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Statistical optimization of immunoaffinity purification of hepatitis B surface antigen using response surface methodology

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Abstract

Response surface methodology (RSM) was successfully applied to find an elution condition enhancing the performance of immunoaffinity (IAF) purification of hepatitis B surface antigen (HBsAg), conducted in batch chromatography. The immunosorbent efficiency measured as HBsAg eluted per milliliter of immunosorbent was selected as the response. Two numerical independent variables were studied: concentration of potassium thiocyanate (KSCN) and ethanol, and a categorical variable formed by two levels of sodium deoxycholate under defined ionic strength (DCS/NaCl). A three-level factorial design of RSM was developed and gets a reduced cubic model which accurately fits the data ($R^2 \approx 0.97$). RSM predicted an optimal region where the response could allow maximum values. At the optimal condition 1.7 M KSCN, 30% (v/v) ethanol, and 0% (w/v) DCS and 1 M NaCl a response of 582 ± 11 μg/ml (559 ± 52 by the model), wherein the measured response does not differs statistically with the maximum experimental value. At optimized condition, a recovery of HBsAg of ∼94% of the adsorbed antigen was obtained, representing a ∼69% more HBsAg than the previously reported. Due to less accumulation of HBsAg this optimum could increase the immunosorbent life in a multiple cycle's operation.

Keywords: Affinity; Chromatography; Hepatitis B surface antigen; Monoclonal antibodies; Optimization; Response surfaces methodology

1. Introduction

Hepatitis B is one of the major diseases affecting the humankind and is a serious global public health problem [1]. Two billion people have been infected with the hepatitis B virus and more than 400 million have chronic infections [1,2]. Since 1982 safe and effective recombinant vaccines based on the hepatitis B surface antigen (HBsAg) are available commercially [3,4].

HBsAg is expressed intracellularly and assembled into virus-like particles in the methylotrophic yeast *Pichia pastoris* [5–8]. Immunoaffinity (IAF chromatography) using a murine monoclonal antibody (mAb) immobilized on a solid matrix [9], is the key step in the purification process of the recombinant antigen allowing to reach a high purity level (>90%) with a reasonable recovery yield (∼70%) [8,9]. Different strategies have been explored to reduce the high cost of the monoclonal antibody and the limited life of immunosorbents at large-scale operation [10–12].

In order to extend the operation cycle of the IAF chromatography columns, very limited success was obtained by manipulating the pH in the eluting conditions [13], observing invariably in cycle-to-cycle operation a fall in the performance and efficacy of the IAF chromatography. Such behavior can be attributed

Abbreviations: ANOVA, analysis of variance; API, active pharmaceutical ingredient; CI, confidence interval; CDR, complementary determining region; CV, coefficient of variations; DCS, sodium deoxycholate; ELISA, enzyme-linked immunosorbent assay; EtOH, ethanol; HBsAg, hepatitis B surface antigen; mAb, monoclonal anti-body; RC, reduced cubic; RSM, response surface methodology; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Nomenclature

e_i	residual ($y_i - \hat{y}_i$)
R^2	coefficient of determination
X_i	independent variable
y	immunosorbent efficiency [as a ratio between the total HBsAg eluted (μg HBsAg) and immunosorbent volume (ml)]
$\hat{y}_i$	estimated response by Design-Expert 7.1.1 trial

Greek symbols

β_i	linear coefficient of Eq. (1)
β_{ii}	quadratic coefficient of Eq. (1)
β_{ij}	interaction coefficient of Eq. (1)
β_0	constant coefficient (intercept) of Eq. (1)
β^{I}_{12}, β^{I}_{13}, β^{II}_{12}, β^{II}_{23}	cubic coefficients of Eq. (1)
η_{IAF}	recovery of HBsAg adsorbed on immunosorbent (%, w/w)

to several factors. These include loss of antibody, degradation of antibody function, support matrix degradation, progressive nonspecific adsorption of contaminants, proteolytic digestion of antibody, and incomplete antigen elution [14]. The frequently harsh elution conditions (3 M KSCN and 1 M NaCl) needed to remove bound antigen seems to be responsible for the loss of antibody function. In most cases, erroneously, only the efficacy for elution was measured, not the actual effect of components of elution on immunosorbent life [14].

Many elution buffers are in use for the retrieval of proteins from affinity columns, after disrupting interactions between receptor and ligand [15]. Together with the widely used chaotropic ions like thiocyanate (SCN^-) [9,12,16], elution at extreme pH [13,14], water–miscible organic solvents [17], denaturing agents [9,18] and detergents [19] have also been successfully employed.

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses widely used for developing, improving, and optimizing processes [20].

RSM defines the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of the independent variables, this methodology generates a mathematical model that accurately describes the overall process, with limited number of experiments [21]. It has been successfully applied to optimizing conditions in some bioprocesses [21,22]. Different types of RSM designs include a three-level factorial design [23], a central composite design [24], a Box-Behnken design [25], and a D-optimal design [26,27].

Here, a three-level factorial design was applied to optimize the elution conditions of IAF batch chromatography for purification of yeast-derived HBsAg. Our approach combines statistical strategies involving the use of an orthogonal design response surface and polynomial regression to establish the best combination of independent variables for maximize the recovery of the recombinant antigen. As the independent variables were selected two numerical: the concentration of potassium thiocyanate (KSCN) and ethanol (EtOH) in the elution buffer, and a categorical one, formed by two-levels of a certain combination of sodium deoxycholate (DCS), and sodium chloride (NaCl). As a response variable was selected the immunosorbent efficiency, expressed as mass of HBsAg eluted per volume of immunosorbent employed. Further, a selecting condition was re-evaluated and compared with the condition employed elsewhere [9,12].

2. Methods and materials

2.1. Materials

All buffers and materials were prepared using high-quality reagents from international suppliers. The immunosorbent with CB HEP 1 anti-HBsAg murine monoclonal antibody immobilized on Sepharose CL-4B (Amersham-Pharmacia Biotech AB, Uppsala, Sweden) at a density of 3.91 mg/ml as described elsewhere [12] was employed during all experiments.

2.2. Starting crude HBsAg preparation

HBsAg was produced by fermentation of a recombinant *P. pastoris* strain (C226) in a medium supplemented with glycerol, and its expression was induced with methanol [8]. The cells were harvested by centrifugation and disrupted using a bed mill (KDL type, WAB, Basel, Switzerland). The disruption buffer contained Tris–HCl (20 mM), EDTA (3 mM), NaCl (0.3 M), KSCN (3 M) and sucrose (10 g/l), at pH 8 [28]. The homogenate was precipitated by adding HCl (1 M) down to pH 4.5 and centrifuged at 10,000 × *g* for 30 min [28]. The supernatant fraction was incubated for 2 h at 8 °C in contact with a diatomaceous earth matrix (Hyflo Super Cell), at pH 4 under continuous stirring [29]. Adsorption was allowed to take place for 2 h and the Hyflo Super Cell was separated by decantation. After washing the matrix twice with two Hyflo Super Cell volumes of 0.2 M KSCN solution, the antigen was eluted in Tris–HCl (20 mM), EDTA (3 mM), sucrose (100 g/l), at pH 8.2 [29]. This procedure gives a semi-purified material of about 10–15% purity that was used as starting material in the present work.

2.3. Analytical determinations

The HBsAg concentration was measured by an enzyme-linked immunosorbent assay (ELISA) system using sheep anti-HBsAg polyclonal antibodies for plate coating, followed by incubation with anti-sheep IgG antibodies-horse radish peroxidase conjugate as explained elsewhere [32]. The HBsAg working standard was calibrated against the Paul Erlich Institute (Frankfurt, Germany) standard. Total protein content was determined by the method of Bradford [33] using bovine serum albumin as standard.

Samples were analyzed by electrophoresis on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels as described by Laemmli [30]. Proteins were stained with Coomassie blue R-250 and purity was analyzed by gel densitometry by using a