBsAg, MHBsAg, and SHBsAg have similar C-terminus regions and different N-terminuses regions. HBsAg is coded by preS1, preS2, and S genes and exhibits antigenic determinants that facilitate the development of protective immunity, group-specific determinant **a**, and two pairs of mutually exclusive subtype determinants **d**/**y** and **w**/**r** [1].

Serum HBsAg can form spontaneously spherical particles that consist of macromolecular aggregates of molecular weight ~$3.5 \cdot 10^6$ Da and contain 75% protein and 25% lipid and carbohydrate. The structure of the spherical HBsAg particles is stabilized by intermolecular disulfide bonds [2].

Current DNA-technology can produce recombinant HBsAg in cells of mammals, bacteria, yeast, and plants [3]. There is also an expression system that uses viruses and insect cells than can produce recombinant proteins with properties analogous to those of the natural ones. This expression system has a number of advantages such as post-translational modifications, high productivity, and a product has no apathogenic effect for humans [4, 5]. HBsAg, β-interferon, and β-galactosidase were expressed using baculovirus system, where *Spodoptera frugiperda* cell line was infected with recombinant nuclear polyhedrosis virus (NPV) of *Autographa californica* containing appropriate genes. The expression system in which silkworm (*B. mori*) larvae and NPV of *B. mori* were used as the expression vector was also used to prepare recombinant proteins in commercial quantities. This expression system has definite advantages over the cellular one. The productivity in larvae is comparable and in certain instances greater than that in cells [6]. Furthermore, large numbers of larvae can be easily bred using natural feed.

In the present work we describe methods for producing recombinant HBsAg (rHBsAg) and isolating and purifying it from *B. mori* larvae infected with baculovirus rBmNPV-Hep-preS1-S constructed earlier by us [7, 8]. The recombinant baculovirus rBmNPV-Hep-preS1-S contained a chimeric gene of the preS1-S region of human HBsAg under the late strong polyhedrin gene promoter.

Larvae infected with rBmNPV-Hep-preS1-S were collected at various times after infection (24, 36, 48, 60, 72, 84, 96 h) in order to determine the optimal intervals required to reach the maximal expression of rHBsAg. The collected larvae were frozen in liquid N_2, homogenized, and analyzed for rHBsAg concentration in the homogenates. It was found that the highest expression occurred 72 h after infection (30-40 μg/mL). At long intervals the level of rHBsAg decreased sharply. Apparently this was due to the action of proteases intrinsic to the larvae.

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TABLE 1. rHBsAg Content after Gel Filtration

Fraction	Protein concentration, μg/mL	E_{492}*
A-1	23	0.015
A-2	27	0.072
A-3	30	0.560
A-4	23	0.106
A-5	30	0.030
Pos. control	60	2.624
Neg. control	60	0.041

*E_{492} is the optical density at 492 nm.

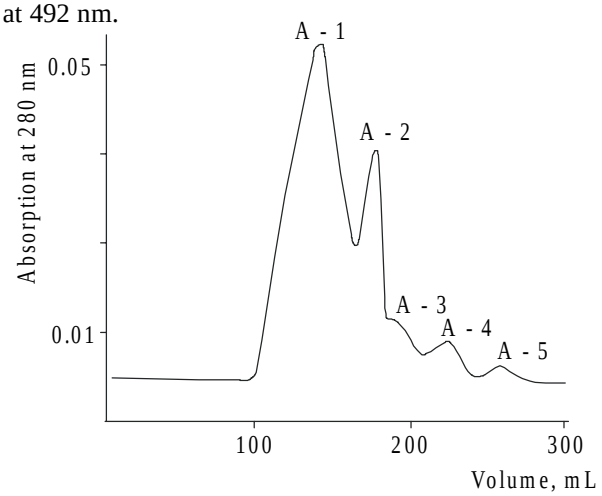

Fig. 1. Chromatogram of HBsAg prepared after gel filtration of the protein-fraction precipitate after sulfate (50%) precipitation.

rHBsAg was purified using the scheme we developed [9]. The proteins precipitated with sulfate (50%) were separated by molecular weight using gel filtration (Fig. 1) to produce five protein fractions. The content of HbsAg in these fractions was determined by enzyme-linked immunosorbent assay (ELISA).

Table 1 shows that the highest amount of rHBsAg was found in fraction A-3. Fractions A-2 and A-4 also contained small amounts of rHBsAg. Taking into account the amino-acid sequence of recombinant protein, it can be assumed that it is hydrophobic. As a result, it associates with other proteins that significantly complicate its isolation and purification.

Fractions A-2, A-3, and A-4 from gel filtration contained rHBsAg, were combined, and were purified by affinity chromatography. Immunoaffinity adsorption chromatography of functionally active proteins that uses immobilized monoclonal antibodies (MAbs) is known in many ways to be a very efficient purification method [10]. The limiting factor of this method is usually the dissociation of adsorbent and ligand. The effectiveness of the dissociation is evaluated from not only the amount of extracted adsorbent but also its specific activity. The dissociating agent may give high quantitative yield of product but may partially or fully denature it, thereby lowering its specific activity [11].

Several affinity columns and various dissociating agents were used to determine the optimal conditions for eluting rHBsAg. MAbs to plasma HBsAg were conjugated to BrCN-Sepharose 4B to synthesize the immunosorbent [12]. The amount of eluted protein was estimated by the Lowry method; its functional activity due to whole antigenic determinants, by ELISA. The results showed that the optimal buffer for eluting rHBsAg was Tris-HCl (20 mM), NaCl (1 M), dextran (0.2 M, MW 60,000), pH 9, which eluted 94% of the adsorbate with full retention of its specific activity (Table 2).

The resulting rHBsAg was analyzed by gel electrophoresis under reducing conditions (SDS—PAGE). The results indicated that the recombinant protein had MW 36 kDa, corresponding to that calculated theoretically from the nucleotide-mass map (Fig. 2). The homogeneity of the prepared rHBsAg was estimated by HPLC (Fig. 3). It was found that the protein was 92% pure.

TABLE 2. Optimal Buffer Composition for Affinity Chromatography

Buffer composition	Extraction, %	Specific activity,* %
20 mM Tris-HCl, pH 11.6	76	98
20 mM Tris-HCl, 1 M NaCl, 0.2 M PG , pH 9	94	98
20 mM Tris-HCl, 3 mM EDTA, pH 7	90	82
0.2 M glycine, pH 3.5	41	67
4.5 M $MgCl_2$	17	84
8 M Urea pH 7.0	100	0
PBS pH 7.0	0	-

*Specific activity is the value characterizing the retention of functional integrity of antigenic determinants.

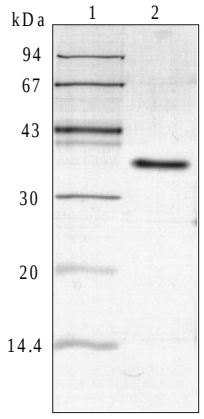

Fig. 2

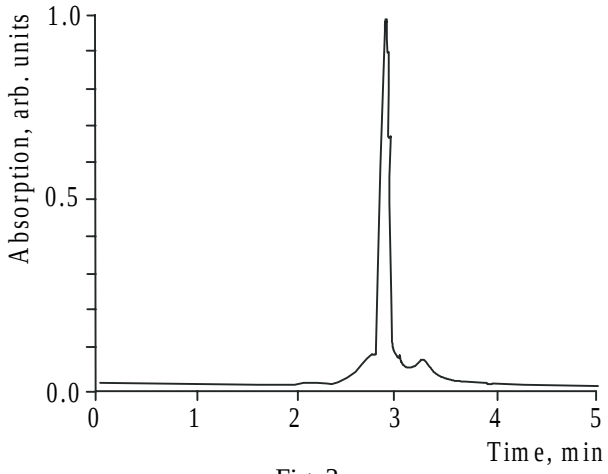

Fig. 3

Fig. 2. Gel electrophoresis (Laemmli) in polyacrylamide gel (12%) under reducing conditions: molecular-weight markers (1), purified rHBsAg formulation (2).
Fig. 3. Chromatogram of purified rHBsAg.

Thus, larvae of silkworm *B. mori* infected with recombinant baculovirus rBmNPV-Hep-preS1-S expressed recombinant HBsAg coded by preS1- and preS2-S regions of human hepatitis B virus. Recombinant HBsAg consisting of a polypeptide with MW ~36 kDa (p36) was purified by gel filtration and affinity chromatography to 92% purity.

EXPERIMENTAL

Reagents (Sigma—Aldrich, Merck), antibody to HBsAg ELISA test system (Ortho, USA), and MAb to plasma HBsAg (Novosibirsk, Russia) were purchased commercially.

Construction of Recombinant Baculovirus. Recombinant baculovirus rBmNPV-Hep-preS1-S was prepared by cotransfection of a mixture of recombinant plasmid p15 containing cDNA of the preS1-S region of human hepatitis B virus and DNA of nuclear polyhedrose virus from *B. mori*. Cotransfection was carried out directly in silkworm larvae [7], which resulted in recombinant baculovirus rBmNPV-Hep-preS1-S with a deleted polyhedrin gene.

Preparation of Recombinant HBsAg in *B. mori* Larvae. Larvae of growth stage V were used for infection by recombinant baculovirus. Virus solution (30 µL) was injected s.c. into the body cavity using a syringe. Shortly (30 min) after infection, larvae were placed on mulberry leaves and maintained at 23-25°C. During the next 3 d, clear symptoms of the infection were not observed. On days 4-5, larvae lost their appetite and gradually perished. Larvae were collected 24, 36, 48, 60, 72, 84, and 96 d after infection and frozen in liquid N_2 in order to determine the maximal level of expression.

Isolation of Recombinant HBsAg. Frozen larvae were ground in a homogenizer (Politron). The homogenate (1 mL) was treated with buffer (3 mL) for extraction (2 mM PMSF, 5 EDTA, 1 DTT, 50 mM Tris-HCl, pH 7, 6) that was cooled on ice. The resulting suspension was homogenized in a homogenizer (glass/Teflon) with cooling (ice) and centrifuged at 14,000 g for 30 min at 4°C. The supernatant was collected as a crude extract of recombinant HBsAg.

The amount of HBsAg at all isolation and purification stages was determined using an antibody to HBsAg ELISA test system (Ortho) and was compared with positive and negative controls and an extract from healthy larvae obtained by the same method as an additional negative control.

Purification of Recombinant HBsAg. A. Salt Precipitation. The crude extract was treated with $(NH_4)_2SO_4$ solution (50% saturated) and centrifuged at 6,000 g for 30 min at 4°C. The resulting precipitate was purified further by gel filtration.

B. Gel Filtration. The precipitate resulting from salt precipitation was dissolved in extraction buffer at a 1:2 volume ratio. If the precipitate did not dissolve completely, then the undissolved material, which consisted of denatured particles, was separated by centrifugation. The separation was performed on a column of TSK HW-50f (Japan) in buffer (20 mM Tris-HCl, 3 mM EDTA, 0.1 M NaCl, pH 7.2). Fractions resulting from the separation were tested for rHBsAg content. Fractions A-2, A-3, and A-4 contained rHBsAg, were combined, and were purified by affinity chromatography.

C. Affinity Chromatography. Immunosorbent was prepared by conjugating MAb to plasma HBsAg with BrCN-Sepharose 4B (Sigma). For this, dialyzed MAb (against 0.1 M sodium phosphate buffer, pH 7.2, with 0.5 M NaCl) was mixed with swelled (in 1 mM HCl) and washed (to remove acid) Sepharose and left overnight at 4°C. The excess of unbounded protein was removed by washing with equilibrating buffer. The amount of immobilized MAb was determined by measuring the total protein by the Lowry method before and after the reaction. Then, the suspension was treated for 2 h with ethanolamine (1 M) at pH 9.0, after which the gel was thoroughly washed with sodium phosphate buffer (pH 7.6 with 1 M NaCl). Immunosorbent (3 mL) containing MAb (5 mg) was placed in a column (5×0.6 cm) and equilibrated with buffer (20 mM Tris-HCl, 3 mM EDTA, 0.1 M NaCl, pH 7.2). The flow rate for adsorption was 20 mL/h; for elution, 30 mL/h. Then the column was charged by recyclization (2 h) with crude rHBsAg (calculated 2 mg HBsAg per 5 mg MAb) obtained by gel filtration in equilibrating buffer. The column was washed to remove nonspecifically bound substances with equilibrating buffer containing NaCl (0.5 M). Bounded rHBsAg was eluted under the following conditions: a) Tris-HCl (20 mM), pH 11.6; b) Tris-HCl (20 mM), NaCl (1 M), polyglucin (0.2 M), pH 9; c) Tris-HCl (20 mM), EDTA (3 mM), pH 7; d) glycine (0.2 M), pH 3.5; e) $MgCl_2$ (4.5 M); f) PBS, pH 7.0 (negative control); g) urea (8 M), pH 7.0 (positive control). The protein elution profile was recorded using a Uvicord instrument at λ 280 nm. The amount of recombinant HBsAg in the eluate was determined using the Ortho test system.

Analysis of Recombinant HBsAg. SDS-PAGE of rHBsAg was performed by the Laemmli method in polyacrylamide gel (12%) under reducing conditions. The separation was monitored by coloring the gel with Coomassie blue and silver nitrate.

HPLC analysis was carried out using a column (ET 250/8/4) of Nucleosil 5 C_{18}, TFA buffer (0.1%), pH 2, and an CH_3CN gradient of 5% (5 min), 5-70% (30 min), 70% (5 min), 70-5% (5 min). The flow rate was 1 mL/min with absorption at 280 nm and 0.08 optical-units sensitivity.

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