Optimization of CB.Hep-1 MAb Coupling to Sepharose

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ABSTRACT

Different conditions of covalent coupling of the CB.Hep-1 monoclonal antibody (MAb) to CNBr-Sepharose CL-4B were studied, with the aim of improving the performance of immunoaffinity chromatography for the purification of the recombinant hepatitis B virus surface antigen (rHBsAg). For this purpose, the immunosorbents containing 2.2, 3.2, 4.2, and 5.2 mg of CB.Hep-1 MAb/mL of gel were examined. The results showed that at a range of ligand density from 3.2 to 5.2 mg of CB.Hep-1 MAb/mL of gel the antigen binding capacity was higher. Other parameters studied were coupling time, coupling pH and ligand concentration in the coupling buffer. Immobilization rates of 75, 120 and 180 min did not show significant differences either in the adsorption capacity or in the ligand leakage. The immunosorbents prepared at the coupling pH 10.0 showed a significant increment in the ligand leakage compared to those prepared at pH 8.3 and pH 9.0. When the concentrations of the CB.Hep-1 MAb in the coupling buffer were examined in terms of adsorption capacity, the highest value was achieved using the concentrations of 4.51–4.57 mg of CB.Hep-1 MAb/mL. In conclusion, the best conditions for the CB.Hep-1 coupling are 3.2–5.2 mg of CB.Hep-1 MAb/mL of gel, 120 min, pH 8.3 and concentrations of 4.51–4.57 mg CB.Hep-1 MAb/mL.

Keywords: immunoaffinity, monoclonal antibody, recombinant hepatitis B surface antigen

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RESUMEN

Optimización del acoplamiento del anticuerpo monoclonal CB.Hep-1. Se estudiaron diferentes condiciones para la inmovilización covalente del anticuerpo monoclonal (AcM) CB.Hep-1 a un soporte Sepharose CL-4B activada con CNBr, con el objetivo de mejorar el funcionamiento de la cromatografía de inmunoafinidad para la purificación del antígeno de superficie del virus de la hepatitis B recombinante (rHBsAg). Para este propósito, se evaluaron densidades de 2,2; 3,2; 4,2 y 5,2 mg de AcM CB.Hep-1/mL de gel. La capacidad de unión al antígeno fue más elevada en el rango de 3,2 a 5,2 mg de AcM CB.Hep-1/mL de gel. Se estudiaron otros parámetros como tiempo de acoplamiento, pH de acoplamiento y concentración del ligando en solución. El procedimiento de inmovilización ejecutado con tiempos de acoplamiento de 75, 120 y 180 min, no causó diferencias significativas en la capacidad de adsorción ni en el desprendimiento del ligando. Los inmunoadsorbentes preparados a pH de acoplamiento 10,0 mostraron un incremento significativo en el desprendimiento del ligando, mientras que a pH 8,3 y pH 9,0 no se observó una dependencia entre el pH y el desprendimiento del ligando. Los valores más altos de capacidad de adsorción se obtuvieron en un rango de concentraciones entre 4,51 y 4,57 mg de AcM CB.Hep-1/mL. En conclusión, las mejores condiciones para el acoplamiento del AcM CB.Hep-1 son: 3,2-5,2 mg de AcM CB.Hep-1/mL de gel, 120 min, pH 8,3 y concentraciones de 4,51-4,57 mg AcM CB.Hep-1/mL.

Palabras claves: anticuerpo monoclonal, antígeno de superficie del virus de la hepatitis B recombinante, inmunoafinidad

*I*ntroduction

Hepatitis B surface antigen (HBsAg) is a particle composed by ~100 repetitive copies of the transmembrane hydrophobic S protein and phospholipids. The assembled particles produced in yeast are highly immunogenic and form the active component used in the commercial hepatitis B vaccines as HEBERBIOVAC HB® [1–4].

Affinity separations are well-established techniques for the purification and recovery of biological molecules [5–8]. The high specificity inherent to immunoaffinity chromatography (IAC) allows a one-step purification to obtain highly pure and concentrated preparations from complex mixtures [9]. Several procedures for HBsAg purification using antibodies have been described [10–12].

CB.Hep-1 MAb is an anti-HBsAg antibody of IgG2b subtype, which is capable of recognizing the monomeric and dimeric forms of the recombinant HBsAg (rHBsAg). Within the S protein chain it specifically recognizes amino acids 121–137 within the *"a"* determinant, referred to as a heat-resistant nonconformation dependent antigenic determinant. Previous experiments have demonstrated that the 121–124 motif is critical for the exposition of the epitope recognized by this MAb [4, 13].

The use of CB.Hep-1 MAb as an immunoligand has been addressed into the most efficient step of rHBsAg purification. On the other hand, several attempts have been done for the optimization of IAC using new elution conditions and a better manufacturing technology [14–16].

The aim of the present work was to optimize CB.Hep-1 MAb coupling to CNBr-Sepharose CL-4B in order to reach the highest throughput in rHBsAg

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immunopurification. This is the first report on the influence of coupling kinetics, coupling pH and ligand concentration on rHBsAg purification. Additionally, the influence of ligand density on the efficiency of rHBsAg adsorption and diffusion into pores resin is discussed.

Materials and Methods

Monoclonal antibody

CB.Hep-1 MAb secreted by the hybridoma cell line 48/1/5/4 was previously generated by Fontirrochi *et al.* [17]. Balb/c mice were subcutaneously immunized 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 titer 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 hybridized in the presence of 45% polyethylene glycol 1450 (Sigma, USA) at a 10:1 ratio, and hybridomas were selected in HAT medium (Sigma, USA). CB.Hep-1 MAb was purified from ascites fluid by protein-A affinity chromatography [18]. The purity of the final antibody preparation was 95% assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The MAb was dialyzed in order to exchange salts 20 mM Tris-150 mM NaCl pH 7.6 with 0.1 M Na_2CO_3-0.1 M $NaHCO_3$-0.5 M NaCl pH 8.3 by gel filtration using pre-packed disposable columns PD-10 (Amersham Pharmacia Biotech, Sweden). Protein concentration was determined according to Lowry *et al.* [19]. The protein was filtered through a 0.2-µm pore-sized membrane (Sartorius, Germany) in aseptic conditions and stored at 4 ºC until coupling. CB.Hep-1 MAb was purified (98%) by the Monoclonal Antibodies Division, Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba [20].

Source of rHBsAg

Recombinant HBsAg was produced by fermentation of a recombinant strain of *Pichia pastoris* (C-226) in saline medium supplemented with glycerol. Expression was induced with methanol. The rHBsAg was recovered and submitted to initial purification steps as previously described [11] and optimized [21, 22]. 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 for 30 min at 10 000 x*g*. The supernatant was mixed with Hyflo Super Cell (a flux-calcined grade of celite filter aid) equilibrated to the same pH (4.0) under continuous stirring. After 2 h of incubation, the Hyflo Super Cell was separated by centrifugation, washed twice with two volumes of 0.2 M KSCN and eluted with 20 mM Tris-HCl,3 mM EDTA and 100 g/L sucrose, pH 8.2. The eluate containing 10–15% rHBsAg was used as starting material for further IAC experiments.

Immunosorbents preparation

Sepharose CL-4B (Amersham Pharmacia Biotech, Sweden) was activated (70–80 µmol/mL) with cyanogen bromide (Merck, Germany) according to Axen *et al.* [23]. Briefly, washings were perfomed with water in order to remove the support preserve. In a reaction bowl CNBr and nitrile acetate (1 mL/g of gel) were mixed adding 4 M NaOH. The pH was controlled in the range 10.5–11 at 18–21 ºC with gentle stirring. The reaction was completed at 15 min. Continuous washings with water (4 mL/mL of gel), 0.1 M acetic acid (2 mL/mL of gel), water (5 mL/mL of gel) and dioxane (2 mL/mL of gel) were performed. Concentration of cyanate esters was determined by a modified König reaction [24].

The activated support was incubated for 15 min with 1 mM HCl (1 mL/mL of gel) and washed 5 mL/mL of gel. Thereafter, the resin was equilibrated with 0.1 M Na_2CO_3-0.1 M $NaHCO_3$-0.5 *M* NaCl, pH 8.3 5 mL/mL of gel. The MAb was covalently coupled at 25 ºC by gentle stirring in sterile flasks using a shaker (Bioblock, 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 washings fractions after the 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 Na_2CO_3-0.1 M $NaHCO_3$-0.5 M NaCl, pH 8.3.

Kinetics of immobilization. The coupling reaction was performed by gentle stirring using a shaker (Bioblock, France) as described above, and coupling efficiency was measured every 15 min (range: 0–120 min of coupling time) and every 30 min (range: 120–240 min of coupling time). The unbound protein in supernatants was separated by centrifugation (Hitachi, Japan) at 1 469 x*g* for 1 min.

Coupling pH measurements. The reagents used to prepare buffers were from Merck, Germany. Samples of CB.Hep-1 MAb were prepared by gel filtration using pre-packed PD-10 columns (Amersham Pharmacia Biotech, Sweden). Coupling was performed with 0.1 M Na_2CO_3-0.1 M $NaHCO_3$-0.5 M NaCl, pH 8.3 (standard procedure), pH 9.0 and pH 10.0.

Concentration of the ligand in coupling buffer. The MAb was concentrated in Amicon DC-2 USA at concentrations from 2.97 to 6.08 mg of CB.Hep-1 MAb/mL pH 8.3 and filtrated through a 0.2 µm pore-sized membrane (Sartorius, Germany) before coupling.

Ligand density. The immunosorbents prepared in the present work contained 5.2, 4.2, 3.2 and 2.2 mg of CB.Hep-1 MAb/mL of gel, as determined from the protein balance.

Immunoaffinity chromatography

The columns C 10/10 (Amersham Pharmacia Biotech, Sweden) were packed with 2 mL of immunosorbents [25], previously washed with 20 mM Tris-3 mM EDTA-1 M NaCl pH 7.0 at a flow rate of 20 cm/h (5 mL/mL of gel). Columns were directly loaded with the semipurified material at a 20 cm/h flow rate and then washed with 20 mM Tris-3 mM EDTA-1 M

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NaCl pH 7.0 at 20 cm/h (5 mL/mL of gel). The adsorbed rHBsAg was eluted with 20 mM Tris-3 mM EDTA-1 M NaCl-3 M KSCN pH 7.0 at 35 cm/h and its concentration determined by direct UV absorption 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, the microtiter plate was coated overnight at 4 ºC with a sheep anti-mouse polyclonal antibody. The plate was blocked for 30 min at 37 ºC; washed and covered with immunoeluates. After incubation for 3 h at 37 ºC with 1% non-fat milk in PBS and subsequent washings, it was incubated with 100 µL of an anti-mouse polyclonal antibody conjugated to horseradish peroxidase (HRP)-streptavidin (Sigma Chemical Co., St. Louis, USA). 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/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%) using Coomassie blue staining for detection of protein bands [26]. The stained gels were scanned using a laser densitometer (Pharmacia LKB, Sweden).

Statistical Analysis

Microsoft Excel and Microsoft Statistica for Windows application were used for the analysis of variances and multiple ranges of Duncan's test of all variables. The significance level was 0.05.

Results and Discussion

Kinetics of immobilization and coupling time

As shown in Figure 1, the coupling of CB.Hep-1 MAb was fast and efficient as generally observed for other proteins [27]. In the first 15 min of reaction, about 88% of the antibody was coupled to the activated matrix, being coupling exponentially increased with time. However, during the following 225 min, the coupling efficiency increased slowly and reached an upper value close to 98%. The coupling efficiency of CB.Hep-1 MAb at 120 min agreed very well with the previously published results [16, 28].

Adsorption capacity (p = 0.456) and ligand leakage (p = 0.517) plotted at 75, 120 and 180 min were similar. Multi-site attachment did not increase because the specific activity of the CB.Hep-1 MAb was the same at the three coupling times, which demonstrated that for this case there was no dependence between coupling time and binding capacity.

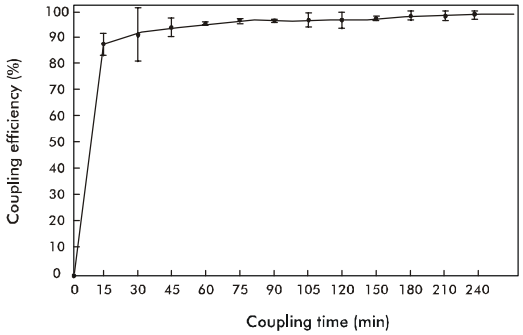

Figure 1. Kinetic of the CB.Hep-1 MAb coupling to CNBr-Sepharose CL-4B.

Coupling pH

The two reactive groups on CNBr-activated sepharose are cyanate esters and cyclic imidocarbonates accounting for 85% and 15%, respectively, of the active species. Cyanate esters are stable at low pH, hydrolyze rapidly in base and couple with a nucleophilic ligand to form an isourea linkage. Conversely, cyclic imidocarbonates hydrolyze in acid but are stable in base and form an *N*-substituted imidocarbonate, since coupling occurs via unprotonated ε-amino from antibody lysines and the rate of coupling is favored at high pH [29–31]. Therefore, coupling efficiency depends on two competitive events: rate of antibody coupling and rate of hydrolysis of reactive groups.

A lower coupling efficiency of CB.Hep-1 to sepharose CL-4B was observed at pH 10.0 (Table). This result is likely related to a rapid hydrolysis of the majority of cyanate esters at basic pH and their conversion into *N*-substituted imidocarbonates. The hydrolysis of C = N double bond at pH 10.0 is in agreement with the hydrolysis rates of imidocarbonates [6, 32–34] and should be responsible for the observed increment of ligand leakage (Figure 2).

At a range of coupling pH of 8.3–9.0, there were no significant differences in the adsorption capacity (p = 0.812) or in the ligand leakage (p = 0.528), which has also been noted for this IAC in other reports [15, 16].

Concentration of ligand in coupling buffer

Immunosorbents prepared at ligand concentration of 4.55 mg of CB.Hep-1 MAb/mL exhibited increments of binding capacity of 24.5% and 35% compared to 2.97 and 6.08 mg of CB.Hep-1 MAb/mL, respectively (Figure 3). The ligand concentration of 4.51–4.57 mg of CB.Hep-1 MAb/mL likely favors its appropriate penetration, homogeneity and distribution in the sepharose matrix during coupling. On the other hand,

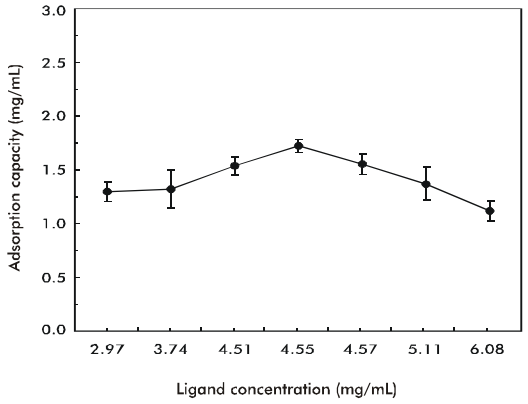

Figure 3. Effect of the ligand concentration over adsorption capacity of CB.Hep-1 immunosorbents. The ligand density used was 2.97 ± 0.03 mg/mL. The concentrations within the optimum range (4.51 mg/mL–4.57 mg/mL) were equal ($p_{[4.55-4.51]}$=0.282), ($p_{[4.55-4.57]}$=0.091), ($p_{[4.57-4.51]}$=0.864). However, when 4.55 mg/mL was compared to concentrations out of this range showed significant differences: ($p_{[4.55-2.97]}$=0.023), ($p_{[4.55-3.74]}$=0.021), ($p_{[4.55-5.11]}$=0.026), ($p_{[4.55-6.08]}$=0.002).

Table. Coupling efficiency of CB.Hep–1 immunosorbents manufactured at different coupling pH value. Significant differences were observed at pH 10.0 (p[pH 10–8.3] = 0.0096), (p[pH 10–9] = 0.0172).

Coupling pH	Coupling efficiency (%)
8.3	99.67
9.0	97.24
10.0	93.20

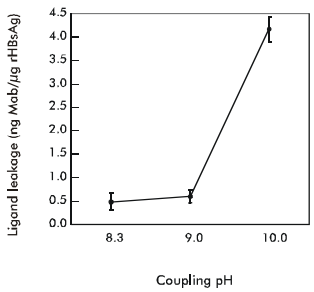

Figure 2. Effect of coupling pH on ligand leakage. Significant differences between pH 10.0 and pH 8.3–9.0 were observed ($p_{[ph\ 10-8.3]}$ =0.0045), ($p_{[ph\ 10-9]}$=0.0002).

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this concentration range seems to minimize any steric or diffusive hindrance when rHBsAg particles are bound to the immobilized MAb molecules. This binding responds to an equilibrium phenomenon between transport and adsorption [35].

Ligand density

The main variables of immunosorbents prepared with different ligand densities showed no statistical differences in the adsorption capacity ($p = 0.143$) (Figure 4). In theory, some rHBsAg particles of 2.4×10^6 Da [36] could not diffuse to the MAb immobilized inside the support pores, given the exclusion range of 6×10^4–2×10^7 Da. Even in the region of the pore-mouth the transport to the ligand takes a considerable time [5, 37]. Therefore, the antigen–antibody interaction possibility seems to be limited by the diffusion rates and accessibility. A similar lack of correlation between ligand density and binding capacity was previously reported by Ostrove and Weiss [38] for protein complexes of large molecular weight, where a marked adsorption increment while decreased ligand density could not be observed. It indicates that protein size and support porosity should be taken into account. These results are in agreement with other reports [39–41].

Although high levels of ligand density could be used for IAC, it is disadvantageous due to its negative influence on immunosorbent costs [42, 43]. Nevertheless, the best immunosorbent behavior was observed at 3.2 mg of CB.Hep-1 MAb/mL of gel, where the highest binding capacity, recovery and rHBsAg purity were overtaken. In addition, the ligand density did not affect significantly the eluted rHBsAg purity ($p = 0.567$), ligand leakage ($p = 0.143$) or total recovery rHBsAg ($p = 0.230$).

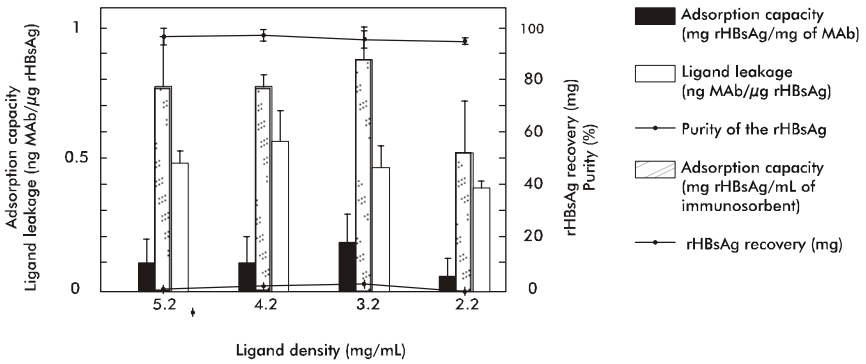

Figure 4. Effect of ligand density on CB.Hep-1 immunosorbent performance.

Conclusions

The results led us to conclude that the optimal conditions for the CB.Hep-1 MAb immobilization are: coupling times from 75 to 180 min, the ligand concentration between 4.51 and 4.57 mg of CB.Hep-1 MAb/mL and pH 8.3. The ligand density ranging from 3.2 to 4.2 mg of CB.Hep-1 MAb/mL of gel allows the enhancement of IAC performance at a minimal amount of immobilized protein.

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