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Journal of Chromatography A, 672 (1994) 25–33

JOURNAL OF CHROMATOGRAPHY A

Immunoaffinity purification of recombinant hepatitis B surface antigen from yeast using a monoclonal antibody

Alberto Agraz*[,a], Carlos A. Duarte[b], Lourdes Costa[c], Lilia Pérez[d], Rolando Páez[a], Vivian Pujol[a], Giuvel Fontirrochi[e]

[a]*Biopharmaceutics Development Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

[b]*Immunotechnology Division, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

[c]*Quality Control Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

[d]*Hepatitis B Vaccine Production Unit, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba*

[e]*Center for Genetic Engineering and Biotechnology, Camaguey, Cuba*

(First received September 13th, 1993; revised manuscript received January 14th, 1994)

Abstract

A murine monoclonal antibody developed for the purification of recombinant hepatitis B surface antigen was immobilized on a chromatographic support and used to adsorb and purify the recombinant antigen from yeast. The adsorption–elution behaviour was first investigated using monoclonal antibody-coated enzyme-linked immunosorbent assay plates and performing adsorption, washing and elution procedures with different elution agents. It was found that 3 *M* KSCN and 8 *M* urea at neutral pH disrupted antigen–antibody interactions in both systems. The procedure for washing the immunoaffinity column was optimized, using different salts and detergents. The best results were obtained by applying the starting material in 1 *M* NaCl and washing with the same buffer. The use of 0.1% sodium deoxycholate in the washing buffer reduced about 20-fold lipopolysaccharide contamination in the eluates as compared with washing without detergent. The relationship between bed height and the adsorption capacity of the column was studied, and it was found that the dynamic capacity decreased twice on reducing its length/diameter ratio tenfold. The recovery of antigen was not affected by increasing the flow-rate up to 25 cm/h but decreased at higher values. Using the optimum conditions, the affinity column was able to purify the recombinant hepatitis B surface antigen to more than 90% purity and a 65% antigen recovery was obtained.

1. Introduction

Immunoaffinity chromatography with monoclonal antibodies (mAbs) has been widely used for the purification of proteins. It has proved to be a powerful tool in several purification procedures, mainly because of the high selectivity of this technique.

Some important aspects have to be considered when an immunoaffinity column is prepared for large-scale purification, including the choice of the solid support, ligand coupling chemistry, ligand coupling density, capacity for the ligand and the product, washing and elution agents, column geometry and flow-rate. In order to improve the performance of the immunoadsorbent, all these parameters must be carefully studied and optimized.

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00047-D

The nature of antigen–antibody binding can vary to a great extent between different mAbs, even when they are directed against the same antigen. For that reason, once a new mAb, specific for the protein of interest, is obtained, the thermodynamic performance of its interaction with the antigen should be investigated in order to evaluate it usefulness as a ligand.

This kind of study is better performed using enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA), where the action of a wide range of agents can be investigated and those best suited for this specific system can be selected. Another correct, although more tedious, approach consists in performing adsorption–elution studies in batch, using microamounts of immunoaffinity supports. Low-affinity mAbs are generally preferred because they allow the use of mild elution steps using low denaturing conditions and agents, such as extreme pH values, amines and organic solvents.

Another important element in this technique is the washing step, which diminishes the proportion of undesired, non-specifically bound molecules (contaminant proteins, endotoxins, etc.). Such molecules can be adsorbed on the immunogel by hydrophobic or electrostatic interactions. Most commonly used washing agents include concentrated salts, detergents and chaotropic salts.

The hepatitis B surface antigen (HBsAg) is a well characterized protein of the hepatitis B virus envelope. About 100 molecules of this M_r 24 000 monomeric form are assembled into 22-nm particles. These particles are stabilized by disulphide bonds [1], and contain carbohydrates and lipids [2]. The gene encoding for the antigenic protein has been cloned and expressed in bacteria [3,4], animal cells [5–8], vaccinia virus [9] and yeast [10–17]. Several purification procedures have been described [1,18–23], some of them using immunoaffinity chromatography.

This paper presents the results of the immunoaffinity purification, using mAb CB-HEP.1, against recombinant hepatitis B surface antigen (r-HBsAg), which is included in a vaccine preparation against the hepatitis B virus. The influence of several chromatographic parameters, such as washing and elution agent, flow-rate and column geometry, on the immunoaffinity performance was also analysed.

2. Experimental

2.1. Materials

All chemicals were of analytical-reagent grade. Sodium deoxycholate was obtained from Fluka (Buchs, Switzerland) and Triton X-100 and Tween 20 from BDH (Poole, UK). Other chemicals were purchased from Merck (Darmstadt, Germany).

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 previously [21]. Briefly, the cells were harvested by centrifugation and disrupted on a bead mill (KDL type; WAB, Basle, 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 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 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 semi-purified material of about 10–25% purity was obtained. This was used as the starting material for all immunoaffinity experiments.

2.3. Monoclonal antibody

Mab CB-HEP.1, secreted by the hybridoma cell line 48/1/5/4, has been characterized previ-

ously [24]. This hybridoma was shown to secrete IgG2b and IgM antibodies having the same specificity against HBsAg. The clone used for this work was selected because of its lower secretion of the IgM component. The antibodies were purified from ascites by protein-A affinity chromatography. The purity of the final antibody preparation (IgG + IgM) was more than 90% and the content of IgM less than 3%, both assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

2.4. Binding and elution of the antigen using 96-well plates

A poly(vinyl chloride) microplate (Dinatech, Cambridge, UK) was coated with 100 μl per well of 10 μg/ml CB-HEP.1 Mab in 0.1 *M* sodium hydrogencarbonate buffer (pH 9.6) overnight at 4°C. mAb solution was removed and the blocking step was performed using 100 μl per well of 1% fat-free milk in phosphate-buffered saline (PBS) for 30 min at 37°C. Subsequently, 100 μl per well of 200 ng/ml purified r-HBsAg diluted in PBS + 1% fat-free milk and 0.5% Tween 20 was added. After incubation for 1 h at 37°C, the plate was washed three times using 0.05% Tween 20 in PBS. Elution from the plate was carried out by adding 100 μl per well of different eluting agents: (a) 8 *M* urea in PBS, (b) 4.5 *M* $MgCl_2$ in PBS, (c) 10% dioxane, (d) 20 m*M* Tris(hydroxymethyl)aminomethane(Tris)–50% ethylene glycol adjusted to pH 11.6 with NaOH, (e) 20 m*M* Tris adjusted to pH 11.6 with NaOH, (f) 0.2 *M* glycine–HCl (pH 3.5), (g) 3 *M* KSCN and (h) 3 *M* GuSCN (where Gu = guanidine). The plate was washed five times in the same manner as described before and incubated at 37°C for 1 h with 100 μl of anti-HBsAg sheep polyclonal antibody–peroxidase conjugate. After washing, the plate was developed using 100 μl of 0.05% *o*-phenylenediamine and 0.015% hydrogen peroxide in citrate buffer (pH 5.0). The reaction was stopped after 20 min with 50 μl of 1.25 *M* H_2SO_4. The plate was finally measured on a Multiskan system (Titertek, Helsinki, Finland) at 492 nm. Each condition was assayed twice in two different tests.

The percentage of elution (E) was calculated as $E = [100 - (M - B/C - B)] \cdot 100$, where M is the absorbance at 492 nm, B is the absorbance without HBsAg and C is the absorbance of a control (eluted with PBS).

2.5. Binding and elution of the antigen using immunoaffinity columns

Sepharose CL-4B (Pharmacia–LKB, Uppsala, Sweden) was activated by the CNBr method and kindly supplied by Dr. L. Rodés (Department of Chemistry of Solid Surfaces, CIGB, Havana, Cuba). The CB-HEP.1 mAb was coupled as recommended by the manufacturer. The ligand coupling density was determined by measuring the total protein before and after the coupling reaction, and it was about 5 mg/ml of gel for all experiments if not specified otherwise.

The gel was packed on a K 16/20 column (5 cm × 1.6 cm I.D.) and equilibrated with 20 m*M* Tris · HCl–3 m*M* EDTA (pH 7.8). The column was loaded with an excess of semi-purified r-HBsAg preparation in the same buffer and washed using about five column volumes of starting buffer containing 1 *M* NaCl. After washing, the column was eluted with the corresponding eluting agent. The elution peak was monitored (280 nm) and collected for all experiments. The eluting agents tested were (a) 8 *M* urea, (b) 4.5 *M* $MgCl_2$, (c) 10% dioxane, (d) 50% ethylene glycol–20 m*M* Tris adjusted to pH 11.6 with NaOH, (e) as (d) without ethylene glycol, (f) 0.2 *M* glycine–HCl (pH 3.5), (g) 3 *M* KSCN and (h) 3 *M* GuSCN. All eluting solutions except (d), (e) and (f) contained 20 m*M* Tris–HCl buffer (pH 7.8) if another pH is not specified. The flow-rate was 25 cm/h. The immunogel was replaced each time and three runs were performed for each condition.

2.6. Optimization of washing step

The immunogel was packed on a K 16/20 column (10 cm × 1.6 cm I.D.) at 25 cm/h and equilibrated using 20 m*M* Tris · HCl–3 m*M* EDTA (pH 7.8). The column was loaded up to 85% of its maximum capacity with semi-purified r-HBsAg. Subsequently, different washing pro-

cedures were tried: (A) first with 0.1% sodium deoxycholate and then with 1 *M* NaCl, (B) first with 10% sucrose and then with 1 *M* NaCl, (C) first with 0.5% Triton X-100, second with 0.3 *M* GuHCl–0.3% Triton X-100, third with 0.5 *M* NaCl–0.2% Triton X-100 and finally with 1 *M* NaCl, (D) first with 0.5% sodium deoxycholate and then with 1 *M* NaCl, (E) first with 1 *M* NaCl, second with 0.5 *M* KSCN and finally with 1 *M* KSCN, (F) first with 0.1% Tween 20 and then with 1 *M* NaCl, (G) first with 1 *M* NaCl, second with 0.1 *M* GuHCl and finally with 0.2 *M* GuHCl, (H) the sample was pre-incubated on 0.1% sodium deoxycholate, applied in this same buffer and then washed with 1 *M* NaCl and (I) 1 *M* NaCl was added to the loading sample, then washed with 1 *M* NaCl. All washing buffers contained 20 m*M* Tris · HCl (pH 7.8). After washing, the column was eluted using 20 m*M* Tris · HCl–3 m*M* EDTA–1 *M* NaCl–3 *M* KSCN (pH 7.8). The purity of the material eluted from each run was determined by SDS-PAGE.

2.7. Washing the column with detergent to reduce endotoxin (ET) levels

Semi-purified r-HBsAg was contaminated with various amounts of bacterial lysate (*Escherichia coli*) to evaluate the ability of the affinity column to remove endotoxins (ETs).

The protocol for the adsorption and elution of the r-HBsAg was similar to that described for previous experiments. Two washing procedures, each involving three consecutive steps, were compared: procedure (a), 1 *M* NaCl + buffer + 1 *M* NaCl; procedure (b), 1 *M* NaCl + 0.1% sodium deoxycholate + 1 *M* NaCl. All buffers contained 20 m*M* Tris · HCl–3 m*M* EDTA (pH 7.8). Samples were analysed for ET content with the Toxicolor LAL test (Seikagaku Kogyo, Tokyo, Japan).

2.8. Influence of ligand density on adsorption capacity

Different amounts of mAb were coupled to 1 ml of activated CL-Sepharose 4B gel. The exact amounts of coupled mAb were calculated from the difference between protein concentrations before and after the coupling procedure. Five columns were prepared with 8.6, 7.1, 4.9, 2.5 and 1.0 mg of mAb/ml of immunogel. Runs were performed by overloading the column with semi-purified r-HBsAg equilibrated in 20 m*M* Tris · HCl–3 m*M* EDTA (pH 7.8), washed with 1 *M* NaCl and eluted with 3 *M* KSCN–1 *M* NaCl in the same buffer.

2.9. Influence of flow-rate and bed height on recovery and productivity of the affinity chromatographic step

Studies were performed on a K 16/20 column (10 cm × 1.6 cm I.D.) packed with the immunogel at linear flow-rates of 10, 20, 50, 70 and 100 cm/h. Three runs were carried out for each condition.

For all experiments, the column was first equilibrated with 20 m*M* Tris · HCl–3 m*M* EDTA (pH 7.8). A semi-purified r-HBsAg sample (about 85% of maximum gel capacity) equilibrated in the starting buffer was applied and the column washed with 1 *M* NaCl in the same buffer. After the washing step, elution was performed using 3 *M* KSCN–1 *M* NaCl in the same buffer.

In order to assess the influence of bed height on the recovery of r-HBsAg, a K 16/20 column was packed with different amounts of immunogel to give bed heights of 20, 10, 5 and 2 cm. Three runs were carried out for each condition, loading the immunogel with 85% of its maximum loading capacity for HBsAg. The linear flow-rate was 20 cm/h. The adsorption, washing and elution steps were carried out as described in previous experiments.

2.10. Determination of r-HBsAg concentration

The concentration of r-HBsAg was determined by a sandwich ELISA using sheep polyclonal antibodies against HBsAg both in the capture antibody and the horseradish peroxidase conjugate. The HBsAg secondary standard was calibrated against an international HBsAg stan-

dard preparation obtained from the Paul Erlich Institute (Frankfurt/Main, Germany).

2.11. Determination of protein concentration

Protein concentration was determined by the Bradford method [25] using bovine serum albumin (BSA) as a standard.

2.12. Electrophoresis

SDS-PAGE of reduced samples was performed according to Laemmli [26]. The gels were stained with Coomassie Brilliant Blue R-250.

3. Results and discussion

3.1. Elution of bound antigen with different agents

The screening for appropriate elution agents using 96-well plates showed that urea, $MgCl_2$, KSCN and GuSCn had the highest elution efficiency, recovering between 65 to 75% of bound antigen (Fig. 1). In contrast, extreme pH buffers and 10% dioxane had a low elution effect. According to these results CB-HEP.1 belongs to the group of antibodies whose interaction with the antigen is better disrupted by chaotropic agents than by extreme pH values.

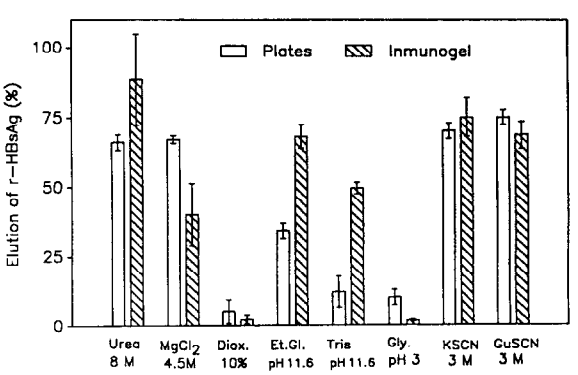

Fig. 1. Elution of r-HBsAg from microplates and Sepharose 4B immobilized mAb using different elution agents. Elution is expressed as % of adsorbed antigen. Diox. = Dioxane; Et. Gl. = ethylene glycol; Gly. = glycine.

In addition, a good correlation of the elution profiles of the different agents using plates and packed immunogel was obtained for most of them (Fig. 1). Only for basic pH buffers was the antigen recovery higher [about twice as much with 50% ethyleneglycol (pH 11.6) and four times as much with Tris adjusted to pH 11.6 with NaOH] on the immunogel than in plates.

In spite of these differences, the results demonstrate that the microplate assay can be very useful for the initial evaluation of a large number of solutions.

Among the solutions tested, 3 *M* KSCN was chosen as the elution buffer, mainly because KSCN is a well known HBsAg particle stabilizer [1].

3.2. Optimization of washing step

Results from the comparison of the different washing procedures are summarized in Table 1. Only with procedures H and I were purity levels higher than 90% achieved. In both instances the washing agent (sodium deoxycholate in H and NaCl in I) was added to the sample before loading it into the column. It is remarkable that the use of NaCl and sodium deoxycholate in other protocols, even at higher concentration, did not produce similar results. This outcome underlines the importance of avoiding the non-specific adsorption of contaminant proteins to the matrix by previously mixing the washing agent with the sample.

The recovery of r-HBsAg in the eluted fraction was adequate for variants A, B and I. On the other hand, variants D and F showed a significant loss of r-HBsAg (41 and 33%, respectively) in the washing fraction, probably owing to the presence of detergents (0.5% of sodium deoxycholate and 1% Tween 20). Variant H showed a low recovery (26%) and only 60% of the applied sample was found in the other fractions.

The same happened with variant E, but in this instance the elution of r-HBsAg was due to the presence of 1 *M* KSCN. The purity of r-HBsAg

Table 1
Washing of the immunoaffinity column using different protocols and solutions

Washing protocol	Amount (mg)				r-HBsAg yield (%)	SDS-PAGE[a] purity (%)
	Applied	Non-bound	Washing	Elution		
A	1.9	0.2	0.1	1.2	63	70–80
B	2.4	0.2	0.2	2.2	91	70–80
C	1.9	0.3	0.1	0.1	5	70–80
D	3.4	0.7	1.4	0.5	14	70–80
E	3.1	1.0	1.4	0.9	29	80–90
F	3.6	0.5	1.2	1.9	52	70–80
G	3.5	0.7	0.6	0.7	20	70–80
H	3.5	0.7	0.5	0.9	26	>90
I	2.9	0.7	0.03	1.9	65	>90

[a] Purity was estimated visually from SDS-PAGE of eluates.

obtained with this variant was higher, but the yield was very low.

With washing protocols C and G, the bound antigen was recovered neither in the washed nor in the eluted fraction. In further studies, GuHCl was found to interfere in the r-HBsAg determination test, so the missing antigen may have been, in fact, in the washed fraction.

Further analysis on a TSK-2000 SW HPLC gel-filtration chromatographic column of the eluted r-HBsAg from protocols H and I showed that some molecule population of the antigen coming from H (0.1% sodium deoxycholate) is overaggregated, judging by the retention time of a peak that eluted before the mean peak of well particulated antigen. The degree of aggregation of r-HBsAg particles was corroborated by electron microscopy (not shown). Changes in the morphology (aspect and size) of the natural HBsAg particles submitted to heat treatment has been described previously [27]. This HBsAg overaggregation is probably induced by the action of sodium deoxycholate on the lipid moieties of the particle.

On the other hand, antigen coming from protocol I had a homogeneous molecule population, showing the expected particle size of about 22 nm, determined by electron microscopy. Fig. 2 shows the SDS-PAGE of purified antigen using washing protocol I.

3.3. Ability of the immunoaffinity column to remove ETs

The use of 0.1% sodium deoxycholate in the washing buffer reduced the ET levels about 25-fold, whereas the washing procedure without detergent reduced ETs only about 1.5-fold (Fig.

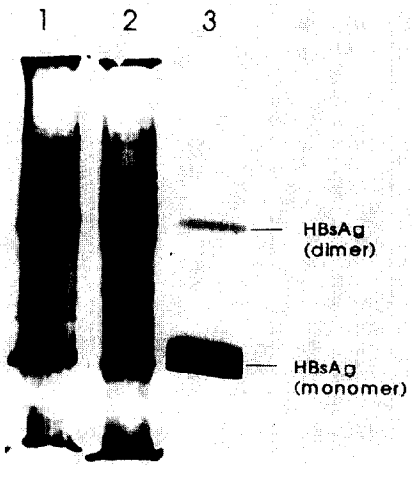

Fig. 2. SDS-PAGE of reduced samples from the immunoaffinity purification using washing protocol I (15% polyacrylamide gel). 1 = Starting material; 2 = non-bound fraction; 3 = eluted fraction. 20 μg of protein were applied per sample.

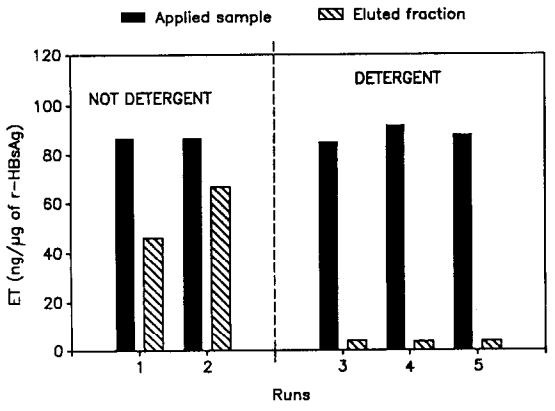

Fig. 3. Effect of detergent on the removal of endotoxins (ETs) from r-HBsAg in the immunoaffinity column. Bars represent the level of Ets in applied and eluted fractions.

3). The recoveries of r-HBsAg in the eluted fraction of runs 1–5 were 57, 65, 62, 57 and 68%, respectively. As can be seen, detergent treatment was able to wash away non-specifically bound lipopolysaccharides (LPSs) without significant losses of adsorbed antigen. This result was predictable, considering the highly hydrophobic nature of LPSs. The use of a washing solution with a high ionic strength must enhance the hydrophobic interactions between LPSs and the matrix or with hydrophobic patches of the adsorbed proteins. Detergents lower this interaction, causing the elution of the contaminating LPSs.

3.4. Effect of ligand density in the productivity per milligram of mAb in the gel

Table 2 shows the results of the adsorption and elution of r-HBsAg from gels prepared with a different ligand density. Although the total adsorbed antigen increased with increasing amount of coupled mAb, this increment was not linear. As a result, the amount of antigen adsorbed per milligram of coupled mAb decreased drastically (fivefold). Consequently, the purification cost per unit of r-HBsAg increased. In this instance, for practical purposes, the use of 1 or 2 mg of mAb/ml of gel can be recommended.

Table 2
Adsorption capacity of the immunoadsorbent at different ligand densities

Ligand coupling density (mg mAb/ml gel)	r-HBsAg eluted (μg)	Antigen purified per mg of coupled mAb (μg r-HBsAg/mg mAb)
8.6	217	25.2
7.1	212	29.8
4.9	196	40.0
2.5	153	61.2
1.0	131	131.0

3.5. Effect of flow-rate on the performance of the immunoadsorbent

Within the range of linear flow-rates studied, high recoveries were obtained up to 25 cm/h (Fig. 4), but the recovery began to decrease almost linearly as the flow-rate increased from 50 to 100 cm/h. Although the gel itself (Sepharose CL-4B) can resist such elevated flow-rates, a diffusion mechanism limits the antigen accessibility to the mAb molecules in the beads, considerably lowering the functionality of the

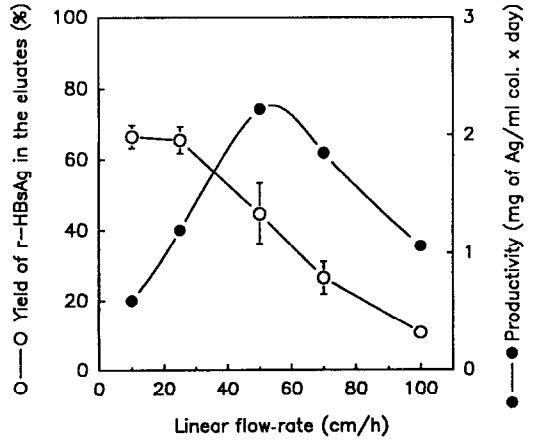

Fig. 4. Influence of linear flow-rate on recovery of r-HBsAg in the eluted fraction. Yield of antigen is expressed as a percentage of the initial r-HBsAg applied to the column. Productivity was calculated on the basis of the average recovery obtained for each linear flow-rate and the time required for completion of a single run.

coupled ligand. Flow-rate is also limited by the slow process of antigen–antibody complex formation. In Fig. 4 the estimated productivity is also shown, based on the average recovery obtained for each linear flow-rate and the time required for completion of a single run. When a high productivity (mg of purified antigen per volume of immunogel per day) is required, 50 cm/h could be used to achieve the highest productivity values, although a recovery of only 44% should be expected at this flow-rate.

3.6. Effect of bed height on adsorption capacity

An unexpected result was obtained when two different bed lengths were compared. When the length/diameter ratio (L/D) of the immunogel column was decreased from 12.5 to 1.25, the amount of antigen that escaped in the non-bound fraction increased proportionally. For a 12.5 ratio $27 \pm 4\%$ of antigen was not retained in the column (found in the non-bound fraction), for a 6.25 ratio $39 \pm 6\%$, for a 3.12 ratio $48 \pm 5\%$ and for a 1.25 ratio $65 \pm 7\%$. This result does not agree with the normal behaviour of affinity chromatography when short and wide columns are used to improve productivity. The observed phenomenon seems to be the result of an inefficient mass transfer of the r-HBsAg particle into the CL-Sepharose 4B gel. The fractionation range reported for Sepharose 4B extends up to M_r $2.0 \cdot 10^7$ and the average molecular mass of the r-HBsAg particle is about $2.4 \cdot 10^6$. The ratio between these values is less than 10, which indicates that some restriction to the free diffusion of the r-HBsAg molecules exists in this system. Other gels with a larger pore diameter could be more suitable for the immunopurification of high-molecular-mass particulate antigens.

3.7. Stability of the immunoadsorbent

Using the optimum running parameters, that is, washing with 1 M NaCl and eluting the bounded antigen with 3 M KSCN, at a linear flow-rate of 25 cm/h, the column was stable for up to 30 runs (with an insignificant decrease in recovery). With further runs the recovery was rapidly affected. Leakage of ligand was measured in the wash and eluates fractions among the runs, using an ELISA for detecting murine IgG. Negligible amounts of IgG were found in such fractions, as compared with the coupled one. From this result it is thought that the cause of inactivation of the immunoadsorbent could be produced by the action of proteases present in the starting material.

4. Acknowledgements

The authors thank the Monoclonal Antibodies Production Unit of CIGB for supplying the mAb CB-HIP.1 and the Hepatitis B Vaccine Production Unit of CIGB for providing the r-HBsAg semi-purified material and performing the r-HBsAg determinations.

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