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Expression and Characterization of Hepatitis B Surface Antigen in Transgenic Potato Plants

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Abstract—Transgenic potato plants expressing the gene of hepatitis B surface antigen (HBsAg) under the control of the double promoter of 35S RNA of cauliflower mosaic virus (CaMV 35SS) and the promoter of patatin gene of potato tubers have been obtained. Biochemical analysis of the plants was performed. The amount of HBsAg in leaves, microtubers, and tubers of transgenic potatoes growing *in vitro* and *in vivo* was 0.005-0.035% of the total soluble protein. HBsAg content reached 1 μg/g in potato tubers and was maximal in plants expressing the *HBsAg* gene under the control of CaMV 35SS promoter. In transgenic plants expressing *HBsAg* gene under the control of tuber-specific patatin promoter, HBsAg was found only in microtubers and tubers and was absent in leaves. Western blot analysis of HBsAg eluted from immunoaffinity protein A-Sepharose matrix has been performed. The molecular weight of HBsAg peptide was approximately 24 kD, which is in agreement with the size of the major protein of the envelope of hepatitis B virus. Using gel filtration, it was determined that the product of *HBsAg* gene expression in potato plants is converted into high-molecular-weight multimeric particles. Therefore, as well as in recombinant *HBsAg*-yeast cells, assembling of HBsAg monomers into immunogenic aggregates takes place in *HBsAg*-transgenic potato, which can be used as a source of recombinant vaccine against hepatitis B virus.

Key words: transgenic potato, hepatitis B surface antigen, gene expression, synthesis, immunoassay, electrophoresis, gel filtration

Antigenic proteins from various pathogens responsible for induction of the immune response in the infected organism are now being identified. These proteins are the basis for the development of manufacturing technologies of new generation subunit vaccines. Transgenic plants are promising for the production of inexpensive and safe vaccines compared to traditional producers. Plant cells have enzymatic systems of post-translational modifications that are required for the assembling of the synthesized monomeric vaccine polymers into immunogenic multimeric forms. From this point of view, plants as potential vaccine producers have an advantage over bacteria, which do not have eukaryotic systems of post-translational protein modification. Gene expression of such proteins in plants allows the full synthesis of target antigens able to induce an active immune response in a plant cell [1]. Viral and bacterial antigens synthesized in plants stimulated formation of immunoglobulins towards corresponding pathogens in animals [2-7]. One of the promising objects for serving as a "biopharmaceutical factory" for production of viral hepatitis B vaccine is the potato plant. In the recent years, experiments have proved the promise of using plants for the production of "edible" vaccines for peroral immunization [8-11]. Plants are safe systems for obtaining therapeutic proteins because they do not contain viruses that are pathogenic for mammals. Transgenic plants are now being created and investigated as produc-

Abbreviations: HBsAg) HBs antigen, hepatitis B surface antigen; CaMV 35SS promoter) double promoter of the 35S RNA of cauliflower mosaic virus; PCR) polymerase chain reaction.

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ers of vaccines against various causative agents of human diseases, including human hepatitis B virus [2, 12, 13]. However, commercially available vaccines based on transgenic plants have not yet been created. Also, there are no publications with either the complete description of the whole work cycle of obtaining transgenic plants synthesizing hepatitis B surface antigen or with the characteristics of its expression under different experimental conditions.

The goal of this work was to obtain transgenic potato plants synthesizing hepatitis B surface antigen as well as its biochemical characterization.

MATERIALS AND METHODS

Construction of plasmids for plant transformation. In this work, we used pDES20 plasmid containing a synthetic gene of recombinant hepatitis B surface antigen of ayw serotype (HBsAg/mayw), 681 bp in size [14]. Flanking of *HBsAg* gene by the specific sequences for molecular cloning was performed by polymerase chain reaction (PCR) using primers containing *Bam*HI (1) and *Bgl*II (2) restriction sites: 1) 5′-CGGGATCCAATGTAAACC; 2) 5′-CGGGTACCATGGAAAACA. The reaction mixture contained 0.1 μg of pDES20 plasmid DNA as a template, 25 mM KCl, 60 mM Tris-HCl, pH 8.5 (at 25°C), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 0.2 mM mixture of deoxynucleotide triphosphates (USB, USA), 0.25 μM of each primer, and 2.5 units of Taq DNA-polymerase (SibEnzyme, Russia). The reaction was performed in 50 μl volume under following conditions: 94°C for 5 min; 30 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 7 min in a Gene Amp® PCR System 2400 amplifier (Perkin-Elmer, USA). The amplified gene was cloned into binary vector for agrobacterial plant transformation pBin-B33 [15] using *Bam*HI restriction site located under promoter of the B33 gene of potato patatin. The plasmid was used for transformation of *Agrobacterium tumefaciens* strain LBA4404 (pAL4404) [16]. For plant transformations (besides already mentioned constructions), we used an earlier obtained *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) containing plasmid with *HBsAg* gene controlled by the 35S RNA double promoter of cauliflower mosaic virus (CaMV 35SS) [17].

Plant transformation. In this work we used potato plants *Solanum tuberosum* L. of Desire cultivar which were grown under sterile conditions. Agrobacterial transformation of leaf and stem explants was performed as described in [15]. The obtained regenerants were grown on Murashige–Skoog medium (MS medium) [18] containing 250 mg/liter cefotaxime, 25 mg/liter kanamycin sulfate, and 0.1 mg/liter indolyl butyric acid for acceleration. Microtubers were obtained on MS medium with the addition of up to 8% sucrose. Plant cultivation in greenhouse conditions was performed in a Biotron artificial climate station.

DNA extraction from potato leaves was performed according to [19]. The leaves were homogenized in 1.5 ml Eppendorf tubes; 0.4 ml of extraction buffer containing 0.2 M Tris-HCl, pH 7.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS was added. The mixture was agitated and then incubated for 1 h at room temperature. Extracts were centrifuged at 12,000 rpm, DNA was precipitated by an equal volume of isopropanol, and the pellet was dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The obtained plant DNA was used as a template for PCR.

PCR analysis of *nptII* and *HBsAg* genes was performed in a reaction mixture containing 0.5-1 μg of genomic plant DNA. PCR conditions were as described above. Oligonucleotides specific for an internal 600 bp gene fragment were used as primers for the *nptII* gene [20]. For the *HBsAg* gene, we used primers mentioned above.

RNA for RNA–DNA hybridization was extracted from tobacco leaves by using hot phenol according to [21]. RNA electrophoresis was performed in 4% polyacrylamide gel containing 6 M urea [22]; thereafter the RNA was transferred onto nylon filter Hybond N^+ (Amersham, UK) by electroblotting using a Transblot apparatus (Bio-Rad, USA) overnight at 20 V potential. RNA hybridization with ^{32}P-labeled DNA-probe being a structural part of *HBsAg* gene was performed under conditions described in [23].

Quantification of hepatitis B surface antigen by immunoenzymatic assay in transgenic plants was performed as follows. Leaves and microtubers of analyzed plants were homogenized in liquid nitrogen and the extraction buffer (0.05 M Na-phosphate buffer, pH 7.5, 0.15 M NaCl, 0.001 M EDTA, 0.3% Tween-20, 0.0004 M phenylmethylsulfonyl fluoride) was added. The extract was centrifuged for 20 min at 3500 rpm. Then the supernatant was transferred into 1.5-ml Eppendorf tubes and centrifuged at 12,000 rpm for 10 min. Content of HBs antigen in the supernatant was determined using monoclonal antibodies (Auszyme Monoclonal diagnostic kits for immunoenzymatic assay (Abbot Laboratories, USA) or Vectogep B (Vector-Best, Russia)). As a positive control, a recombinant HBs antigen obtained from yeast cells was used [14]. Assay was performed in accordance to the operation manual of the test system.

Immunoprecipitation and immunoblotting of HBs antigen. HBs antigen from analyzed protein extracts was immunoprecipitated using mouse monoclonal antigens against native HBs antigen on protein A-Sepharose CL-4B sorbent (LKB Pharmacia, Sweden). The pellet was dissolved in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS, 2% dithiothreitol, 0.05% bromophenol blue) and boiled for 5 min. Then the proteins were fractioned by electrophoresis in 15% polyacryl-

amide gel [24] and transferred onto Immobilon™ PVDF (polyvinylidene difluoride) membrane (Millipore, USA) by electroblotting and using a MiniTrans-blot® apparatus (Bio-Rad) in buffer containing 0.025 M Tris-HCl, 0.193 M glycine, and 20% ethanol for 2 h at 100 V. The membrane was rinsed in TBS-T buffer (0.01 M Tris-HCl, pH 7.5, 9% NaCl, 0.1% Tween-20 (Sigma, USA)); blocking was performed in the same buffer with 5% skim milk powder for 12 h. The membrane was treated with rabbit polyclonal antibodies against HBsAg monomer in a dilution of 1 : 5000 (obtained from Combiotech AOZT, Russia). As a secondary antibodies, we used goat antibodies (1 : 10,000) conjugated with horseradish peroxidase (Pierce, USA). Development of the membrane was performed using the ECL chemiluminescence system (Pierce).

Analytical gel filtration of HBs antigen. Three milliliters of standard cell-free extract was applied on a 1.5 × 20 cm column with Sephadex G-25 (LKB Pharmacia) equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, and elution was performed using the same buffer. The void volume fraction was concentrated 3 times in a vacuum lyophilizer and used for high-performance gel filtration. For this purpose, 100 μl of the fraction was applied on a Superose-6 (HR10/30) HPLC column (LKB Pharmacia) equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, and elution was performed using the same buffer for 1 h at 0.4 ml/min flow rate. The eluted material was detected by measuring absorbance at 280 nm. Fractions of 0.8 ml of the eluted material were collected in vials; quantitative content of HBs antigen was determined using immunoenzymatic assay. As molecular weight standards, gel filtration of dextran blue D2000 (Pharmacia) with molecular weight of 2000 kD, thyroglobulin (669 kD), and ferritin (443 kD) was performed on the column under the same conditions.

RESULTS AND DISCUSSION

The aim of this investigation was to obtain transgenic potato plants synthesizing hepatitis B surface antigen (HBsAg) and investigation of its expression. We have produced two groups of plants containing the *HBsAg* gene controlled by the constitutive double promoter CaMV 35SS [17] and by the tissue-specific promoter of potato B33 tubers patatin gene [15]. Patatin is one of the main storage proteins in potato tubers. Promoters of reserve proteins in seeds and in vegetative reproductive organs of plants are strong promoters and determine high level of synthesis (in specific organs and tissues) of not only corresponding storage proteins but also the products of foreign genes [25]. Hence, the *HBsAg* gene (expressed under the control of this promoter) should exhibit tissue-specific expression in transgenic potato plants and be synthesized mainly in the tubers. For PCR amplification of the synthetic *HBsAg* gene encoding HBsAg/mayw polypeptide, we used recombinant pDES20 plasmid, which is employed in yeast cells producing hepatitis B recombinant vaccine [14]. The structure of this gene is modified by taking into account the codons most frequently used in intensively expressed eukaryotic genes. Amplified *HBsAg* gene was flanked by specific sequences for cloning into the vectors for plant transformation. For the convenience of *HBsAg* gene insertion under patatin promoter, the synthetic primers containing *Bam*HI and *Bgl*II restriction sites at 5′-termini were used. The analysis of nucleotide sequence of the obtained gene confirmed its identity with the original gene sequence. *HBsAg* gene was inserted into the plant transformation plasmid pBin-B33 using the unique *Bam*HI restriction site. Structures of the obtained plasmids employed for potato plants transformation are presented in Fig. 1.

Transgenic plants were obtained using the leaf explant agrobacterial transformation technique. As the result of transformation and subsequent selection on medium with kanamycin, 12 and 23 lines of potato regen-

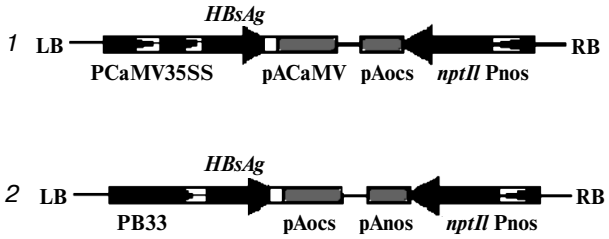

Fig. 1. Scheme of the plasmids containing *HBsAg* hepatitis B surface antigen gene controlled by: *1*) double 35S promoter of cauliflower mosaic virus (CaMV 35SS); *2*) B33 patatin gene promoter from potato (PB33). Designations: Pnos) promoter of nopaline synthase gene; pACaMV, pAocs, and pAnos) signals of polyadenylation 35S cauliflower mosaic virus RNA, octopine synthase and nopaline synthase genes, accordingly; *HBsAg*) hepatitis B surface antigen gene; *nptII*) neomycin phosphotransferase II gene; LB, RB) left and right borders of T-DNA.

Fig. 2. PCR analysis of transgenic potato plant DNA using primers towards *HBsAg* genes (lanes *1-6*) and *nptII* (lanes *7-12*). *1*, *7*) DNA of non-transgenic plants; *2-5*, *8-11*) DNA of transgenic plants containing the *HBsAg* gene; *6*, *12*) DNA of plasmid containing the *HBsAg* and *nptII* genes.

erants were selected, transformed correspondingly by the recombinant vectors containing target *HBsAg* gene controlled by CaMV 35SS and B33 promoters as well as selective gene marker *nptII* conferring the resistance to kanamycin. Both genes are localized within the same genetic region flanked by 25-bp imperfect direct repeats present at the boundaries of the T-DNA. During agrobacterial transformation, vir-function of Ti-plasmid determines the excision of this genetic region of binary plasmid vector by its flanking boundaries, and after that it is transferred into the plant cell and integrated into its nuclear genome [16]. Therefore, based on resistance of the transformed cells and regenerated plants to kanamycin a conclusion can be made not only about the transfer of the *nptII* gene into the plant, but also about the target gene *HBsAg*. Among regenerant plants selected on kanamycin medium for further research, we have chosen the lines exhibiting the best growth and physiologic characteristics.

The presence of the *HBsAg* gene and the antibiotic resistance marker gene (neomycin phosphotransferase II or *nptII*) in the transgenic plant genome was detected by the PCR technique. This analysis revealed that genomes of all investigated plants contained DNA, the size of which corresponded to that of the *HBsAg* gene and to the inner fragment of the *nptII* gene (Fig. 2).

Expression of the *HBsAg* gene in transgenic plants was demonstrated via RNA–DNA hybridization. For this procedure, total RNA was extracted from potato leaves and microtubers and hybridized with ^{32}P-labeled *HBsAg* gene used as a probe. The presence of those gene transcripts was detected in the cells of all investigated lines of transgenic potato plants (Fig. 3).

The quantitative content of surface HBs antigen in tissues of the obtained transgenic plants was investigated using test systems for immunoenzymatic detection. The amount of antigen in leaves and microtubers of transgenic potato plants from different lines and two groups grown *in vitro* and *in vivo* was 0.005-0.035% of total soluble protein

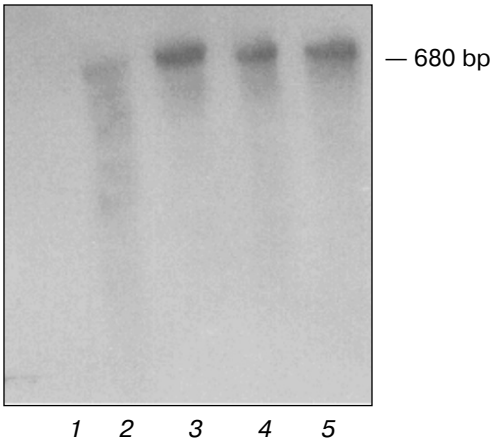

Fig. 3. Analysis of *HBsAg* mRNA in leaves (*2*, *3*) and microtubers (*4*, *5*) of transgenic potato plants via RNA–DNA hybridization. ^{32}P-labeled *HBsAg* gene was used as a probe. *1*) RNA from non-transformed plant leaves.

(Tables 1-4). The content of HBs antigen in potato tubers reached 1 μg/g of tuber mass (Table 1) and was the highest in plants expressing the antigen controlled by the double promoter CaMV 35SS. Antigen amount in transgenic potato plant leaves and microtubers containing the *HBsAg* gene controlled by double promoter 35S of cauliflower mosaic virus and grown *in vitro* in culture was 0.008-0.035% of total soluble protein. Expression of the *HBsAg* gene under the double promoter CaMV 35SS results in accumulation of surface antigen HBsAg in transgenic potato plants at least ten times higher concentration compared to its amount in transgenic tobacco plants expressing the *HBsAg* gene controlled by the single promoter CaMV 35S [17]. The data on quantitative HBs antigen content in transgenic potato tissues correspond to the data presented by other laboratories [2, 13, 26].

Microtubers of transgenic potato plants contain HBs antigen at the same level as their leaves. This fact is not

Table 1. Amount of HBs antigen in leaves and microtubers of transgenic potato plants with *HBsAg* gene under the control of CaMV 35SS double promoter, growing *in vitro**

Potato lines	Amount HBs antigen, ng/g tissue raw weight		Ratio of HBs antigen towards total soluble protein, %	
	leaves	microtubers	leaves	microtubers
2–1	1504 ± 94	330 ± 43	0.032	0.011
2–4	640 ± 34	697 ± 65	0.008	0.023
2–5	747 ± 85	751 ± 55	0.009	0.025
2–7	701 ± 63	1036 ± 101	0.009	0.035

* Average values from three independent experiments and standard deviations.

Table 2. Amount of HBs antigen in leaves and microtubers of transgenic potato plants with *HBsAg* gene under the control of CaMV 35SS double promoter, growing in greenhouse conditions*

Potato lines	Amount HBs antigen, ng/g tissue raw weight		Ratio of HBs antigen towards total soluble protein, %	
	leaves	tubers	leaves	tubers
2–1	669 ± 65	302 ± 43	0.01	0.009
2–4	1516 ± 88	371 ± 38	0.031	0.012
2–5	423 ± 44	665 ± 67	0.008	0.022
2–7	383 ± 33	776 ± 70	0.006	0.025

* Average values from three independent experiments and standard deviations.

Table 3. Amount of HBs antigen in leaves and microtubers of transgenic potato plants with *HBsAg* gene under patatin promoter, growing *in vitro**

Potato lines	Amount HBs antigen, ng/g tissue raw weight		Ratio of HBs antigen towards total soluble protein, %	
	leaves	microtubers	leaves	microtubers
2–1–2	0	243 ± 24	0	0.009
2–1–3	0	216 ± 19	0	0.006
3–1–6	0	181 ± 21	0	0.005
3–1–8	0	201 ± 26	0	0.01

* Average values from three independent experiments and standard deviations.

Table 4. Amount of HBs antigen in leaves and microtubers of transgenic potato plants with *HBsAg* gene under patatin promoter, growing in greenhouse conditions*

Potato lines	Amount HBs antigen, ng/g tissue raw weight		Ratio of HBs antigen towards total soluble protein, %	
	leaves	tubers	leaves	tubers
2–1–2	0	341 ± 34	0	0.01
2–1–3	0	456 ± 59	0	0.018
3–1–6	0	326 ± 31	0	0.01
3–1–8	0	440 ± 46	0	0.014

* Average values from three independent experiments and standard deviations.

unexpected since it is known that 35S promoter of cauliflower mosaic virus is constitutive and does not exhibit noticeable tissue or organ specificity [27].

In accordance with the specificity of gene expression under tuber-specific patatin promoter, the HBs antigen was detected only in microtubers and was absent in leaves of the transgenic potato plants. Antigen amount in microtubers from different lines of this transgenic potato growing under *in vitro* and *in vivo* conditions was 0.005-0.018% of total soluble protein amount, 200-400 ng/g of tuber (Tables 3 and 4). These data correlate to the reports about productiveness of HBs antigen by transgenic pota-

to plants expressing *HBsAg* gene controlled by patatin promoter, which were further used for oral immunization experiments in mice [10].

The synthesis of HBs antigen in plants from different lines was different, which can be associated with both the presence of various copy number of the *HBsAg* gene in their genomes and individualities of chromosome locus in which it is integrated. We could not detect any dependency between the amount of HBs antigen and amount of the corresponding mRNA in transgenic plant cells; however, low level of synthesis of *HBsAg* transcripts does not always mean low level of protein synthesis [2].

In this work, purified concentrated HBs antigen sample was obtained. For this purpose, we performed the immunoprecipitation from plant extracts on protein A-Sepharose using monoclonal mouse antibodies against HBs antigen. After rinsing from Sepharose and denaturing under reducing conditions, the protein was analyzed by the immunoblotting technique. Specially obtained polyclonal rabbit antibodies against the monomeric form of HBs antigen was used for immunoblotting. The picture of immunoblotting for HBs antigen from transgenic potato plants is presented in Fig. 4. Besides monomeric forms of HBs antigen, the analysis also reveals dimeric forms, which can be explained by incomplete degradation of multimeric HBs antigen complex under the conditions of the experiment. The use of polyclonal antibodies towards monomeric HBs antigen and high sensitivity of this technique due to the chemiluminescence ECL substrate allow unspecific detection of molecular weight marker proteins during enzyme immunoassay. In this analysis the molecular weight of HBs antigen from synthesizing plants and yeast was determined as 24 kD, which corresponds to the molecular weight of the protein part of the monomeric form of hepatitis B HBs antigen [28]. These results along with the possibility of direct immunoenzymatic determination of the native HBs antigen in cell-free extracts from transgenic plants indicate that HBs antigen synthesized in potato plants is able to aggregate into oligomers, which are known to have significantly greater immunogenic and antigenic activity compared to single HBs polypeptides [2]. Also noted is the stability of *HBsAg* gene products in transgenic plants: in plant extracts, these products preserve antigenic activity for up to seven days at 4°C without noticeable decrease.

Direct proof of the existence of a multimeric form of HBs antigen in transgenic potato plants was obtained in the experiments on analytical gel filtration of the *HBsAg* gene expression product. We used homogenate fraction from leaves and microtubers of transgenic plants that were purified free from pigments and low molecular weight protein substances. HBs antigen was detected in a fairly narrow elution zone of high molecular weight compounds with molecular weights of approximately 2000 kD (Fig. 5). Considering that monomeric form of HBs antigen is 24 kD, it can be concluded that *HBsAg* gene expression

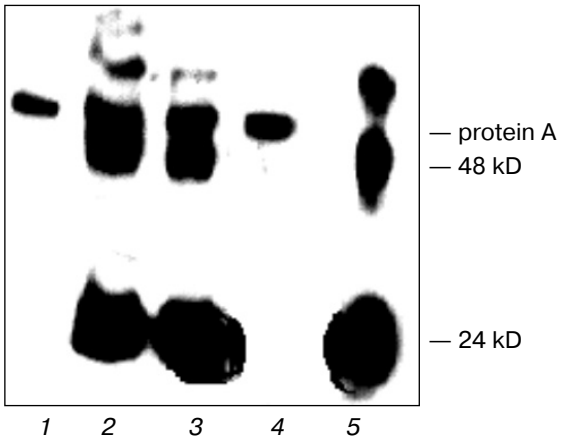

Fig. 4. Immunoenzymatic blot analysis of hepatitis B surface antigen (HBsAg) precipitated from protein extracts by immunoprecipitation on protein A-Sepharose CL-4B sorbent. *1*) Protein extract from non-transformed potato leaves; *2*) extract from transgenic potato leaves; *3*) extract from recombinant yeast producing HBsAg; *4*) negative control (protein A-Sepharose CL-4B); *5*) positive control (antigen without immunoprecipitation). Polyclonal antibodies against HBsAg monomer diluted 1 : 5000 were used. Antibody binding was visualized using an ECL kit (Pierce).

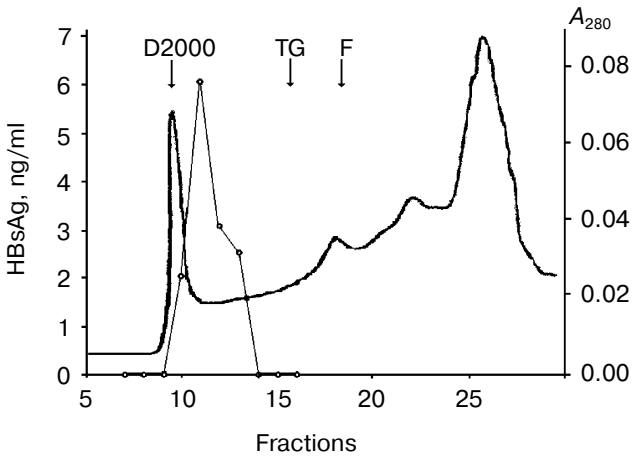

Fig. 5. High performance gel filtration of HBs antigen extracted from transgenic potato leaves on a Superose-6 column (HR 10/30). Elution by 0.05 M Na-phosphate buffer (pH 7.5) containing 0.15 M NaCl for 60 min at 0.4 ml/min flow rate. The thick line indicates absorbance, and the thin line shows detection of HBs antigen. D2000) dextran blue (2000 kD); TG) thyroglobulin (669 kD); F) ferritin (443 kD).

product in transgenic plant cells is forming multimers including not less than 70-80 monomers. Our data regarding analytical gel filtration of HBs antigen synthesized by transgenic potato cells correspond to sedimentation and electron microscopy characteristics of HBs antigen extracted from transgenic tobacco cells by other

authors [2]. During gel filtration, HBs antigen is eluted in the zone, which is virtually free from the presence of other proteins, which makes the gel filtration technique on this carrier an efficient stage in the industrial production of hepatitis B vaccine based on transgenic plants. The asymmetric profile of HBs antigen observed during gel filtration can be caused by certain proteolytic degradation of its multimeric aggregates or by the presence of incomplete assembling products in transgenic plant cells. This hypothesis is consistent with sedimentation analysis data of HBs antigen obtained from various transgenic plants, where dispersion of aggregated HBs antigen particles by size was observed [2, 12, 29]. Hence, due to enzymatic systems of post-translational protein modification in transgenic potato plant cells, HBs antigen assembling into multimeric aggregates occurs. Those multimers have significantly higher immunogenic and antigenic activity compared to the original monomeric form and have potential for use as a substance for hepatitis B vaccine production.

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