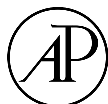

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Biochemical and Biophysical Research Communications 308 (2003) 94–100

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Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants ☆

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Received 16 June 2003

Abstract

The application of bioengineering to plants for production of biological products for human and animal use has expanded in recent years. The reasons for this expansion are several and include advances in the technology for novel production systems and the need for very large quantities of therapeutic proteins. The process of growing pharmaceutical proteins in plants, extracting, and purifying is a hard task considering the lack of available information concerning these topics. In this work, a recombinant murine monoclonal antibody specific for the hepatitis B surface antigen, expressed in stably transformed transgenic *Nicotiana tabacum* plants, was purified by means of a recombinant protein A Streamline chromatography as the main purification step. The antibody expression level varied with the age of the plants and the number of harvests from 40 to 15 μg/ml and the maximum process yield was about 25 mg of plantibody/kg of biomass. Protein A Streamline chromatography was successfully used in the purification process yielding a recovery of about 60% and a plantibody SDS–PAGE purity of over 90% but unexpectedly, previous clarification steps could not be totally avoided. The amino acid sequence recognized by this affinity purified plantibody was similar to its murine counterpart verifying the potentiality of plants to replace animals or bioreactors for large-scale production of this monoclonal antibody.

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Keywords: Plantibody; Monoclonal antibody; Transgenic plant; Expanded bed adsorption; Tobacco

The tremendous importance of the hepatitis B disease due to its association with the hepatocellular carcinoma implies the need for a cost-effective and safe vaccine [1]. Several procedures have been used to purify the hepatitis B surface antigen for vaccinal purposes. Immunopurification using HBsAg specific murine monoclonal antibodies has been one of the most successful strategies for such a purpose [2,3]. However, ethical and regulatory requirements and the relatively high cost have compelled to a radical and rapid replacement of the murine monoclonal antibodies produced in mice [4].

The continuous production of monoclonal antibodies from mouse splenic lymphocytes after immortalization by fusion with a plasmocytoma cell line was first reported by Köhler and Milstein [5]. This gave research a powerful and versatile tool, since the specificity of antibodies is exquisite and extremely sensitive. Murine ascites production was the time-honored technique for producing small-scale and research laboratory quantities of Mabs. Nevertheless, there are several problems associated with the scale-up of this production [6–9].

Nowadays in vitro production of monoclonal antibodies is the most applied choice to solve problems associated with the use of production in mice. The main

☆ *Abbreviations:* HBsAg, hepatitis B virus surface antigen; Mab, monoclonal antibody; MAP, mouse antibody production.

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0006-291X/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/S0006-291X(03)01335-4

advantages of the in vitro production are: it does not utilize animals for production; no animal care issues or IACUC approval is required; avoids or significantly decreases the need for laboratory personnel experienced in animal care and use; no host contributed immunoglobulin or typical ascitic-fluid contaminants; and higher immunoreactivity with ascites (90–95%) in some cases and under most circumstances eliminates the need for testing mouse antibody production [10–12]. However, the cost factor is still high, which has boosted the search for other alternatives.

Biotechnology is writing a new story by using the recombinant DNA technology to express and produce biological molecules in plants for pharmaceutical applications [13]. This seems to be an attractive alternative to produce some kind of molecules such as: cytokines, hormones, monoclonal antibodies, enzyme, and vaccines (Table 1). In 1989, a mouse antibody was expressed in a genetically transformed tobacco plant opening the alternative to produce large amounts of therapeutic antibodies cheaply [14]. Plant expression systems are becoming increasingly attractive because of a heightened awareness of the need to remove animal and human blood-derived components from biological processes, where they are often used as media components. Biologics made in plants pose relatively few concerns and they will be regulated similar to biologics produced in other systems [13]. However, there are issues still to be resolved relating to the environmental control, downstream processing, and post-translational modifications of proteins.

In this study, a recombinant antibody specific for the HBsAg was purified from *Nicotiana tabacum* plants (plantibody) in order to test its ability to immunopurify a yeast rec-HBsAg to be putatively used as a vaccine in humans. The purification system was mainly based on recombinant protein A expanded bed adsorption chromatography, which has been widely used for monoclonal antibody purification [15–18]. In this chromatography, a strong interaction among the protein A and the Fc fragments of the antibodies is normally produced, allowing high recovery and purity. Additionally, its main advantage is the capacity to be introduced as primary recovery operation to handle unclarified materials, yielding a combination of clarification, concentration, and adsorptive purification in a single step. This work permits a better estimate of the real advantages that the process of obtaining biologics from plants has and the possibility of further process cost analysis.

Materials and methods

Tobacco plants were grown in a greenhouse under controlled conditions according to standard production practices of the Center for Genetic Engineering and Biotechnology of Havana, Cuba, during last summer. Seedlings were manually transplanted at the rate of 10 plants/square meter. Leaves were then harvested at the age of 8–15 weeks after transplanting and transported at room temperature to be selected, weighted, and ground.

Extraction and clarification. Tobacco leaves were wetted by dropping phosphate-buffered saline, pH 8, with ascorbic acid at 4 °C. Extraction buffer in the ratio of 400 ml per every kilogram of leaves was used to extract proteins. This material was then processed using a Fitzmill Comminutor (The Fitzpatrick, USA). Subsequently the green fraction was removed from the juice by centrifugation at 1500 rpm in a basket centrifuge Rina (Riera Nadeu, Spain). The supernatants which still contained a small amount of suspended green particles were centrifuged again at 16,000 rpm (retention time 0.16 h), 4 °C in a tubular centrifuge CEPA (Carl Padberg, Germany) and simultaneously pumped to the recombinant protein A Streamline column.

Affinity chromatography. Expanded bed preparative chromatography using 800 ml of recombinant protein A Streamline adsorbent (Amersham-Biosciences, Uppsala, Sweden) was used in a 10-cm inner diameter Streamline column. Equilibration was carried out with five times adsorbent volume in 2.5-fold bed expansion. The following buffers and flow rates were employed: equilibration and wash buffer: 150 mM phosphate-buffered saline, pH 8 (300 cm/h), and elution buffer: 100 mM citric acid, pH 3 (100 cm/h). Soon after the elution, the

Table 1
Examples of pharmaceutical antibodies produced in transgenic plants

Antigen	Plant	Antibody	Application	Company
Streptococcus surface antigen SAI	Tobacco	sIgA/G (CaroRX)	Therapeutic (topical)	Planet Biotechnology, CA
Non-Hodgkin's lymphoma idiotypes	Tobacco	Virus vector scFv	Vaccine	Large Scale Biology, CA
Human IgG	Alfalfa	IgG	Diagnostic	—
Carcinoembryonic antigen	Tobacco, pea, tomato, rice, and wheat,	scFv	Therapeutic/diagnostic	—
Carcinoembryonic antigen	Tobacco	Diabody	Therapeutic/diagnostic	—
Herpes simplex virus	Soybean, rice	IgG	Therapeutic (topical)	EPIcyte
Respiratory syncytial virus	Corn	IgG	Therapeutic (inhaled)	EPIcyte
Clostridium difficile	Corn	IgG	Therapeutic (oral)	EPIcyte
Sperm	Corn	IgG	Contraceptive (topical)	EPIcyte
Various	Corn	IgG	Therapeutic/diagnostic	Integrated Protein Technologies, Monsanto, MO
Colon cancer antigen	Tobacco	IgG	Therapeutic/diagnostic	—

pH of the eluted fractions was adjusted to 7.6. This was performed with stirring to prevent local concentration pH peak and the regeneration was done after each cycle using 2 M NaCl/1 mM NaOH/20% alcohol.

Gel filtration chromatography. Buffer exchange was carried out by means of a gel filtration chromatography using Sephadex G-25 coarse (Amersham-Biosciences, Uppsala, Sweden). This chromatography was performed in a BP 113/120 column using as equilibration buffer 20 mM Tris/150 mM NaCl, pH 7.6. The volume of the samples ranged from 1 to 3 L and the flow rate used was 129 cm/h.

Estimation of specific antibody activity by enzyme-immunoassay and protein determination. A polystyrene microplate (Costar, Cambridge, USA) was coated with 10 μg per well of recombinant HBsAg in 0.1 M $NaHCO_3$ buffer for 20 min at 50 °C. After this step samples were added to the plate in 0.05% Tween 20 in phosphate-buffered saline and incubated for 1 h at 37 °C. After several washings with 0.05% Tween 20/phosphate-buffered saline, the plate was incubated for 1 h at 37 °C with a horseradish peroxidase conjugate (Sigma Chemical, St. Louis, USA). The reaction was then revealed using 100 μl/well of 0.05% *O*-phenylenediamine and 0.015% H_2O_2 in citrate buffer, pH 5.0, and stopped with 50 μl/well of 1.25 M H_2SO_4. The absorbance was measured in a Multiskan ELISA Reader (Labsystems, Helsinki, Finland) using a 492-nm filter [19]. In all cases, the protein concentration was determined by the method described by Lowry et al. [20], using bovine serum albumin as standard material.

Purity measured by SDS–PAGE and HPLC-gel filtration. Sample purity was analyzed by gel electrophoresis on a 12.5% (w/v) SDS–polyacrylamide gel [21] followed by Coomassie staining. A HPLC-gel filtration column TSK G3000 PW (600 mm × 7.5 mm I.D., TosoHaas, Japan) was also used to determine the purity of antibody. The chromatographic mobile phase was phosphate buffer, pH 7.0, and 10 μl of the samples dissolved in phosphate buffered saline was directly applied onto the system. The volumetric flow rate employed was 0.2 ml/min and the absorbance was measured at 226 nm.

Western blotting. Samples analyzed by gel electrophoresis on a 12.5% SDS–polyacrylamide gel were transferred to nitrocellulose and immunoblotted using a peroxidase-conjugated goat anti-mouse IgG and diaminobenzidine as color substrate for identification of immunoglobulins.

Determination of the amino acid sequence recognized by the plantibody. A cellulose membrane which contains several peptides of the HBsAg was washed two times with dimethylformamide/ethanol/water and with 0.05% Tween/TBS (0.137 mM NaCl/0.0026 mM KCl/0.05 mM Tris), pH 7.0, three times for 10 min per wash. Subsequently the membrane was blocked with 5% milk in TBS and incubated with the plantibody solution for 3 h at room temperature. The membrane was then washed in 0.05% Tween–TBS four times and incubated with an anti-mouse alkaline phosphatase conjugate in 5% of milk/0.5% Tween 20/TBS for 30 min. After several washings with 0.05% Tween–TBS again, 5-bromine-4 chlorine-3 indol phosphate was added to the membrane for 10–30 min. The reaction was stopped washing the membrane with TBS. The amino acid sequence of the peptides is shown below.

1.MENITSGFLGPLB	13.RWMCLRRFIIFLB	25.GNCTCIPIPSSWB
2.GFLGPLLVLQAGB	14.RFIIFLFILLLCB	26.PIPSSWAFAKYLB
3.LVLQAGFFLLTRB	15.FILLLCLIFLLVB	27.AFAKYLWEWASVB
4.FFLLTRILTIPQB	16.LIFLLVLLDYQGB	28.WEWASVRFSWLSB
5.ILTIPQSLDSWWB	17.LLDYQGMLPVCPB	29.RFSWLSLLVPFVB
6.SLDSWWTSLNFLB	18.MLPVCPLIPGSTB	30.LLVPFVQWFVGLB
7.TSLNFLGGSPVCB	19.LIPGSTTTSTGPB	31.QWFVGLSPTVWLB
8.GGSPVCLGQNSQ	20.TTSTGPCKTCTTB	32.SPTVWLSAIWMMB
9.LGQNSQSPTSNHB	21.CKTCTTPAQGNSB	33.SAIWMMWYWGPSB
10.SPTSNHSPTSCPB	22.PAQGNSMFPSCCB	34.WYWGPSLYSIVSB
11.SPTSCPPICPGYB	23.MFPSCCCTKPTDB	35.LYSIVSPFIPLLB
12.PICPGYRWMCLR	24.CTKPTDGNCTCIB	36.PFIPLLPIFFCLB
		37.LLPIFFCLWVYIB

Results and discussion

Several procedures have been used to purify the HBsAg for vaccinal purposes. Immunopurification, using rec-HBsAg specific murine monoclonal antibodies, has been one of the most used [2,3]. However, relevant ethical and safety concerns beside the high cost of this procedure have compelled to a radical and rapid replacement of Mab produced in mice. The limitations, which constrain the present and perspective use with the in vivo method becoming a not so attractive method, are: requiring animal facilities, support services, trained personnel, and daily monitoring to minimize pain and distress; use of pristane which may produce residual contamination, reactive endogenous murine retroviruses and promote the production of IgG auto-antigens. Ascitic fluid is a more complex mixture than blood plasma, having the same difficulties associated with the use of serum for in vitro cultures. There are some other non-regulatory disadvantages which also limit the use of the ascites: higher range of variability (inter-animal) in some cases; the reduced Mab immunoreactivity and the higher contamination level with biochemically identical immunoglobulins, growth factors, rodent plasma proteins, cytokines, bacteria, and viruses.

Currently vast experience has been gathered in the manufacture of biological molecules in bacteria, yeast, and mammalian cells [22] but results on the large-scale production of recombinant antibodies derived from plants have not been published yet.

Using plants to synthesize foreign proteins at high level is the topic of intense research activity. In the area of human health, considerable effort has been put into expressing antibodies in plants (Table 1). As in many systems a critical aspect is the amount of the antibody to be produced. This generally requires a high level of expression of the candidate in the target plant tissue. However, the expression level is not the unique factor to be considered. Also of relevant importance is stability of the protein in the plant and also in the crude material. In the case of the plantibody CB.Hep1 specific for the HBsAg the concentration of this protein varied with the age of the plant and with the number of harvests (Table 2). The concentration maximized at 8–9 weeks reaching values near of 40 μg/ml but this parameter fell down 2-fold just 1 week later, which may have a great impact on the recovery of downstream process, purity of the purified antibody, and process yield.

The initial purification of a target molecule has traditionally been addressed by adsorption chromatography using a conventional packed bed adsorbent. This necessitates clarification of the crude feed before application to the chromatography column combining several methods such as centrifugation and micro-filtration. Both are time-consuming processes and could compromise on the stability and recovery of the desired mole-

Table 2
Results of Mab adsorption on expanded bed adsorption chromatography

Batches	Plant age (weeks)	Extract IgG concentration (μg/ml)	Leaves (kg)	Yield (%)	Purified IgG (mg)	Process concentration factor (fold)	Affinity process time (h)	Mab concentration after elution (μg/ml)
1	8	41.00	50	30.00	732	21.4	8.0	93.60
2	9	40.90	60	64.60	1582	24.0	5.1	400.99
3	10	17.40	60	72.20	689	27.5	5.0	344.55
4	11	28.60	60	62.20	941	21.2	4.4	376.73
5	12	26.20	100	51.50	716	26.5	4.0	398.02
6	12	29.90	100	57.70	690	16.0	4.0	276.06
7	14	22.04	100	62.60	1269	26.2	8.1	362.72
8	15	22.61	115	44.20	1038	52.0	7.1	371.28
9	16	15.62	208	46.90	1833	78.1	13.4	272.24

cule. Fresh tobacco plants contain 80–90% of water and a vast conglomerate of sugars, amino acids, vitamin starch, cellulose, pigments, alkaloids, polyphenols, etc. Since fresh tobacco contains a lot of water much water, it can easily be ground up in its own juice. This juice can be easily removed from the insoluble part and the remaining soluble part contains proteins in three different forms. The insoluble part also contains the plant chloroplasts and cell nucleus [23]. Taking into consideration the complexity of this mixture, several experiments at analytical scale were done first to decide which purification strategy could be more appropriate to extract the desired protein with high purity and recovery (data no shown). These preliminary studies led us to choose Streamline as the method for large scale purification.

Streamline adsorbent is expanded in a single-pass operation in which the target proteins are purified from crude extracts without the need of clarification, concentration, and initial purification. The expansion of the adsorbent particles increases the bed volume in the bed, which allows the passage of cells, cell debris, and other particulates during application of the crude feed to the column [24,25]. Fluidized beds have been used in industry for many years to recover antibiotics [26,27], purification of *Escherichia coli* homogenate, lysate, inclusion body or secreted products [28,29], yeast homogenate and secreted products [17,30], whole myeloma and hybridoma fermentations [18], as well as milk and animal tissue extracts [31,32]. But the literature is lacking the information about the use of fluidized beds for large-scale purification of antibodies from plant crude extracts.

In this study, the process recovery was about 60% but we did not quantify plantibody in the expanded bed adsorption non-bound and wash fractions, reason why we speculate that some kind of overestimation should occur in the starting material sample. Unexpected initial clarification steps could not be totally avoided because of the strong interaction of the green components with the Streamline nets and the adsorbent provoking precipitation and blocking the column very often. This phenomenon has a negative influence on the recovery and the purity of the purified antibody as well. So, in this sense the advantages of the Streamline technology could not be exploited completely, showing a similar behavior to that of packed bed adsorption chromatography. Additionally all elution fractions of the purification cycles were characterized by a light yellow color that might have been produced by plant pigments or phenols. It was not possible to remove this yellow color by any other procedures tested such as: recombinant protein A packed bed adsorption, ion exchange, differential precipitation, gel filtration, and ultra-filtration. However in favor of this affinity chromatography we say that the Streamline column resists a higher flow rate allowing a faster purification process which is quite important in product derived from plant crude a high purification capacity (Table 2) and also the recombinant protein A packed bed adsorption gel could not be cleaned completely after few purification cycles using the recommended regeneration solution.

Figs. 1 and 2 show that the purity of the purified antibody from the expanded bed adsorption method was consistently over 90% but it was never higher than that of the antibody purified from ascitic fluid by recombinant protein A packed bed adsorption. The most common bands in the expanded bed adsorption purified antibody were two bands, one band between heavy and light chain and in some cases a band at a molecular weight of 10–15 kDa. Those contaminants have been reported by other authors but none of them substantially affect the final purity of the plantibody [33]. We have also observed aggregation in the expanded bed affinity purified antibody. Fig. 3 shows these samples analyzed by immunoblotting using a peroxidase labeled goat anti-mouse IgG to detect the recombinant antibody. The band of 35 kDa seems to be a degradation of the heavy chain as reported by Fischer et al. [34]. The presence of this band was also confirmed by SDS–PAGE under non-reducing conditions (data not shown). The 10–15 kDa band was not detected by immunoblotting, which means that it corresponds to a plant contaminant or to fragment degradation that has not recognized this goat anti-mouse IgG. Figs. 4A and B

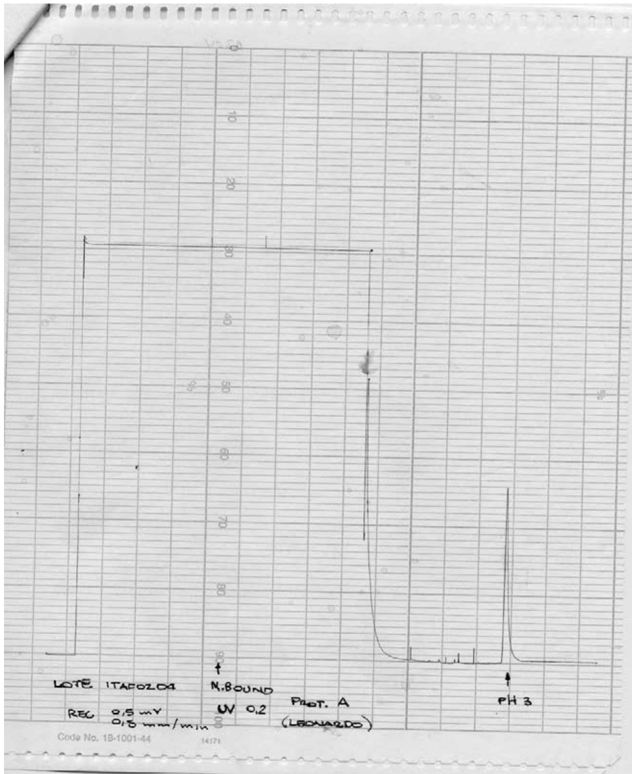

Fig. 1. Expanded bed adsorption protein A affinity chromatogram. This run uses a 100-cm inner diameter and 10 cm of bed height. The flow rate used was 300 cm/h and the column was loaded with tobacco plant clarified extract.

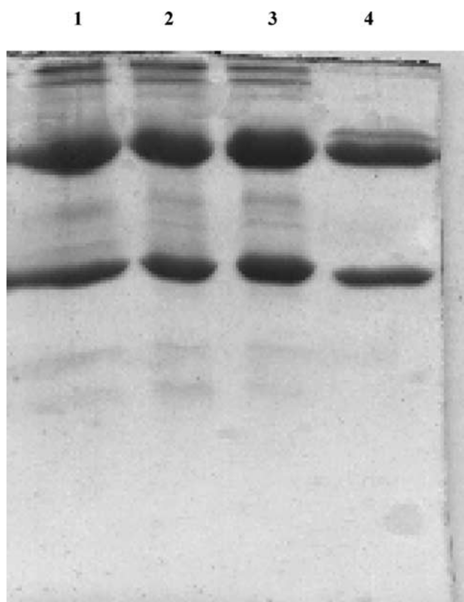

Fig. 2. Coomassie blue stained SDS–PAGE antibody purified by expanded bed adsorption method. Lane 1, purified antibody (experiment 1); lane 2, purified antibody (experiment 2); lane 3, purified antibody (experiment 3); and lane 4, mouse monoclonal antibody purified by packed bed adsorption.

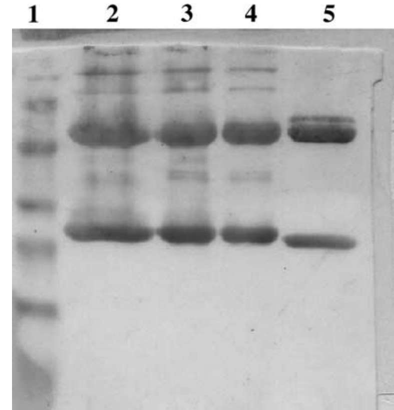

Fig. 3. Immunoblot analysis of the plant derived antibody. Protein samples were purified by expanded bed adsorption, separated by 12.5% of SDS–PAGE, and transferred onto nitrocellulose. Lane 1, molecular weight marker (myosin, 209 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49 kDa; carbonic anhydrase, 34.8 kDa; soybean trypsin inhibitor, 28.9 kDa; lysozyme, 20.6 kDa; and aprotinin, 7.10 kDa); lane 2, purified antibody (experiment 1); lane 3, purified antibody (experiment 2); lane 4, purified antibody (experiment 3); and lane 5, mouse monoclonal antibody purified by packed bed. Antibodies were detected with a horseradish peroxidase labeled goat anti-mouse IgG.

reveal HPLC-gel filtration chromatograms of the recombinant antibody and its mouse monoclonal antibody produced in mice. Streamline recombinant protein A purified antibody showed no detectable differences in the purity measured by this procedure in comparison to that of the mouse antibody. The level of purity was over 95%, which corresponds to the requirements of purity of many regulatory agencies. Further optimization works should be done to minimize the effects mentioned above which irreversibly foul the column thereby reducing the lifetime of the matrix and increasing the cost of the process and to elucidate the differences observed between SDS–PAGE and HPLC-gel filtration chromatography.

Detailed information on the antigen determinant and sequence identified by both antibodies were obtained from the peptide assay. To determine the amino acid sequence recognized by the plant derived antibody as the criterion of specificity and identity, several overlapping peptides of the hepatitis B surface antigen were fixed to a cellulose membrane facing the mouse and plant derived antibodies. Our results indicate that the recombinant antibody was similar to its mouse counterpart recognizing the same hepatitis B surface antigen peptides. The peptides recognized by both antibodies were 20 and 21, respectively, corresponding with the following amino acid sequences: peptide 20.TTSTGP CKTCTTB and 21.CKTCTTPAQGNSB, which means both antibodies interacting with the sequence CKTCTT which is shared between both peptides (Figs. 5A and B). This region has been reported as being quite immunogenic corresponding with one of the most important antigen determinants of the HBsAg [35]. Furthermore,

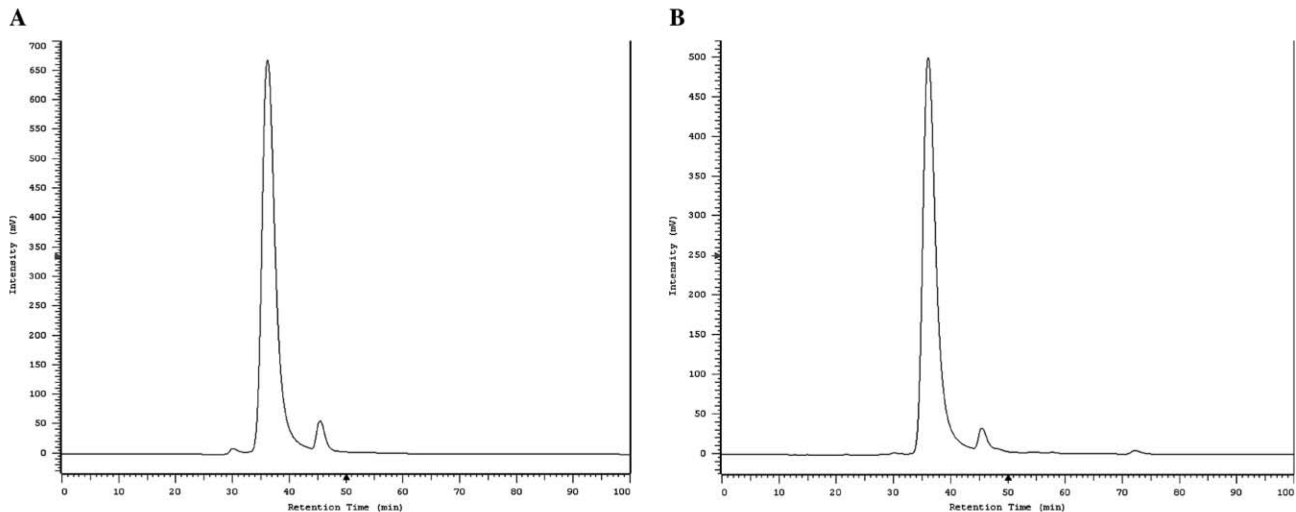

Fig. 4. Purity measured in high performance liquid chromatography gel filtration. (A) Mouse monoclonal antibody specific for the hepatitis B surface antigen. (B) Plant derived antibody specific for the hepatitis B surface antigen.

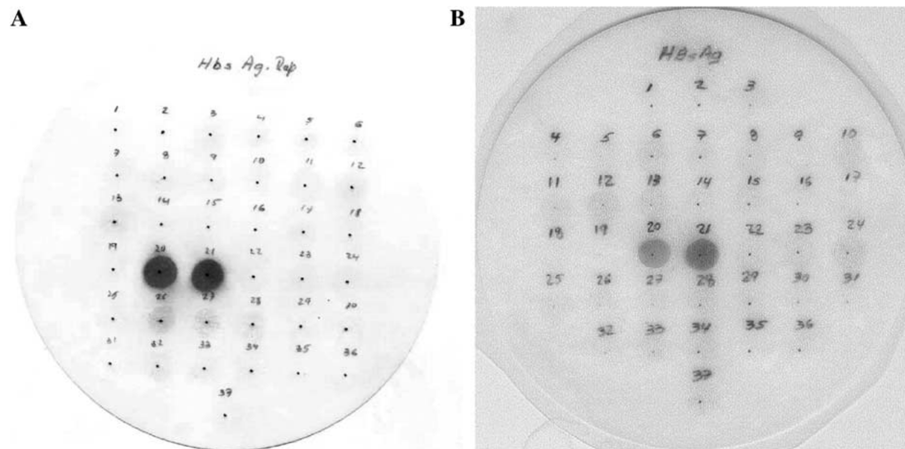

Fig. 5. Hepatitis B surface antigen's peptides recognized by the plantibody and the monoclonal antibody CB.Hep-1. (A) Plantibody and (B) mouse monoclonal antibody counterpart.

indirectly no post-translational modification related with the antigen binding site of this plant derived antibody was observed.

Conclusions

This study shows that this plant expressed recombinant antibody produced in tobacco is similar to its counterpart murine monoclonal antibody CB.Hep-1 specific for the Hepatitis B surface antigen. The amount of protein extracted from tobacco leaves maximized at 8–9 weeks. In spite of the fact that expanded bed adsorption chromatography proved the high recovery and purity of this recombinant antibody, previous purification steps could not be totally avoided in this process scale, because real interactions of plant components with the column and the stationary phase were detected.

Acknowledgments

For their generous contribution the authors thank Raudel Sosa, René Gallo, Milagro Font, Alberto Nuñez, and Julio Cesar Sánchez for process control assistance. They are also indebted to Nadia Ramirez and Meylin Rodríguez for providing the transgenic tobacco clone expressing the antibody and to Marlen Pérez and Roberto Pérez for growing plants.

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