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Optimisation of the coupled monoclonal antibody density for recombinant hepatitis B virus surface antigen immunopurification

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Abstract

Using immunosorbents based upon cyanogen bromide-Sepharose CL-4B, we have examined different ligand densities in coupling of monoclonal antibody (MAb) to find the best performance, for recombinant hepatitis B surface antigen (rHBsAg) purification. Three replicates of 5 and 15 cycles of densities ranges: 2.17–2.19, 3.18–3.62, 4.06–4.17, and 5.13–5.40 mg/ml (control); or 1.81–2.47, 3.17–3.41, 4.16–4.28, and 5.16–5.19 mg/ml (control), respectively were evaluated in terms of binding capacity, antigen recovery, ligand leakage and purity of antigen, and compared to the control. Adsorption and antigen recovery of immunosorbents manufactured were not different statistically, eventhough increased 8.08 and 9.90% at a range of 3.17–3.41 mg/ml. At this range, efficiency expressed as productivity and MAb saving was optimal. Ligand leakage and purity of antigen showed similar behaviour among all densities. Aspects related to ligand density in antigen immunoaffinity purification are discussed.

Keywords: Monoclonal antibody density; Immunopurification; Recombinant hepatitis B virus surface antigen

1. Introduction

Immunoaffinity chromatography (IAC) is a powerful technique in production of biologically active molecules, in which usually great amounts of monoclonal antibodies (MAbs) are coupled to a polymeric support for obtaining selective bioseparation immunosorbents [1,2].

Ligand density or quantity of molecules chemically bound to a resin is an important factor influencing immunosorbent efficiency and costs. High ligand densities used for purification are related to crowding of adjacent immobilised MAb molecules on the surface of bed particle and diffusion troubles inside the pores during chromatographic process. This phenomenon known as steric hindrance causes a decrease of antigen (Ag) binding due to a limited specific activity of the crowded ligand [3,4].

Conversely, low ligand density provides an intrapore atmosphere sufficiently available with a better penetration and higher adsorption probability of the target protein into the matrix, minimising steric restriction, and favouring its movement and binding [5–7].

The MAbs used for immunosorbents technology are expensive and after coupling often show low adsorption efficiency (1–10%). Consequently, IAC should be optimised according to a ligand density in order to increase the Ag capture capacity and total recovery [8–10].

CB.Hep-1 MAb has been successfully immobilised to Sepharose for specific recognition and adsorption of recombinant hepatitis B virus surface antigen (rHBsAg) [11]. But, its industrial application requires reducing man-

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ufacture and coupling costs to save time and financial resources.

In this work, we comparatively study different ligand densities to select the best range for immunosorbent production.

2. Experimental

2.1. Monoclonal antibody

CB.Hep-1 MAb secreted by the hybridoma cell line 48/1/5/4 was previously generated by Fontirrochi et al. [12]. In brief, BALB/C mice were immunised subcutaneously with a first dose of 50 μg of natural hepatitis B surface antigen (nHBsAg), in Freund's complete adjuvant, followed 15 and 21 days later by similar doses in Freund's incomplete adjuvant. Three days before the fusion, the animals with the highest anti-nHBsAg antibody title received an intraperitoneal injection of 50 μg of antigen in phosphate buffered saline and spleen cells were fused with the myeloma cell line Sp2/0-Ag14. Spleen cells and myelomas were hybridised in the presence of 45% polyethylene glycol 1450 (Sigma, St. Louis, MO, USA), at a 10:1 ratio, and hybridoma selected in HAT medium (Sigma, St. Louis, MO, USA). CB.Hep-1 MAb was purified from ascites fluid by protein-A affinity chromatography [13]. The purity of the final antibody preparation was 95% assessed by sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS–PAGE) under reducing conditions. The MAb was dialysed in order to exchange salts 20 mM Tris–150 mM NaCl, pH 7.6 with 0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.3, by gel filtration using pre-packed disposable columns PD-10 (Amersham-Pharmacia Biotech, Uppsala, Sweden). Protein concentration was determined according to Lowry et al. [14]. Protein solution was filtered through a 0.2 μm pore-sized membrane (Sartorius, Göttingen, Germany) in aseptic conditions and stored at 4 °C until its coupling. CB.Hep-1 MAb was purified (98%) in Monoclonal Antibody Department, Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba) [15].

2.2. Source of rHBsAg

rHBsAg was produced by fermentation of a recombinant strain of *Pichia pastoris* (C-226) in saline medium supplemented with glycerol, and its expression was induced with methanol. The rHBsAg was recovered and submitted to initial purification steps as previously described [16] and optimised [17,18]. Briefly, the cells were harvested by centrifugation and disrupted on a bed mill (KDL type: WAB, Basel, Switzerland). The disruption buffer contained 20 mM Tris–HCl, pH 8.0, 3 mM EDTA, 0.3 M NaCl, 3.0 M KSCN and 10 g/l sucrose. The homogenate was submitted to acid precipitation by adding 1 M HCl down to pH 4.0 and centrifuged at 10,000 × *g* for 30 min. The supernatant was placed in contact with Hyflo Super Cell (a flux-calcined grade of celite filter aid) equilibrated to the same pH (4.0) under continuous stirring. Adsorption was allowed to take placed for 2 h and Hyflo Super Cell was separated by centrifugation. After washing the matrix twice with two Hyflo Super Cell volumes of 0.2 M KSCN solution, the antigen was eluted with 20 mM Tris–HCl–3 mM EDTA-100 g/l sucrose pH 8.2. With the described procedure, a semi-purified material of about 10–15% purity was obtained. This starting material was used for immunoaffinity chromatography.

2.3. Immunosorbents preparation

Sepharose CL-4B (Amersham-Pharmacia Biotech) was activated (70–80 μmol/ml) with cyanogen bromide (Merck, Damstadt, Germany) according to a modified procedure previously described [19]. Briefly, washings with water in order to remove to the preservation solution from the support were made. In a reaction bowl, cyanogen bromide and nitrile acetate (1 ml/g of Sepharose) were mixed adding 4 M NaOH. The pH was controlled in a range from 10.5 to 11 at 18–21 °C with gentle stirring. The reaction was completed at 15 min. Continuous washings with water (4 ml/ml of Sepharose), 0.1 M acetic acid (2 ml/ml of Sepharose), water (5 ml/ml of Sepharose) and dioxane (2 ml/ml of Sepharose) were performed. Concentration of cyanate esters was determined by a modified König reaction [20].

The activated support was incubated for 15 min with 1 mM HCl (1 ml/ml of gel) and consecutively washed with the same solution at 5 ml/ml of gel. Later, washings using 0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.3, at 5 ml/ml of gel were achieved. MAb was covalently coupled at 25 °C by gentle stirring in sterile flasks using a shaker (Bioblock Scientific, Strasbourg, France). Coupling efficiency (δ) was determined by an indirect method, following the formula: $\delta(\%) = \chi/\lambda \times 100$. Where χ is the amount of coupled protein determined as the difference between the original amount of ligand (λ) and the amount detected in the filtration and washing fractions after coupling.

Excess of reactive groups was blocked adding 0.1 M glycine, pH 8.0. Immunosorbents were washed alternating five washings with 0.1 M sodium acetate–0.5 M NaCl pH 4.0 and 0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.3.

2.4. Immunoaffinity chromatography

IAC was performed in columns C 10/10 (Amersham-Pharmacia Biotech) packed with 2 ml of immunosorbents [21], previously washed with 20 mM Tris–3 mM EDTA–1 M NaCl, pH 7.0, at 20 cm/h flow rate 5 ml/ml of gel. Columns were directly loaded at 20 cm/h flow rate. Washings using 20 mM Tris–3 mM EDTA–1 M NaCl, pH 7.0, at 20 cm/h flow rate 5 ml/ml of gel were performed. Elution was carried out using 20 mM Tris–3 mM EDTA–1 M NaCl–3 M KSCN, pH 7.0 at a flow rate of 35 cm/h flow rate. The eluted antigen concentration was determined by UV measurement [A_{280} (1 cm, 1 mg/ml) = 5].

The amount of ligand leakage was measured by a validated murine IgG-specific sandwich ELISA. Briefly, a plate was coated overnight at 4 °C with a sheep anti-mouse polyclonal immunoglobulin. The plate was blocked 30 min at 37 °C, the wells were washed and the eluted samples from the immunosorbents were added. The plate was incubated for 3 h at 37 °C with 1% non-fat milk in PBS. After three washings, it was incubated with 100 μl of horseradish peroxides (HRP)–streptavidin conjugate anti-mouse polyclonal immunoglobulin (Sigma). The reaction was revealed using 100 μl/well of 0.05% ortho-phenylenediamine (OPD) and 0.015% hydrogen peroxide (H_2O_2) in citrate buffer, pH 5.0. After 20 min, the reaction was stopped with 50 μl per 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.

The antigen purity was determined by SDS–PAGE (12.5%) with Coomassie blue staining [22] using a laser densitometer (LKB Pharmacia Biotechnology, Uppsala, Sweden).

2.5. Statistical analysis

GraphPad Prism [23] and Microsoft Excel programs were used as tools for statistical analysis. Gaussian distribution and variants homogeneity were verified by Kolmogorov–Smirnov and Bartlett test, respectively. Analysis of variances (test of F for α=5%) was achieved according to procedures recommended for chromatographic processes [24,25]. The significance level (α) was 0.05, and the statistica for Windows application was used.

Experimental groups of ligand density were statistically compared in terms of adsorption capacity, recovering of rHBsAg, ligand leakage, and purity of rHBsAg (Table 1). All experiments were reproduced three times.

The hypothesis test was formulated assuming equal variances:

$H_o : \mu_1 = \mu_2$ (null hypothesis)
$H_i : \mu_1 \neq \mu_2$ (alternative hypothesis)

where μ_1 is mean of variables from control treatment; μ_2 is mean of variables from each experimental treatment.

2.5.1. Logical deductions

If the null hypothesis (H_o) is accepted it will imply that a reduction of ligand density with respect to the control treatment does not affect process variables. On the contrary, if it is rejected, then the result will be satisfactory only when $\mu_1 \neq \mu_2$ for binding capacity, recovery of rHBsAg and purity, and $\mu_1 > \mu_2$ for ligand leakage.

Table 1
Different ranges of ligand densities reached in coupling efficiency

Treatments	Ligand density (mg/ml)	
	Control	Experimental
5 cycles	5.13–5.40	4.06–4.17, 3.18–3.42, 2.17–2.19
15 cycles	5.16–5.19	4.16–4.28, 3.17–3.41, 1.81–2.47

3. Results and discussion

3.1. Binding capacity and recovery of rHBAsg

Significant differences were not observed neither in adsorption capacity of immunosorbents ($P = 0.845$) during five chromatographic cycles (Fig. 1) nor in rHBsAg recovery ($P = 0.186$), although the best behaviour was observed at 3.18–3.42 mg/ml, probably because a more correct spacing of adjacent molecules of coupling MAb.

Figs. 2 and 3 show similar binding capacities ($P = 0.888$) [>0.514 mg of rHBsAg/ml of gel] as well as Ag

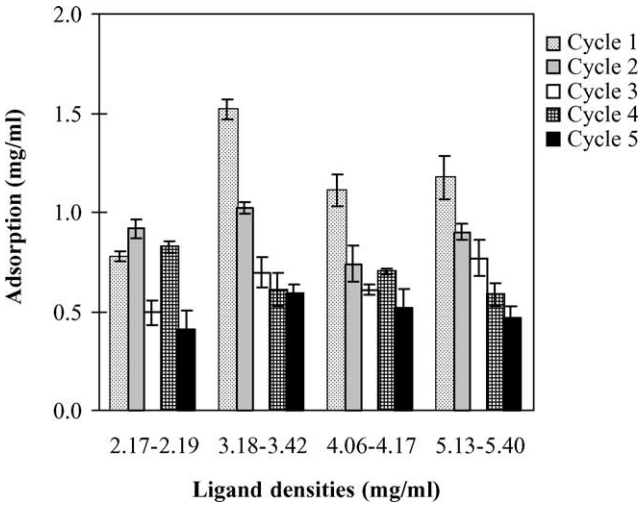

Fig. 1. Performance of immunosorbents for five cycles of immunoaffinity chromatography.

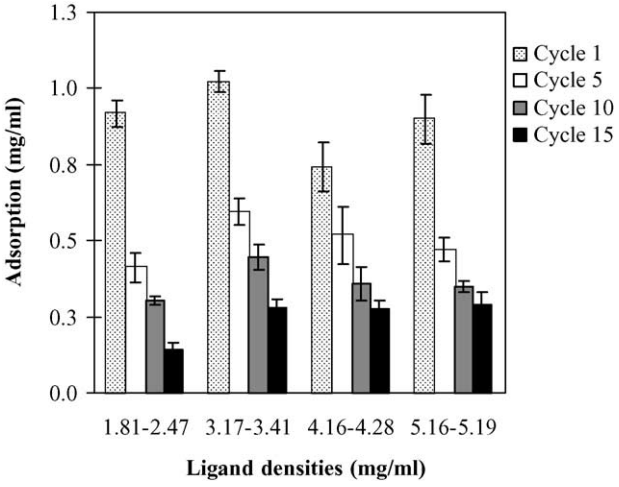

Fig. 2. Binding capacity of different densities of monoclonal antibody coupled for recombinant hepatitis B virus surface antigen purification.

recoveries ($P = 0.488$) for 15 cycles. However, in the range from 3.17 to 3.41 mg/ml increments up to 8.08 and 9.90% were evidenced respectively in comparison with the control treatment. Densities of 1.81–2.47 mg/ml showed the lowest rHBsAg recovery and productivity, likely due to the amount of randomly MAb coupled in this range with partial or total uncapable to adsorb rHBsAg became a critical factor.

A sequence of four steps were involved in adsorption: (i) transport of protein from fluid to adsorbent particle; (ii) transport through liquid layer surrounds the particle; (iii) diffusion of protein in to the particle pores; and (iv) interaction between protein and binding site [26,27]. Usually, step 1 is infinitely fast [28], while step 3 is too slow and decisive in total speed of adsorption [27].

Differences of steric hindrance among ligand densities seem to be small with respect to the great diffusional limitations caused by a large size of the rHBsAg, so that makes its access in to the active site of ligand difficult.

rHBsAg diffusion in to the pores of adsorbent is severely restricted [29]. Mean diameter of an aggregated rHBsAg molecule is 22–27 nm [30] and Sepharose pores have a maximum exclusion limit up to 2×10^7 Da (~36 nm) [31], while the CB.Hep-1 MAb diameter (15×10^4 Da) is assumed as 7 nm [29].

Solute diffusivity inside the bed is about 15–20% of the free molecular diffusivity, which indicates that solute and pores have the same order of dimensions. Thus, intraparticle diffusivity of rHBsAg is only 11% (~9 times smaller) and exhibits a low transfer coefficient, so it is likely that adsorption occurs initially in a rapid way on external sites of stationary face, and later, when those sites have been occupied, it takes place slower inside the particle.

In purification of high molecular weight complexes, a limited protein intraparticle diffusion hinders to observe a marked effect of ligand density over binding capacity

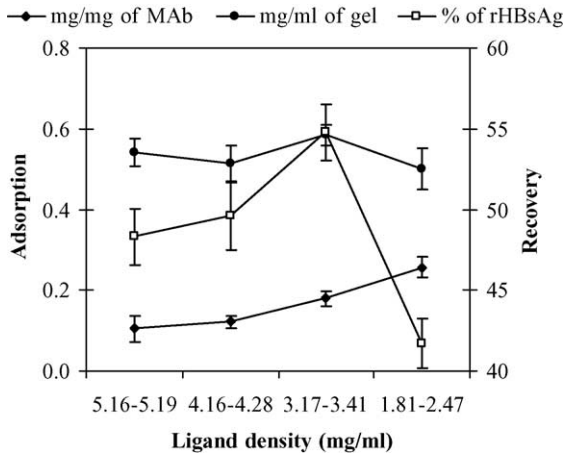

Fig. 3. Comparison of average binding capacity and antigen recovery of immunosorbents during immunoaffinity chromatography.

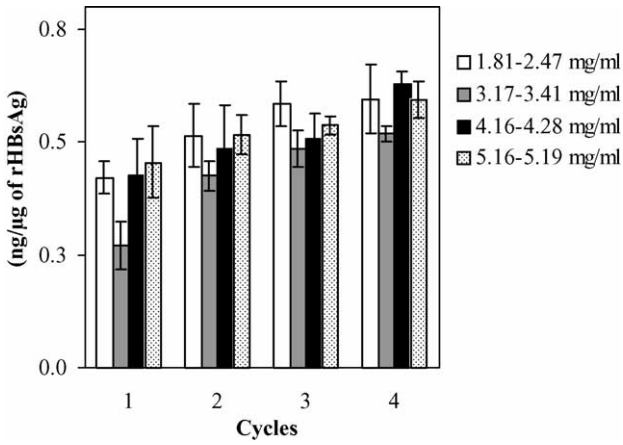

Fig. 4. Ligand leakage in recombinant hepatitis B virus surface antigen recovered with immunosorbents.

[32,33]. Nevertheless, in this case a light increase of adsorption could be observed at low density, presumably due to interstitial spaces of the support with less crowding, favouring a more homogeneous MAb coupling throughout their surface and an easier Ag–Ag binding to improve a stechiometric Ag:MAb ratio [34].

3.2. Ligand leakage and purity of the antigen

The presence of ligand contamination in pharmaceutical products should be kept at a given level so far as MAb can be associated with the human answer mouse antibody (HAMA) [35–37].

CB.Hep-1 immunosorbents prepared with varying ligand density showed a stable behaviour of the MAb presence detected in rHBsAg purified for a half live time of 15 cycles (P=545) with a maximum value of 0.629 ng/µg rHBsAg (Fig. 4). Additionally, the purity of rHBsAg recovered did not evidence statistical differences (P=0.864) and values always were above to 80% in agree with other records [38,39]. The 24 kDa major bands corresponded to the monomer and the 48 kDa to the dimer as previously reported Wampler et al. [40] and Fernández de Cossío [11] (Fig. 5).

3.3. Productivity

The main IAC paramenters were involved in productivity. It was defined according to the concepts described before [41], as the quotient of the product resulting from multiplying capacity, recovery, and purity divided by average time of chromatographic run performed for different ligand densities.

Densities around 2 mg/ml led to productivities below to control, influenced mostly by an decrease of the antigen recovery. On the contrary, the immunopurification process was more efficiently at densities of 3.17–3.42 mg/ml (Fig. 6).

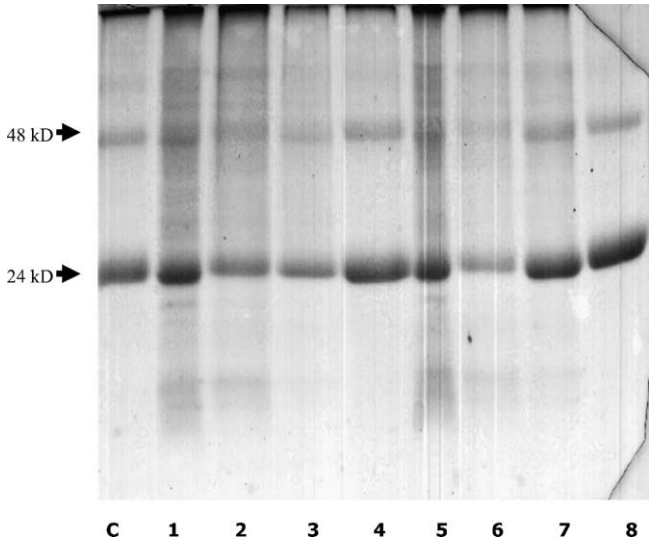

Fig. 5. SDS–PAGE in reducing conditions of recombinant hepatitis B virus surface antigen purified with CB.Hep-1 MAb at densities 5.19 mg/ml (Lanes 1–4; cycles 15, 10, 5 and 1) and 3.17 mg/ml (Lanes 5–8; cycles 15, 10, 5 and 1) for 15 cycles. C: pattern of recombinant hepatitis B virus surface antigen.

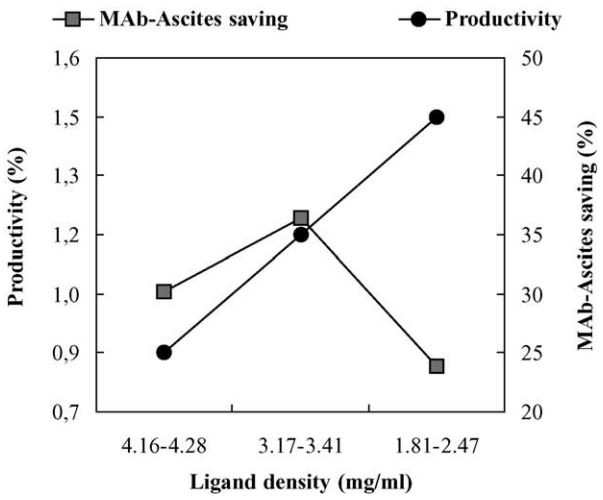

Fig. 6. Productivity of immunoaffinity columns for 15 cycles. Variables values were referred to the control density.

4. Conclusions

Ligand densities from 1.81 to 5.40 mg/ml did not cause significant differences in immunosorbents behaviour, likely due to a great size of rHBsAg, which made it too difficult to observe a marked effect of reduction of the ligand density on adsorption capacity.

The results obtained suggest for bench scale CB.Hep-1 MAb coupling, to use an optimal range of ligand density of 3.17–4.28 mg/ml, preserving specific functional activity of the ligand, to reach an appropriate productivity for 15 cycles of IAC, and a satisfactory reduction of the MAb cost. On the other side, ascites consume, amount of animals necessary for MAb production, mice manipulation and debris vertiment to the environment were reduced.

This experience in the rHBsAg purification allowed us to propose the achievement of new probes for further IAC process scales up with a more profitable ligand density range.

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