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Stirrer tank: an appropriate technology to immobilize the CB.Hep-1 monoclonal antibody for immunoaffinity purification

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Abstract

The CB.Hep-1 monoclonal antibody was coupled to CNBr-activated Sepharose CL 4B at three different immobilization scales for purification of recombinant hepatitis B surface antigen. Standard laboratory apparatus to obtain immunosorbents of 1 l (scale I) and 3 l (scale II) as well as a stirrer tank to prepare 6 l immunosorbents (scale III) were used. The binding capacity at scale III was 2- and 1.5-fold higher with respect to the scales II and I, while a reduction in the ligand leakage of 5- and 2-folds was observed. Immunosorbents from scale II showed a significantly reduced adsorption, and an increased ligand leakage. Differences in the coupling efficiency were not observed. Antigen purity eluted from the immunosorbents was always above 85%. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Stirrer tank; Immobilisation; CB.Hep-1 monoclonal antibody

1. Introduction

Immunoaffinity chromatography (IAC) is a powerful technique used to purify proteins [1]. The covalent coupling of antibodies to the chromatographic supports is usually performed to CNBr-agarose supports activated [2,3]. However, in order to obtain highly reproducible and reliable results, (i) coupling efficiency, (ii) adsorption capacity, (ii) purity of the product and (iv) ligand leakage during either antigen-binding or antigen-release, have to be carefully controlled. Ligand leakage deserves special attention in pharmaceutical preparations, which are used in therapies requiring large and repetitive doses. The monoclonal antibody (MAb) used in this study is obtained from mice; thus, product contamination could be associated with the human anti-mouse answer (HAMA) [4,5]. A further disadvantage of ligand leakage is the reduced binding capacity of the immunosorbent towards the target antigen.

Stirrer tanks and reactors have been used in molecular bioseparation [5–8]; nevertheless reports on the application of these technologies for MAb coupling are scarce. In the present work, a comparison of the immunosorbents prepared for the purification of the recombinant hepatitis B surface

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antigen (r-HBsAg) using either glass-made filter funnels or a stirrer tank at three different scales is made.

2. Experimental

All reagents used in these experiments were supplied by Merck (Frankfurt, Germany).

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. [9]. It recognizes the 'a' determinant of the r-HBsAg [10]. BALB/c mice were immunized subcutaneously with a first dose of 50 mg of natural hepatitis B surface antigen (nHBsAg), in Freund's complete adjuvant, boosted 15 and 21 days later by similar doses in Freund's incomplete adjuvant. Three days before the fusion, the animal 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. CB.Hep-1 was purified from ascitis fluid by protein-A affinity chromatography [11]. The purity of the final antibody preparation was 95%, assessed by sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE) under reducing conditions. The MAb was dialyzed in order to exchange the buffer 20 m*M* Tris–150 m*M* NaCl, pH 7.6, with coupling buffer 0.1 *M* Na_2CO_3–0.1 *M* $NaHCO_3$–0.5 *M* NaCl, pH 8.3, by gel-filtration chromatography. Prepacked disposable columns PD-10 (Amersham-Pharmacia Biotech, Uppsala, Sweden), with 9.1 ml of swollen Sephadex G-25 *M* were used. The protein concentration (5 ± 0.2 mg/ml) was determined according to Lowry et al. [12]. Finally, the MAb was filtered through a 0.2-μm pore-sized membrane (Sartorius, Goettingen, Germany) and stored at 4°C.

2.2. Source of r-HBsAg

r-HBsAg 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 r-HBsAg was recovered and submitted to initial purification steps as described and optimized previously [13–15]. Briefly, the cells were harvested by centrifugation and disrupted on a bed mill (KDL type: WAB, Basel, Switzerland). The disruption buffer contained 20 m*M* Tris–HCl, pH 8.0–3 m*M* 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 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 pH 4.0 under continuous stirring. Adsorption was allowed to take place for 2 h and the 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 m*M* Tris–HCl–3 m*M* EDTA–100 g/l sucrose, pH 8.2. With the described procedure, a semipurified material of about 10–25% purity was obtained. This was used as the starting material for IAC.

2.3. Immunosorbents preparation

Sepharose CL 4B (Amersham-Pharmacia Biotech) was activated with CNBr according to the procedure reported by March et al. [16]. Immunosorbents were prepared of 1-l (scale I) and 3-l (scale II) with glass-made filter funnels (Standard Laboratory Apparatus, Schott, Germany), and a mechanical stirrer (IKA-RW20, Kehl/Rhein, Germany). A 70-l stirrer tank AISI 316 (Amersham-Pharmacia Biotech) in scale III was used to prepare 6-l immunosorbents (Fig. 1).

The support was wetted in 1 m*M* HCl for 15 min, and washings with 0.1 *M* Na_2CO_3–0.1 *M* $NaHCO_3$–0.5 *M* NaCl, pH 8.3, were performed. The MAb was coupled by covalent bonds on the support at pH 8.3 for 2 h at 25°C by gentle stirring according to the conditions previously optimized [17,18]. In order to obtain ligand densities of 4.94 mg/ml of immunosorbent (scales I and II) or 4.95 mg/ml of immunosorbent in scale III. The coupling efficiency (φ) was determined by an indirect method, following the formula: $\varphi\ (\%) = \delta/\chi.\ 100$, where δ is the mount of coupled protein determined as the difference between the original amount of ligand (χ) and the amount

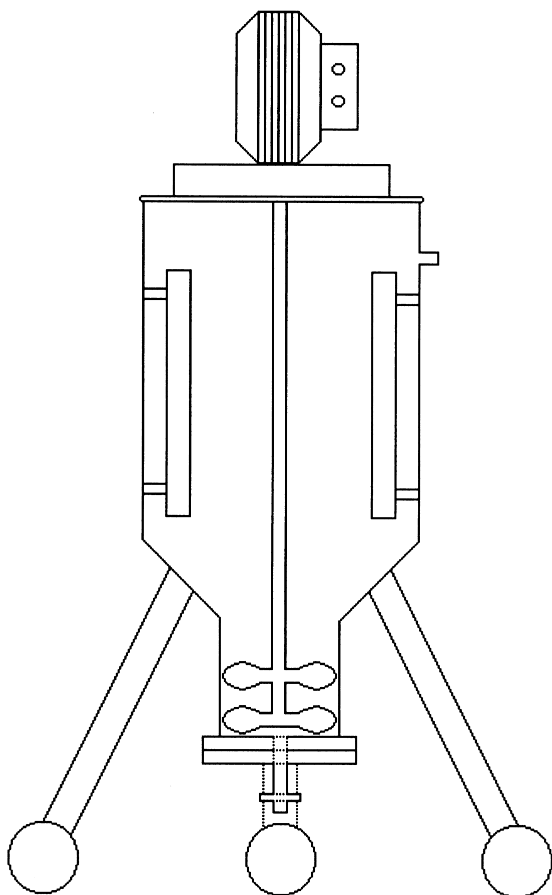

Fig. 1. Schematic of the stirrer tank employed in the immobilization of CB.Hep-1 MAb (scale III) for r-HBsAg purification.

detected in the filtration and washings fractions after the coupling.

Free reactive groups were blocked by adding of 0.1 *M* glycine, pH 8.0. Five alternate washings with 0.1 *M* $C_2H_3O_2Na$–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, were carried out. Finally, the immunosorbent was washed and stored in phosphate buffered saline–0.01% Tiomersal, pH 7.2, at 4°C.

2.4. Immunoaffinity chromatography

Samples (5 ml) of the immunosorbents were packed at bed height of 3.18 cm on columns C 10/10 (10 cm×1 cm, I.D.) (Amersham-Pharmacia Biotech) at hydrostatic pressure using a flow-rate of 0.688 ml/min previously optimized by Agraz et al. [19]. Washings with 25 ml of 20 m*M* Tris–3 m*M* EDTA–1 *M* NaCl, pH 7.0 were carried out. The columns were loaded in saturation conditions with 10 mg of starting material. Washings were performed using 20 m*M* Tris–3 m*M* EDTA–1 *M* NaCl, pH 7.0, 5 ml/ml of immunosorbent, at a 0.393 ml/min flow-rate. The elution was carried out using 20 m*M* Tris–3 m*M* EDTA–1 *M* NaCl–3 *M* KSCN, pH 7.0, at a flow-rate of 0.688 ml/min in all cases [20]; followed by buffer exchange to PBS, using Sephadex G25 Coarse (Amersham-Pharmacia Biotech). 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, St. Louis, MO, USA). The reaction was revealed using 100 μl/well of 0.05% *o*-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 using SDS–PAGE (12.5%) with Comassie blue staining [21]. The samples were denatured by treatment with 2-mercaptoethanol and SDS for 20 min at 100°C.

2.5. Statistical analysis

Coupling efficiency, binding capacity and ligand leakage were evaluated by multiple ranges of Duncan's test. The Student's *t* test was used to compare the purity of immunopurified antigen. For all the cases the significance level (α) was 0.05, and the STATISTICA for Windows application was used.

3. Results and discussion

3.1. Coupling efficiency

The immunosorbent coupling efficiency is shown in Fig. 2. The results demonstrated that this parameter neither depended on the scale nor on the introduced changes in the immobilization technique. These results coincided with those obtained by Pfeiffer et al. [22] and Valdés et al. [23].

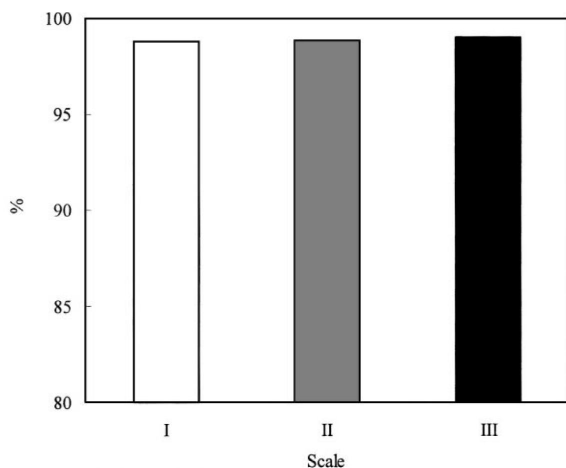

Fig. 2. Coupling efficiency of CB.Hep-1 immobilized at three scales on Sepharose CL-4B activated with cyanogen bromide. Each bar represents the mean value of seven immunosorbents. This parameter showed values above 90% in all cased. For scales I, II and III, significant differences were not observed.

3.2. Binding capacity

The binding capacity of the immunosorbents is summarized in Table 1 and Fig. 2. At scale II a significant decrement to 33.65% was observed. However, a highly significant increment was obtained at scale III, compared with the mean values of scales II and I (1.5 and 2-fold, respectively). This could be justified by a more appropriate configuration of the stirrer tank and using a constant agitation system to prepare immunosorbents at this scale. Probably a more effective swollen of the support was achieved with the acid treatment, minimizing the susceptibility towards a nucleophilic attack by the antigen due to the formation of protonated imidocarbonates intermediate, which could be the responsible for a low-specific activity and purity of antigen [24–26].

Additionally, these results led to an inference that another influencing factor was a greater effectiveness of the non-covalently bound protein removing washings, which is normally the main source of non-specific interaction [27].

On the other hand, the constant agitation system in the stirrer tank probably increased the interaction between ligand molecules and the support active

Table 1
Statistical comparison of binding capacity (mg of eluted rHBsAg/ml of gel) of immunosorbents prepared on three immobilization scales (average of three runs)

	Scale		
	I (Scale factor 1)	II (Scale factor 3)	III (Scale factor 6)
	0.968	0.247	0.908
	0.660	0.606	0.963
	0.725	0.330	1.372
	0.525	0.361	0.850
	0.520	0.367	0.986
	0.795	0.698	1.156
	0.595	1.008	1.081
Mean	0.684	0.517	1.045
Probability	0.0194[a]	0.0003[b]	0.0016[c]

[a] Statistical comparison between scales I and II showed significant differences.
[b] Difference between scales II and III was highly significant.
[c] Difference between scales I and III was highly significant.

groups during immobilization. Similar results were achieved with tanks used in IAC to purify diverse proteins where high yields and exploitation efficiency have been reached from immunogels by facilitating mass transference [5,8]. Furthermore, a reduction in steric hindrance and arising decrease of diffusion limitations during IAC, allowed increasing binding capacity [7].

3.3. Ligand leakage

Several causes provoke ligand leakage: the cleavage of chemical bond between the ligand and polymeric support or spacer arm due to hydrolysis or aminolysis [28]; the cleavage and releasing of the ligand bound to a fragment of the polymeric support induced by chemical and shear forces [29]; the release of ligand by proteolysis [30] and the coelution of ligand coupled via non-covalent bonds. All these events may occur simultaneously after scaling-up [31,32].

At scale II the ligand leakage increased two fold (Fig. 4). Insufficient stirring and washing conditions in glass-made filter funnel appear to be related to a reduced multiple attachment and, in consequence, a lower binding capacity (Fig. 3). However, scale III showed a lesser ligand leakage than scales I and II, where the coupling was more controlled (Fig. 4). This behavior appears to be influenced by washings performed with stirrer tank technology with better mass transference [31–33].

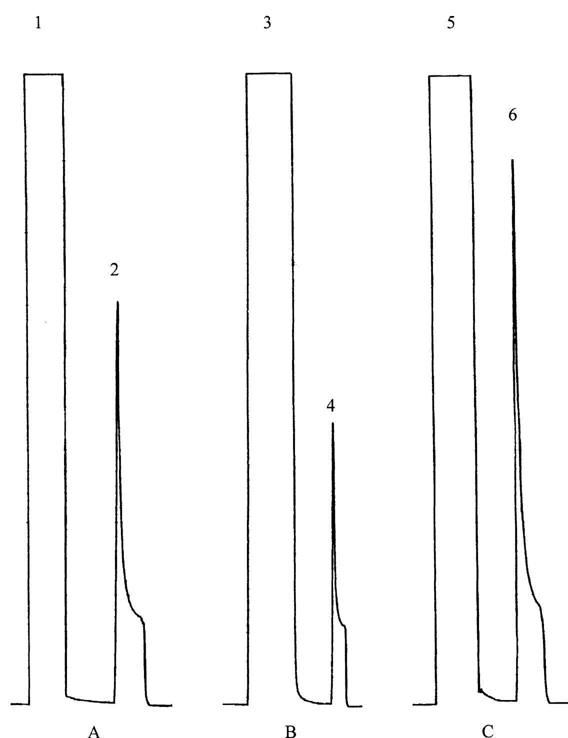

Fig. 3. Typical profiles for r-HBsAg purification by IAC using immunosorbents prepared at scale I (A), scale II (B) and scale III (C). The examples correspond to the first run for one randomly taken immunosorbent. Each profile shows the break-through fraction (1, 3 and 5) and the fraction containing r-HBsAg (2, 4 and 6).

3.4. Purity of the antigen

Fig. 5 shows that there were not significant differences in the purity of the eluted antigen between scales II and III, although an increment of a 3% in scale III was observed. This could be explained by a more efficient reduction of non-specific antigen–antibody interactions and improved antigen purity. In consequence, either non-specific binding of impurities to the immunosorbents or their release from it, were lesser at the same loading and elution buffer.

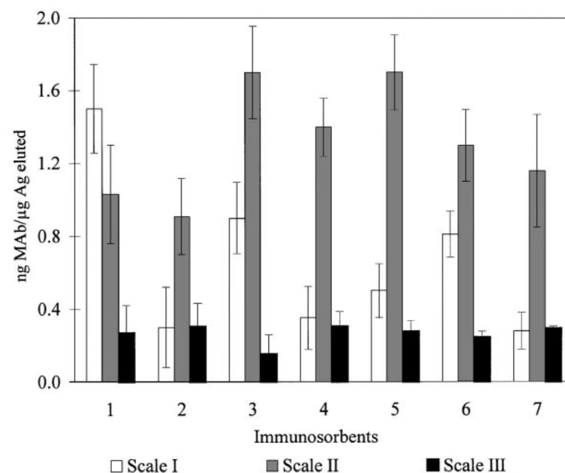

Fig. 4. Leakage of CB.Hep-1 MAb during r-HBsAg purification. Each bar represents the average of three runs. At scale II, the ligand leakage showed the highest mean value.

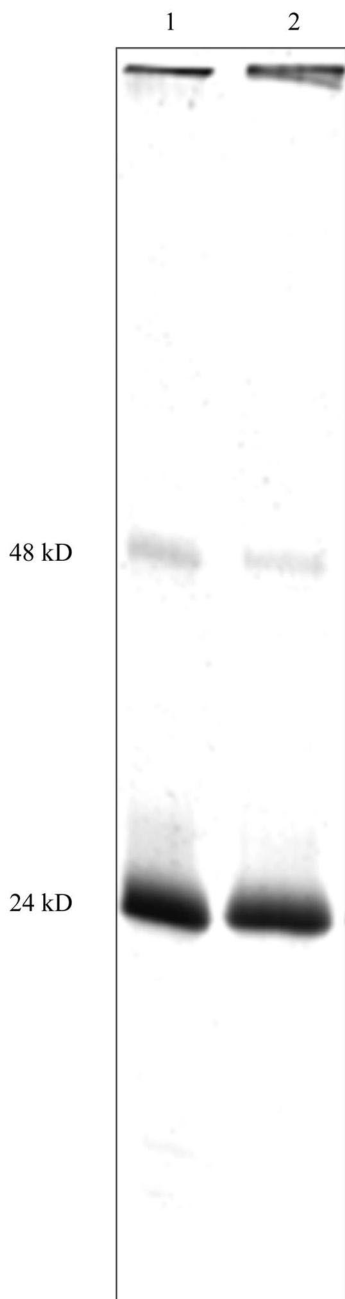

Fig. 5. SDS–PAGE (12.5%) of r-HBsAg (24 kD) purified by immunoaffinity chromatography with CB.Hep-1 immunosorbents at scale II (lane 1, 89%) and III (lane 2, 94%). The samples were taken from the first run with immunosorbent 4 (Fig. 4). The 24-kD major band corresponds to the monomer and the 48-kD to the dimer, as reported by Fernández de Cossío et al. [13] and Wampler et al. [34].

4. Conclusions

These results indicate that the use of a stirrer tank for CB.Hep-1 MAb chemical immobilization provides the best binding capacity, a significant reduction of the ligand leakage during IAC and an increment of the final product purity, while it allows for an increase in the stability of the immunosorbent. The stirrer tank is an appropriate technology for large-volume of immunosorbents where the operations and analytical control costs could be reduced with respect to the glass-made filter funnel technique.

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