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Journal of Chromatography B, 852 (2007) 1–7
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Comparison of different ligand densities in immunoaffinity chromatography of the plantibody HB-01 coupled to Sepharose CL-4B to purify the rHBsAg

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Received 10 May 2006; accepted 3 December 2006
Available online 17 December 2006

Abstract

This paper evaluates the immunopurification behavior of a plantibody HBsAg specific plantibody coupled to Sepharose CL-4B at different ligand densities. Results show no significant differences in the adsorption and elution capacities, and rHBsAg recovery of immunosorbents at 3.43, 4.45, and 5.31 mg/mL of ligand densities compared to its mouse-derived mAb counterpart consistently used in the rHBsAg purification process. Therefore, plantibody ligand densities higher than 3.43 mg/mL do not improve the immunopurification behavior of this immunosorbent, but increase the antibody consumption and the Hepatitis B vaccine cost. Immunosorbent of 2.23 mg/mL of ligand density demonstrated a poor performance. The IgG leached detectable level never exceeded the approved limit (3 ng IgG/μg rHBsAg). Values close to this limit were only observed at the ligand density of 5.31 and 2.27 mg/mL. In the case of the ligand density of 2.23 mg/mL the IgG leached value was high (2.90 ng IgG/μg rHBsAg) due to a low level of eluted antigen. In conclusion, it supports feasibility of using this plantibody at 3.43 mg/mL of ligand density for large-scale immunopurification of rHBsAg for human use, avoiding the biosafety and ethical concerns of the massive use of animals for this purpose.

Keywords: Plant-derived antibody; Plantibody; Monoclonal antibody; Hepatitis B virus surface antigen; Immunoaffinity; Immunopurification

1. Introduction

Hepatitis B virus infection is the most common cause of liver disease worldwide [1,2]. Vaccines against this virus have been available since the 1980s and more than 1 billion doses have been employed [3–5]. The immense majority of these vaccines use the rHBsAg as the active pharmaceutical ingredient, which is purified using several procedures, such as immunopurification chromatography based on mouse mAb [6,7]. This affinity separation is a well-established technique for the purification and recovery of biological molecules [8,9], but the regulatory and ethical constrains of mAb production in animals break the massive application of this technique for large-scale production of biological products, forcing the researchers to evaluate other production alternatives.

Many pharmaceutical proteins of mammalian origin have been synthesized in plants [10]. It has gained a great importance in the last years [11] since it represents a cost-effective system for the large-scale production of pharmaceutical antibodies and

Abbreviations: rHBsAg, recombinant Hepatitis B virus surface antigen; mAb, mouse-derived monoclonal antibody; plantibody, plant-derived antibody

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doi:10.1016/j.jchromb.2006.12.018

provides an additional level of safety compared with animals, because plant viruses are not known to infect humans [12].

Within plants the main advantages of tobacco plant include the nature technology for gene transfer and expression, the high biomass yield, the potential for rapid scale-up owing to prolific seed production, and the availability of large-scale biomass infrastructure for processing [11]. In this work, different ligand densities of the plant-derived antibody HB-01 expressed in *Nicotiana tabacum* plants specific for the aminoacid sequence CKTCTT of the HBsAg "a" determinant were evaluted [13,14]. The determination of the optimal ligand density contributes valuable information for reducing the product cost and the ligand level leached from the matrix and for making a precise estimation of the required production capacity.

2. Experimental

2.1. Antigen source

A recombinant strain of *Pichia pastoris* was fermented in common inorganic salts (e.g. $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$) supplemented with glycerol and vitamins (e.g. biotin, riboflavin). Methanol was continuously added at a flow of 2.9–10.9 g L^{-1} h^{-1} to induce the synthesis of rHBsAg. The level of methanol never reached values higer than 2% because it is a toxic level for this yeast. The rHBsAg was purified according to the procedure described by Pentón et al. [5].

2.2. Monoclonal antibody source

The murine B-lymphocyte hybridoma (CB.Hep-1) was previously generated by Fontirrochi et al. [15]. It was obtained by a fusion of Sp2/0-Ag14 myeloma cells and spleen cells of a BALB/c mouse immunized with a natural HBsAg. The hybridoma CB.Hep-1 secretes an IgG-2b mAb, directed against the "a" determinant of the HBsAg [16]. This mAb is routinely produced by ascites to be employed as an immunoligand at 3.8 mg/mL of ligand density in the rHBsAg purification process. The antigen obtained by this procedure is the active pharmaceutical ingredient of the commercially available HB vaccine (Heberbiovac^{TM} HB, HeberBiotec S.A., Cuba) [5].

2.3. Plant-derived antibody HB-01 production

Transgenic tobacco plants expressing the antiHBsAg plantibody HB-01 generated as described by Ramírez et al. [13] were used for this study. Seedlings were transplanted in Zeolite and leaves were harvested and wetted with phosphate buffered saline (PBS), pH 8.0 (400 mL of PBS 150 mM/Kg of leave) and 0.56 mM ascorbic acid at 4 °C to be ground by a Fitzmill Comminutor (The Fitzpatrick Company, USA). The green fraction was removed from the supernatant by filtration using a Rina basket centrifuge at 1051.38 × *g* (Riera Nadeu S.A., Spain) and the supernatant was centrifuged again at 19,635 × *g* in a CEPA tubular centrifuge (Carl Padberg, Germany). The purification of plantibody HB-01 was carried out by means of an affinity chromatography using a recombinant protein A Streamline column (Amersham-Biosciences, Uppsala, Sweden) and finally a buffer exchange from affinity elution buffer to 20 mM Tris-HCl/150 mM NaCl pH 7.2 was carried out by gel filtration chromatography using Sephadex G-25 (Amershan-Biosciences, Uppsala, Sweden) [14].

2.4. Immunoaffinity matrix

Sepharose CL-4B (Amersham-Biosciences, Uppsala, Sweden) was activated by the CNBr method [17]. The mouse-derived antibody and the plantibody were coupled as recommended by the manufacturer (Amersham-Biosciences, Uppsala, Sweden). The amount of coupled antibody was determined by measuring the total protein before and after the coupling reaction. In all cases, the antibody concentration to start the immobilization process was similar to the expected ligand densities.

2.5. Immunoaffinity chromatography

Gels (12.1 mL) were packed into PD10 columns (Amersham-Bioscences, Uppsala, Sweden) and equilibrated with the buffer containing 20 mM Tris–HCl/3 mM EDTA/3 mM NaCl pH 7.2. Adsorption and elution flow rates were 20 and 35 mL h^{-1}, respectively. These flow rates were chosen to improve the adsorption and to speed up the elution. The columns were loaded with 7 mg of a purified rHBsAg preparation diluted in the equilibrium buffer in each purification cycle. After washing, the bound antigen was eluted with 20 mM Tris/3 M potassium thiocyanato/3 mM EDTA, pH 7.0, and monitored at 280 nm. All experiments were done at 22 ±2 °C.

2.6. Gel filtration chromatography

The eluted antigen buffer exchange to water was performed by gel filtration chromatography in a PD10 column with Sephadex G-25 (Amersham-Biosciences, Uppsala, Sweden). The column equilibrium was done with 3-column volumes of purified water and the volume of the applied samples was 2.5 mL. The buffer exchange to purified water was done because this antigen is quite stable under these conditions, allowing the evaluation of the concentration and purity by SDS-PAGE without the interference of any buffer.

2.7. Estimation of antigen-specific antibody activity by enzyme linked immunoassay

A polystyrene (PE) microplate (Costar, Cambridge, USA) was coated with 10 μg per well of 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 PBS and incubated for 1 h at 37 °C. After several washes with 0.05% Tween 20/PBS the plate was incubated for 1 h at 37 °C with an anti-mouse IgG-horseradish peroxidase conjugate (Sigma Chemical, St. Louis, USA). The reaction was revealed using 100 μL/well of 0.05% orthophenylenediamine 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 [18].

2.8. Estimation of leached antibody by enzyme linked immunoassay

A sandwich ELISA determined the concentration of IgG leached from the matrixes. Briefly, a plate (Costar, Cambridge, USA) was coated with sheep anti-mouse polyclonal immunoglobulin overnight at 4 °C. The plate was blocked with 1% fat-free milk in PBS for 30 min at 37 °C. Wells were washed and the eluted samples from the immunosorbents were added and incubated for 3 h at 37 °C with 1% non-fat milk dried powder in PBS. After washing the plate, it was incubated with 100 μL/well of a goat anti-mouse polyclonal immunoglobulin-horseradish peroxidase conjugate at a work dilution 1:9000 (Sigma Chemical, St. Louis, USA). The reaction was then revealed using 100 μL/well of 0.05% orthophenylenediamine 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.

2.9. Protein determination

Protein concentration was performed by the method described by Lowry et al. [19], using bovine serum albumin as standard material. Absorbance measurement at 280 nm was used for the quantification of the purified antibodies and antigens. The molar extinction coefficients used were 1.37 for mAb CB.Hep-1 and plantibody and 5.0 for rHBsAg [20].

2.10. SDS-PAGE

Samples were analyzed by electrophoresis on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels as described by Laemmli [21]. Separated proteins were stained with Coomassie blue R-250 and then analyzed by gel densitometry.

2.11. Western blot

Proteins were transferred by a semi-dry electrophoretic transfer with 25 mM Tris/192 mM glycine/20% methanol at 25 V for 20 min onto a nitrocellulose membrane (Scheiler & Schuell, Daseel, Germany) [22]. After incubating with 1% non-fat milk dried powder in PBS for 1 h at 37 °C the membrane was washed three times in PBS and incubated for 1 h at room temperature with 100 μL of a goat anti-mouse IgG conjugate to horseradish peroxidase (Sigma Chemical, St. Louis, USA). Bands were visualized by reacting with the substrate solution (5 mg of 3,3-diaminobenzidine/10 μL of 30% H_2O_2/10 mL PBS). The reaction was stopped with deionized water.

2.12. HPLC gel filtration

A HPLC-GF column TSK G3000 PW was used to determine the purity of the mAb and also of the plantibody. The mobile phase of the chromatography was PBS pH 7.0 and the volumetric flow rate employed was 0.2 mL min^{-1}. In both cases, the buffer of the samples was previously exchanged from 20 mM Tris-HCl/150 mM NaCl pH 7.2 to PBS pH 7.0 using a PD 10 column loaded with Sephdex G-25 (Amersham-Biosiences/Uppsala, Sweden).

2.13. Isoelectrofocussing

A high-resolution electrophoretic technique was applied to resolve proteins and peptides based on their isoelectrofocussing. The conditions used were a linear pH gradient, ranging from 5.0 to 8.0 (PHASTGEL IEF, Amershan-Biosciences, Uppsala, Sweden); and a homogeneous polyacrylamide gel (5%T, 3%C). An inner standard of mAb CB.Hep-1 was used as a reference material.

2.14. Statistical analysis

Adsorption and elution capacities, recovery and leached IgG were evaluated by ANOVA (simple factor). The significance level (a) was 0.05, and the STATISTICA for Window application was used.

3. Results and discussion

Immunoaffinity chromatography offers a high yield and purity in a single purification step simplifying a further downstream process. In this case, this technique acquires a higher meaning because this antibody is directed against the "a" determinant of the HBsAg, which is important to raise a protective human immune response against this virus [5,14,16,23]. Therefore, it is an additional guarantee of the immunological properties of the purified active pharmaceutical ingredient of the Hepatitis B vaccine.

In order to purify the rHBsAg, the plantibody HB-01 was obtained from tobacco leaves with a yield of up to 12 mg IgG/Kg of biomass, SDS-PAGE (Fig. 1) and HPLC-GF (Fig. 2) purity over 95%. Differences in the SDS-PAGE and HPLC-GF purity were not observed between mAb CB.Hep-1 and plantibody HB-01. Differences in the isoelectrofocussing pattern were observed (Fig. 3). The pattern was characterized by the presence of 7–8 bands with isoelectric pH value ranged from 7.5 to 6.8. The average theoretical isoelectric pH was 6.9, and this value was obtained from the analysis of the amino acid sequence (Fig. 4).

The nature of the matrix to which proteins are attached is important in several respects. It must be physically and chemically stable under the experimental conditions, must have satisfactory flow properties and must be free from non-specific adsorption effects. The open pore structure of Sepharose-4B allows the use of this matrix for gel filtration chromatography of proteins up to 20 million Daltons and also displays virtually all the desirable features of a matrix for protein immobilization. The immunopurification behavior of the immunosorbents was evaluated using 12.1 mL of matrix (IgG-Sepharose CL-4B) that represents the 0.1% of the real production scale (12 L) and several experiments were previously done at this scale (0.1%)

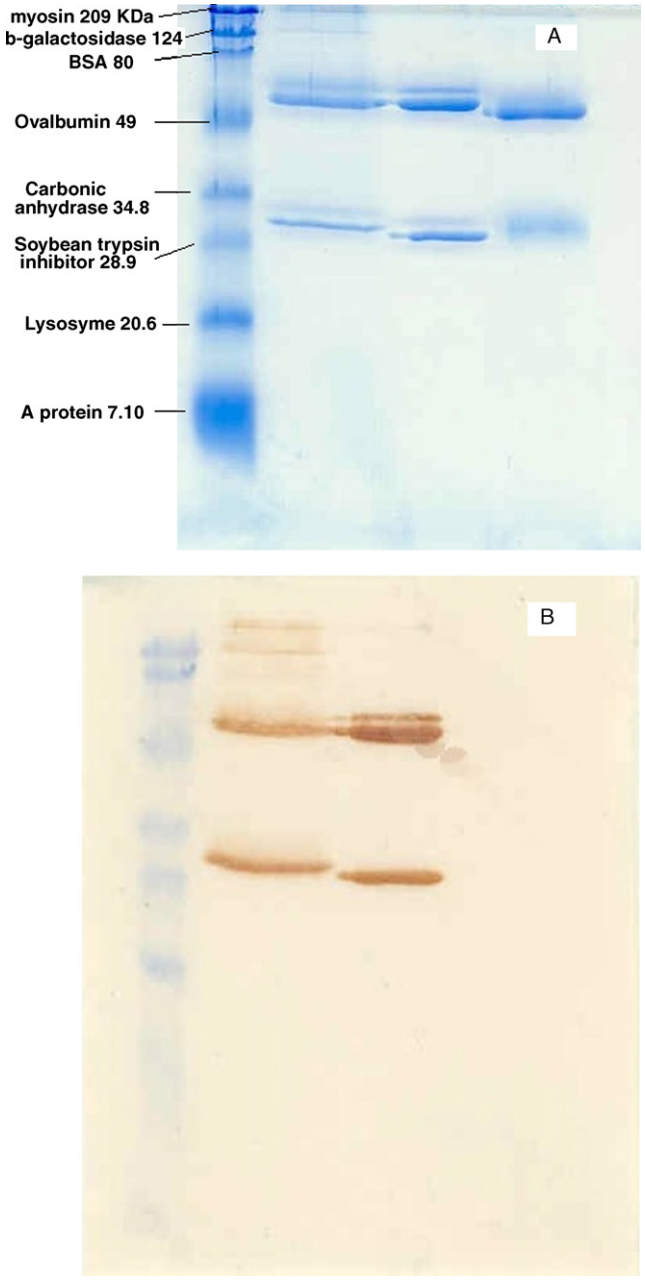

Fig. 1. Coomassie blue stained SDS-PAGE of plantibody HB-01 purified by expanded bed adsorption method and mAb CB.Hep-1 purified by packed bed adsorption. (A) Lane 1, molecular weight marker lane 2, plantibody HB-01 (5 μg); lane 3, mAb CB.Hep-1 (5 μg), lane 4, goat IgG (5 μg). (B) Immunoblot analysis, samples were transferred onto nitrocellulose and antibodies were detected with a horseradish peroxidase labeled goat anti-mouse IgG. Lane 1, pre-stained molecular weight marker; lane 2, plantibody HB-01 (5 μg); lane 3, mAb CB.Hep-1 (5 μg), lane 4, goat IgG (5 μg, negative control).

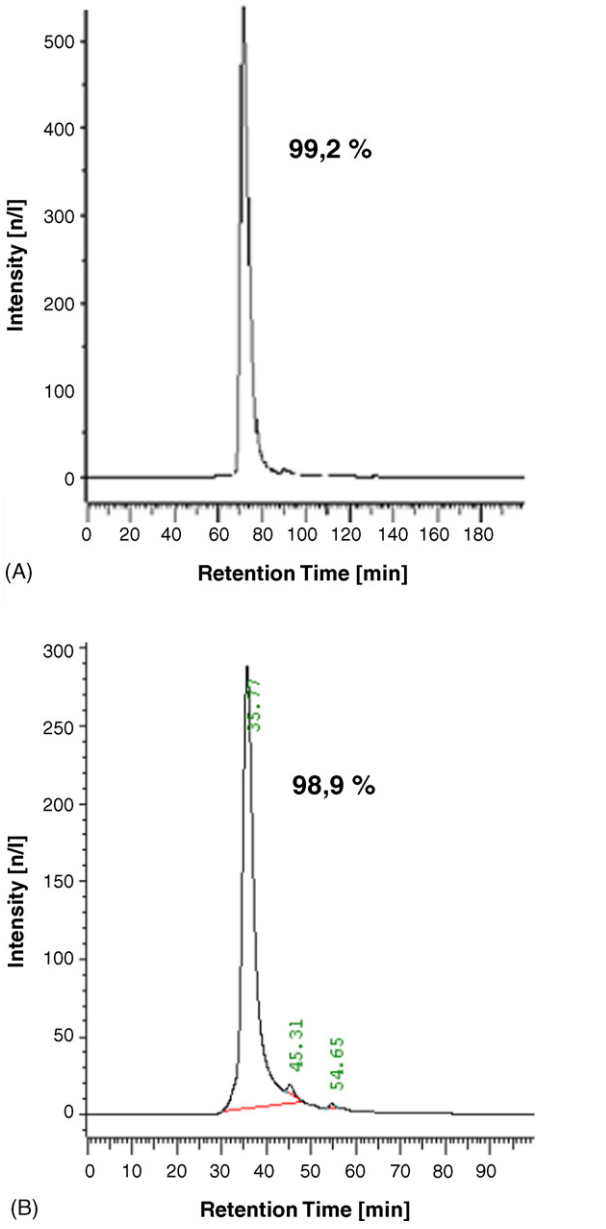

Fig. 2. HPLC gel-filtration profile of the mAb CB.Hep-1 (A) and plantibody HB-01 (B).

demonstrating a rHBsAg recovery of about 30–50% and biocomparability between antigens purified at both scales (data not shown).

On the other hand, the most popular, randomly oriented immobilized ligand activation procedure for agarose is the cyanogen bromide method. A large number of reaction schemes have been proposed for the coupling of proteins and other biopolymers to insoluble matrices, and the cyanogen bromide method results in an active product to which substances may be covalently coupled easily under mild conditions. Cyanogen bromide reacts with the hydroxyl groups of Sepharose to form imidocarbonate and carbamate groups. During the subsequent coupling of the protein to the active product, the imidocarbonate groups react with amino groups belonging to the protein with the formation of stable covalent linkages [17].

In this study, we select the cyanogen bromide method to immobilize the mAb CB.Hep-1 and the plantibody HB-01; this selection was made based on the number of (k) (lysine) present in the amino acid sequence of the mAb CB.Hep-1 and plantibody HB-01 molecules (Fig. 4). The coupling efficiency was over 92% for a final ligand density of 2.23, 3.41, 3.82, 4.45 and 5.31 mg/mL that is coincident with the coupling efficiency reported for this method. Nevertheless, this parameter

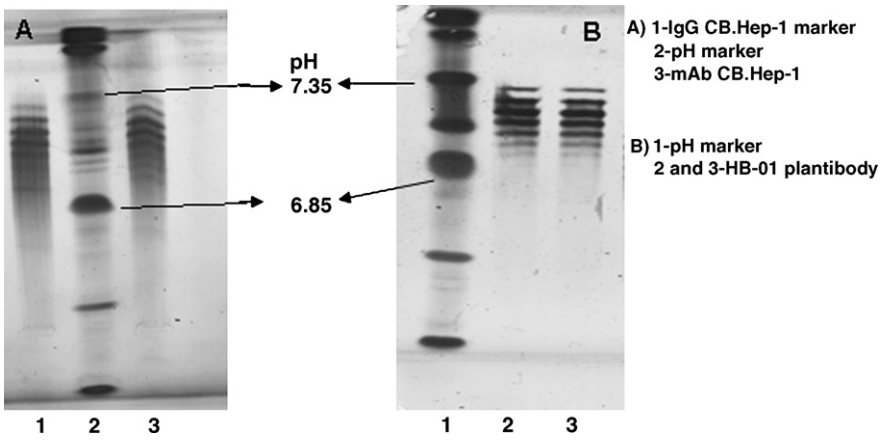

Fig. 3. Isoelectrofocussing pattern of the mAb CB.Hep-1 (A) and the plantibody HB-01(B). (A) Sample per lanes: (1) IgG CB.Hep-1 marker; (2) pH marker; and (3) CB.Hep-1 mouse antibody; (B) sample per lanes: (1) pH marker; (2 and 3) plantibody HB-01.

MDIVMSQSPSSLAVSVGE**K**VALSC**K**SSQSLLYLNNH**K**NYLAWFQQ**K**PGQSP**K**LLIYWASTRDSGVPDR
FTGSGSGTDFTLMISSV**K**AEDLAVYYCQQYYNYPYTFGGGT**K**LEI**K**RADAAPTVSIFPPSSEQLTSGGA
SVVCFLNNFYP**K**DINV**K**WK IDGSERQNGVLNSWTDQDS**K**DSTYSMSSTLTLT**K**DEYERHNSYTCEATH
KTSTSPIV**K**SFNRNECSR**K**DEL

(A) Plant-derived antibody HB-01 Light Chain

MEV**K**LDETGGGLVQPGRPM**K**LSBVASGFTFSDFWMNWVRQSPE**K**GLEWVAQIRD**K**PDNYAIYYSESV
KGRFTISRDDSRSSVFLQMNSLRPEDTGIYYBTAGFDYWGQGTTLTVSSA**K**TTPPSVYPLAPGBGDTT
GSSVTLGBLV**K**GYFPESVTVTWNSGSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWPSQTVTBSVAHP
ASSTTVD**K**K LEPSGPISTINPBPPBPPB**K**EBH**K**BPAPNLEGGPSVFIFPPNI**K**DVLMISLTP**K**VTBVVVDV
SEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTIRVVSTLPIQHQDDWMSG**K**EFK BK VNN**K**DLPSPIE
RTIS**K**I**K**GLVRAPQVYILPPPAEQLSR**K**DVSLTBLVVGFNPGDISVEWTSNGHTEENY**K**DTAPVLDSDGS
YFIYS**K**LNM**K**TS**K**WE**K**TDSFSBNVRHEGL**K**NYYL**K**K TISRSPG**K**SR**K**DEL

(B) Plant-derived antibody HB-01 Heavy Chain

Fig. 4. Aminoacid sequence of plantibody HB-01. In bold (K) the potential linkage points with CNBr activated matrix. (A) Aminoacid sequence of light chain with 16 potential linkage points, 6 of them inside the variable region of each chain. (B) Aminoacid sequence of heavy chain with 30 potential linkage points, 6 potential linkage points inside the variable region of each chain.

was always inferior in the case of the plantibody HB-01 in comparison with the mAb purified from ascitic fluid (Table 1); thus, further experiments should be conducted to investigate this difference.

Each column was purified between 4.03 and 30.69 mg of rHBsAg in 12 purification cycles (Table 1) for a rHBsAg recovery between 4.52 and 59.37%. The higher recovery values were observed at the ligand densities of 3.41, 4.45, 5.31 mg/mL and the control (mouse mAb column, 3.82 mg/mL). The statistical analysis of the immunopurification parameters (adsorption capacity, elution capacity, and recovery) did not show significant differences ($p = 0.89$, 0.74 and 0.51, respectively) corroborating the similitude between the mouse-derived immunoaffinity column and the plantibody immunoaffinity columns behavior at these ligand densities. Conversely, only 8.11 mg rHBsAg were adsorbed and 4.03 mg eluted from the matrix coupled with plantibody HB-01 at a ligand density of 2.23 mg/mL showing significant differences ($p = 5.96715\text{E-}11$) with the rest of ligand densities evaluated. This may be explained by the fact that a low antibody amount in regard to the number of active group could compromise also the antigen recognition site with the matrix where there are several potential linkage points with the activated groups (Fig. 4).

The reason why the rHBsAg recovery is relatively low (30–50%) is still unclear. We hypothesize that rHBsAg particles size makes this immunochromatography inefficient. As

Table 1
Immunopurification behavior of the plantibody HB-01columns in 12 purification cycles

Ligand density (mg/mL)	Coupling efficiency (%)	Total adsorbed rHBsAg (mg)	Total purified rHBsAg (mg)	Recovery of rHBsAg (%)	Average of leached IgG (ngIgG/μgrHBsAg)	IgG leached (%)
2.23	92.21	8.11 ± 0.26	4.03 ± 0.16	4.52 ± 2.26	2.90 ± 2.93	0.098
3.4	92.48	65.55 ± 1.81	30.68 ± 0.89	46.80 ± 12.79	0.13 ± 0.08	0.023
4.45	94.62	45.69 ± 0.83	24.93 ± 0.85	54.56 ± 12.32	0.79 ± 0.50	0.009
5.31	98.05	42.09 ± 1.03	24.99 ± 0.32	59.37 ± 4.94	3.29 ± 0.99	0.031
3.82 (mAb column)	98.10	63.46 ± 2.20	30.69 ± 1.26	48.33 ± 17.91	0.88 ± 0.50	0.037

can be extrapolated from the Table 1, about 45% of the applied antigen pass through the column and can be quantified in the non-bound fraction, even using a low linear flow rate during the adsorption process. We have also demonstrated that there is rHBsAg which is retained into the matrix in every purification cycle and that the matrix regeneration buffers (Buffer 1: 0.1 M Tris-HCL + 0.5 M NaCl pH 8.5 and Buffer 2: 0.1 M Tris + 0.5 M NaCl + 0.1 M AcNa pH 4.0) are strong to eliminate unspecifically bound proteins, carbohydrates and lipids but not the retained rHBsAg.

Several factors can cause a loss of column capacity during a repeated operation. One of the most important is the irreversible denaturalization of the antibody, usually caused by harsh elution conditions [24]. The plantibody HB-01 columns (ligand density: 3.41, 4.45, and 5.31 mg/mL) showed rapid elution capacity decrease similar to that of the mAb column. These rapid decreases seem to depend on the use of the chaetropic agent in the elution buffer and on the following of the matrix with non-specific adsorbed antigen, perhaps provoked by the lower ion exchange character of the adsorbent. Other factors, such as contaminants cannot be considered because the applied rHBsAg was always pure (≥95%).

Ligand leached contributes impurities to the product. Taking into consideration that the antigen eluted from these columns is intended for pharmaceutical use, this parameter raises great importance. This immunoaffinity chromatography is the first step of the chromatographic purification process of the rHBsAg, for that reason an IgG level ≤3 ngIgG/μgrHBsAg has been established as the approved maximum co-elution level, because the subsequent downstream process is able to assure a non-immunogenic level of IgG in the Hepatitis B vaccine [22]. In this work, the average IgG detectable level never exceeded the approved limit. Table 1 and Fig. 5 show clearly the average amount of the IgG leached from the matrixes. A value close to the limit was observed only at the ligand density of 5.31 mg/mL, constituting this another aspect against the use of high ligand densities and also in the immunosorbent of ligand density 2.23 mg/mL, but in such case the ratio IgG/rHBsAg was affected by the low level of antigen eluted from the column. The percentage of IgG leached for this immunosorbent was only 0.098% (Table 1). In addition, the ligand leached was not the major cause of the elution capacity decrease because the total leached IgG was lower than 0.029% of the total coupled IgG

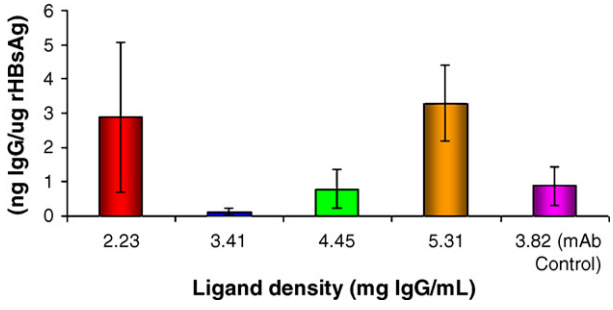

Fig. 5. Antibody leached from the immunochromatography matrixes. The approved value of IgG leached for the immunochromatography eluates in 3 ngIgG/μgrHBsAg.

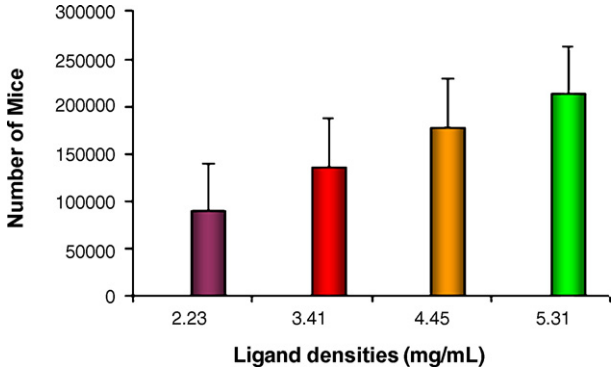

Fig. 6. Total amount of mice required for the production of 200 L of immunosorbents. This volumen correspond with our installed production capacity.

for plantibody HB-01 columns and 0.037% for mAb CB.Hep-1 column (Table 1).

These results validate the use of this plantibody for the immunopurification of the rHBsAg for human use, opening a new alternative to overcome constrains of mAb CB.Hep-1 production in mice. Fig. 6 shows the amount of mice required for the production of 200 L of this immunosorbent. Therefore, as soon as the plantibody HB-01 is introduced in the large-scale production of the active pharmaceutical ingredient of this Hepatitis B vaccine, thousands of mice will not be required for this purpose.

4. Conclusion

These results support the feasibility of using the plantibody HB-01 for the large-scale immunopurification of the rHBsAg for human use at a ligand density of 3.47 mg/mL because it demonstrated the same immunopurification behavior of the mouse-derived antibody column routinely used for this purpose.

Acknowledgment

Authors greatly acknowledge the rHBsAg Production Department for providing the purified recombinant Hepatitis B surface antigen.

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