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Enhancing recovery of recombinant hepatitis B surface antigen in lab-scale and large-scale anion-exchange chromatography by optimizing the conductivity of buffers

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ABSTRACT

In biopharmaceutical science, ion-exchange chromatography (IEC) is a well-known purification technique to separate the impurities such as host cell proteins from recombinant proteins. However, IEC is one of the limiting steps in the purification process of recombinant hepatitis B surface antigen (rHBsAg), due to its low recovery rate (<50%). In the current study, we hypothesized that ionic strengths of IEC buffers are easy-to-control parameters which can play a major role in optimizing the process and increasing the recovery. Thus, we investigated the effects of ionic strengths of buffers on rHBsAg recovery via adjusting Tris-HCl and NaCl concentrations. Increasing the conductivity of equilibration (Eq.), washing (Wash.) and elution (Elut.) buffers from their initial values of 1.6 mS/cm, 1.6 mS/cm, and 7.0 mS/cm to 1.6 mS/cm, 7 mS/cm and 50 mS/cm, respectively yielded an average recovery rate of 82% in both lab-scale and large-scale weak anion-exchange chromatography without any harsh effect on the purity percentage of rHBsAg. The recovery enhancement via increasing the conductivity of Eq. and Wash. buffers can be explained by their roles in reducing the binding strength and aggregation of retained particles in the column. Moreover, further increase in the salt concentration of Elut. Buffer could substantially promote the ion exchange process and the elution of retained rHBsAg.

1. Introduction

Hepatitis B virus infection (HBV), one of the major infectious diseases that may lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC), is endemic in many parts of the world [1]. Globally, about 350 million people are chronic carriers of HBV, and still, this infection is one of the global health problems. The most effective prevention of HBV infection is immunization with Hepatitis B vaccine. The Hepatitis B surface antigen (HBsAg), 22 nm spherical or filamentous particle with about 100 subunits stabilized with disulfide bonds, is the main part of Hepatitis B vaccine [2]. So far, rHBsAg has been genetically expressed in bacteria [3], mammalian cells [4,5] and yeast [6,7] amongst which, *Pichia pastoris (P. Pastoris)* yeast has received major attention due to the high potential of this host cell for high-level heterologous gene expression [8–11].

After intracellular expression of rHBsAg by *P. Pastoris* in the fermentation process, multiple stages required to extract and concentrate rHBsAg particles in the downstream sections. One of these sections is the clarification section where methods such as centrifugation and precipitation are applied [11–13]. For further purification, various types of separation units such as chromatography, filtration, and ultracentrifuge are integrated [14,15]. For large scale rHBsAg production [11,12], affinity chromatography (AFC) is the preferred method for the purification processes [16,17]. Besides, ion-exchange chromatography (IEC) and size exclusion chromatography are employed to remove impurities such as endotoxins, DNA, host cell proteins and lipids [12,18–20]. Nowadays, IEC using commercial adsorbents such as Diethylaminoethyl (DEAE) is one of the most popular and frequently used chromatography techniques for large-scale purification of therapeutic vaccines such as HBsAg because of feasibility to automate and scale-up [12,19,20]. However, the purification step of IEC has a low recovery (<50%) because of the irreversible aggregation or dissociations of HBsAg particles and is the bottleneck in the production process of rHBsAg [21]. Some

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studies have investigated the effect of different variables involved in the IEC, including the properties of the absorbent, pH and flow rate, on the rHBsAg recovery process [22–24]. Given their results, increasing the pore size and decreasing the ligand densities in DEAE adsorbent yields a significant improvement in the recovery of rHBsAg [22–24]. However, these studies were not comprehensive and did not take into account some other properties of IEC that could have an influence on the rHBsAg recovery like ionic strengths of IEC buffers.

We hypothesized that the ionic strengths of IEC buffers might have a major impact on the recovery of rHBsAg particles by influencing binding strength, dispersion, and elution of retained rHBsAg. The ionic strength of Elut. buffer can change the recovery by affecting the rHBsAg-resin attraction. Parallel to that, the ionic strengths of Eq. and Wash. buffers were considered additional factors since they could influence the binding strength and dispersion of virus-like particles and other impurities in the column. Thus, we aimed to investigate the effect of the conductivity of Eq., Wash. and Elut. buffers on the recovery of IEC via adjusting Tris-HCl and NaCl concentrations. After finding the optimum condition for rHBsAg recovery in lab-scale, we employed the obtained optimum values in the large-scale IEC.

2. Material and methods

2.1. Materials

All buffers were prepared with analytical grade chemicals purchased from Merck (Germany) company and water for injection (WFI) from the Pasteur Institute (Iran). For the lab-scale IEC, we used PD-10 empty column from BIO-RAD company (USA). In the large-scale, we applied K-Prime® 40-ll chromatography system with Merck Millipore Quick Scale column. Weak anion media (DEAE-Cellulose DE-52) was purchased from Whatman (U.K) company.

2.2. Fermentation and cell disruption

The rHBsAg was produced in fermenter by the recombinant strain of *P. pastoris* [11,12]. The fermentation medium contained ammonium sulfate, glycerol, magnesium sulfate, EDTA, dipotassium hydrogen phosphate, calcium chloride and trace elements. The fermentation was carried out in the fed-batch mode in presterilized medium (3L) at 29 °C/500–600 rpm and pH around 4.5 via adding 20% w/v ammonia solution [11,12]. The methanol induction started when glycerol was depleted and the dissolved oxygen (DO) did not increase further. After methanol induction, the cells were harvested by disk stack centrifuge and washed twice with deionized water [13]. The composition of the disruption buffer was 20 mM Tris-HC1, 5 mM EDTA, 0.3 M NaCl, 3 M KSCN, 1% sucrose and pH 8.0. In the bead mill technique, Dyno-Mill type KDL bead mill (WAB, Switzerland) was applied, using beads with a diameter of 0.5–0.75 mm and a packing density of 85% [11–13].

2.3. Clarification and purification

The pH of the disrupted cell suspension from the KDL mill was adjusted to 4.5 using 1 M HCl, under continuous stirring in an ice bath to decrease most of *P. pastoris* proteins and enhance the purity of rHBsAg [13]. The centrifugation was applied as 12,000 g for 30 min at 4 °C suspension to introduce cell-free lysate sample [13]. Afterward, the pH was readjusted to 7.5 using 1 M NaOH.

In the first stage of the purification process, substances like DNA, pigments and some host proteins were separated in the anion exchange chromatographic phase [18]. In the second stage, Immunoaffinity (IAF) chromatography was employed, which its CnBr activated Sepharose media was coupled to anti-hepatitis B Monoclonal Antibodies. Due to the high selectivity of this method, above 95% of DNA and carbohydrates, and lipids were separated from rHBsAg [11,12,16,17]. Gel filtration with Sephadex G-25M column was applied before introducing the product to the second IEC to desalt the eluent. During the second IEC, remained impurities such as host cell proteins were separated to reach HBsAg with around 95% purity [11,12].

2.4. Analytical assays

Samples of rHBsAg were analyzed before and after IEC experiment. The total protein concentration was measured according to Bradford assay using Bovine Serum Albumin (BSA) as the standard [25]. We also determined the rHBsAg concentration by sandwich ELISA where sheep polyclonal antibodies against rHBsAg were coated on the plate and conjugated with horseradish peroxidase [11]. The homogeneity and purity of rHBsAg were detected, respectively, by Size Exclusion High-Performance Liquid Chromatography (SEC-HPLC) with TSK-PW3000 column and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 12% gel as described by laemmli [26]. As for size standards in protein electrophoresis, we used Thermo Scientific Unstained Protein Molecular Weight Markers which were a mixture of seven purified proteins (β-galactosidase, Bovine serum albumin, Ovalbumin, Lactate dehydrogenase, REase *Bsp*98I, Lysozyme). According to the intensity of the Coomassie-bands in the SDS-PAGE, the purity of rHBsAg among other proteins was measured with AlphaEase® FC software. The recovery (R) percentage of rHBsAg was calculated by using equation (1). In this equation, C represents the concentration of the intended substances and V accounts for the volume of the sample.

$$R_i = \frac{C_{HBsAg,outlet} \cdot V_{i,\ outlet}}{C_{HBsAg,\ injection} \cdot V_{i,\ injection}} \times 100 \quad (1)$$

2.5. Characterization of IEC inlet material

According to the SDS-PAGE analyses (Fig. 1) and the ratio of rHBsAg concentration to the total protein concentration (ELISA assay/Bradford assay), the average purity of rHBsAg after IAF column was 82 ± 2%. As shown in Fig. 2, the SEC-HPLC analyses (homogeneity analyses) of IEC inlet sample demonstrates three major peaks. In this chromatogram, the first peak (retention time of 21.5 min) and the third small peak (retention time of 44.0 min) indicate the aggregated rHBsAg particles and monomeric rHBsAg particles, respectively. The main peak in the chromatogram (retention time of 28.6 min) represents the matured and multimeric rHBsAg particles which is broad due to the heterogeneity of the particle assembly as well as the presence of some other protein impurities (~20%) [27–29].

2.6. Lab-scale IEC

Table 1 shows the composition of IEC buffers and the injection sample. The Tris-HCl concentration of Eq. buffer and NaCl concentration in Wash. and Elut. buffers were the variable parameters. We performed the process at the ambient temperature under gravity-flow condition. In the first step, weak anion exchange media with 10 ml packed bed volume (1.5 * 5.65 cm) was equilibrated with a 5-fold column volume of Eq. buffer. Subsequently, 50 ml of rHBsAg sample was injected into the column and washed with 2-