Protein Expression and Purification 74 (2010) 257–263

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Regulation of protein multipoint adsorption on ion-exchange adsorbent and its application to the purification of macromolecules

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ARTICLE INFO

Article history:
Received 13 May 2010
and in revised form 21 June 2010
Available online 15 July 2010

Keywords:
Multipoint adsorption
Ligand density
Steric mass-action model
Bovine serum albumin
Hepatitis B surface antigen
Macromolecule

ABSTRACT

Ion-exchange chromatography (IEC) using commercial ionic absorbents is a widely used technique for protein purification. Protein adsorption onto ion-exchange adsorbents often involves a multipoint adsorption. In IEC of multimeric proteins or "soft" proteins, the intense multipoint binding would make the further desorption difficult, even lead to the destruction of protein structure and the loss of its biological activity. In this paper, DEAE Sepharose FF adsorbents with controllable ligand densities from 0.020 to 0.183 mmol/ml were synthesized, and then the effect of ligand density on the static ion-exchange adsorption of bovine serum albumin (BSA) onto DEAE Sepharose FF was studied by batch adsorption technique. Steric mass-action (SMA) model was employed to analyze the static adsorption behavior. The results showed that the SMA model parameters, equilibrium constant (K_a), characteristic number of binding sites (υ) and steric factor (σ), increased gradually with ligand density. Thus, it was feasible to regulate BSA multipoint adsorption by modulating the ligand density of ion-exchange adsorbent. Furthermore, IEC of hepatitis B surface antigen (HBsAg) using DEAE Sepharose FF adsorbents with different ligand densities was carried out, and the activity recovery of HBsAg was improved from 42% to 67% when the ligand density was decreased from 0.183 to 0.020 mmol/ml. Taking the activity recovery of HBsAg, the purification factor and the binding capacity into account, DEAE Sepharose FF with a ligand density of 0.041 mmol/ml was most effective for the purification of HBsAg. Such a strategy may also be beneficial for the purification of macromolecules and multimeric proteins.

Introduction

Ion-exchange chromatography (IEC)[1] using commercial adsorbents (DEAE, Q, CM, SP, etc.) is a widespread technique used for protein purification. It is a simple and rapid method based on the selective desorption of the proteins that have been adsorbed with different strength to the adsorbent [1]. To improve the capacity, most of the commercially available ion-exchange adsorbents are highly activated with ionic groups, so that they can adsorb the maximum percentage of proteins from a given extract [2]. In IEC, protein adsorption often involves multipoint binding, which generally has little or no side-effect on protein desorption and protein structure disruption. However, most of protein macromolecules, especially for multimeric and large proteins, such as β-galactosidase, bovine liver catalase, hepatitis B virus surface antigen (HBsAg), etc., because of the large surfaces to interact with the ionic support, may promote a very intense multipoint adsorption on the surface of the solid absorbent [1–3]. It would make the further desorption difficult. What is more, the conformation of protein macromolecules may be partially destroyed and their bioactivity was reduced by the intense multipoint adsorption and the harsh elution conditions during the adsorption–desorption process in IEC [3]. In the study of IEC and hydrophobic interaction chromatography (HIC) of cytochrome *c*, bovine serum albumin (BSA), immunoglobulin G and fibrinogen, Goheen and Gibbins found a linear relationship between the amount of protein recovered and the log of its molecular mass [4], which may also be due to the large size of the protein and its intense multipoint adsorption.

The ligand density of the adsorbent plays an important role on the chromatographic behavior in adsorption chromatography, including IEC [1–3], HIC [5] and immobilized metal ion affinity chromatography (IMAC) [6]. In principle, the change of ligand

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[1] *Abbreviations used:* IEC, ion-exchange chromatography; HBsAg, hepatitis B virus surface antigen; HIC, hydrophobic interaction chromatography; BSA, bovine serum albumin; IMAC, immobilized metal ion affinity chromatography; IAC, immunoaffinity chromatography; GFC, gel filtration chromatography; SMA, steric mass-action; CCS, cell culture supernatant; STD-R, stoichiometric displacement theory for retention; ISEC, inverse size-exclusion chromatography; CHO, Chinese hamster ovary.

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doi:10.1016/j.pep.2010.07.002

density would provide a very useful means to alter protein binding intensity, retention and capacity [1–3,5,6]. The adsorbents with a high ligand density provide an intense multipoint binding, an intense retention and a high capacity, while the adsorbents with a low ligand density provide a mild binding, a moderate retention and a lower capacity. Pessela and his co-workers took advantages of multipoint adsorption of large proteins, selectively adsorbing and purifying large proteins (β-galactosidase, bovine liver catalase and antibody–antigen complex) with lowly activated ion-exchange adsorbents or IMAC adsorbents [1,2,6]. Other researchers also studied the effect of ligand density on the purification of protein or antibody in HIC, IEC and immunoaffinity chromatography (IAC) [5,7–10]. However, there is still little research dealing with the regulation of protein multipoint adsorption and its application to the purification of macromolecules.

The downstream process for a large scale purification of HBsAg was generally composed of HIC, IEC and gel filtration chromatography (GFC) [11,12]. Among them, IEC with the lowest step recovery (<50%) is still a bottleneck of the whole purification process [12]. The low recovery is mainly caused by the complex assembly structure of virus-like HBsAg particles, which are easily destroyed or aggregated during the process of purification [13]. It has been reported that HBsAg particle is a virus-like particle with a diameter of 22 nm and is composed of about 100 subunits [14,15]. This fact may promote a very intense multipoint adsorption on the surface of DEAE Sepharose FF (GE, 0.130 mmol DEAE/ml absorbent), a commercially available ion-exchange absorbent broadly used in the purification of HBsAg. In our previous investigation [3], the chromatographic efficiency of HBsAg was improved by using lowly activated ion-exchange adsorbent. However, the range of ligand density studied was not wide enough, and the mechanism of ligand density effects on HBsAg structure and recovery was not well illuminated.

In this article, DEAE Sepharose FF adsorbents with controllable ligand density were prepared. BSA was chosen as the model protein to evaluate the effect of ligand density on the adsorption behavior. Then the static adsorption equilibrium data were analyzed using steric mass-action (SMA) model [16], and the feasibility of regulating protein multipoint adsorption by adjusting the ligand density was discussed. Finally, the effect of ligand density on IEC of HBsAg was studied, and the advantage of using DEAE Sepharose FF adsorbent with a lower ligand density for the purification of protein macromolecules was discussed.

Materials and methods

Materials

DEAE–HCl was purchased from Nanxiang Chemical Company (Shanghai, China). BSA was purchased from Roche (Germany). Cell culture supernatant (CCS) contained HBsAg was a kindly gift from North China Pharmaceutical Group Corporation (NCPC, Shijiazhuang, China). Sepharose 6 FF was purchased from GE Healthcare (USA). Other reagents were all of analytical grade from local sources.

Preparation of DEAE Sepharose FF adsorbents

DEAE Sepharose FF adsorbents were synthesized according to Eq. (1).

$$\mathrm{M-OH + ClCH_2CH_2N(C_2H_5)_2 - HCl \overset{NaOH}{\rightarrow} M-O-CH_2CH_2N(C_2H_5)_2 - HCl} \quad (1)$$

where M represents the matrix of Sepharose FF. A series of experiments were carried out to study the effects of DEAE–HCl concentration (0.5–3.0 M), NaOH concentration (2.0–10.0 M), reaction time (0.5–6.0 h) and reaction temperature (40–70 °C) on the ligand density of ionic adsorbents synthesized. Then a series of DEAE Sepharose adsorbents with controllable ligand density were prepared using corresponding reaction time under the optimal conditions. The ligand density was quantitated by acid–base titration.

Adsorption equilibrium

A series of adsorption equilibrium experiments of BSA onto DEAE Sepharose FF with different ligand densities (0.020, 0.041, 0.078, 0.104, 0.123, 0.161 and 0.183 mmol/ml) were performed using the stirred batch adsorption method in 0.01 M phosphate buffer (pH 7.0) at 25.0 °C. Generally, about 150 mg of drained gel was pre-equilibrated in the buffer and then added to the flasks containing 15 ml of buffered protein solution with different concentrations (0.1–2.0 mg/ml) and ionic strength (0–0.15 M sodium chloride). The flasks were shaken at 170 rpm for 10 h in a water bath at 25.0 °C. After centrifugation (3000*g*, 10 min, 25.0 °C), the protein concentration in the supernatant was determined with a UV–Vis spectrophotometer at 280 nm. The amount of protein adsorbed onto the DEAE Sepharose FF was then calculated by the mass balance.

The static adsorption data of BSA onto DEAE Sepharose FF were fitted to SMA model equation (Eq. (2)) by nonlinear least squares regression using Origin 7.0 software [17]:

$$c = \left(\frac{q}{k_a}\right)\left(\frac{c_s}{\Lambda - (\upsilon+\sigma)q}\right)^{\upsilon} \quad (2)$$

where K_a is the equilibrium constant, υ is the characteristic charge, σ is the steric factor, Λ is the ligand density of the adsorbent, c_s is the salt concentration in the liquid phase, c is the protein concentration in the liquid phase, and q is the adsorbed protein concentration. The SMA parameters of K_a, υ and σ can be estimated by fitting the equation to experimental data.

Ion-exchange chromatography of hepatitis B surface antigens

HBsAg in CCS was pre-purified by HIC, and the HBsAg fraction eluted from HIC was desalted by hollow-fibre ultrafiltration using a 500 kDa molecular weight cut-off membrane (Millipore, USA), which was performed as described previously [3]. The desalted sample was stored at 4 °C for the further purification by IEC.

Seven DEAE Sepharose FF absorbents with different ligand densities (0.020–0.183 mmol/ml) were used in IEC. The IEC operation was performed as described previously [9]. The desalted sample was loaded onto seven IEC columns (75 mm × 16 mm I.D., CV = 15 ml) pre-equilibrated with buffer A (20 mM sodium phosphate, pH 7.0), respectively. According to the difference of the DEAE ligand density, different elution gradients were optimized [3]. The absorbent with a ligand density of 0.183 mmol DEAE/ml absorbent was pre-equilibrated with buffer A and eluted stepwise with 13% buffer B (20 mM sodium phosphate, pH 7.0, added 1.0 M NaCl) and 100% buffer B in sequence. The other absorbents with lower ligand density were pre-equilibrated with buffer A and then eluted stepwise with lower gradients (7–13% buffer B) and 100% buffer B in sequence. The eluted fractions were collected for the further analysis.

HBsAg was quantitatively assayed by a commercially available HBsAg ELISA kit from Kehua, Shanghai, China [11]. Protein concentration was determined according to Bradford's method with slight modification [11]. High performance size-exclusion chromatography with on-line multi-angle-laser-light-scattering and refractive index detectors (HPSEC-MALLS-RI) was performed as described previously [14].