Chinese Journal of Chemical Engineering 24 (2016) 151–157

Contents lists available at ScienceDirect

Chinese Journal of Chemical Engineering

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

Biotechnology and Bioengineering

Experimental and *in silico* studies on three hydrophobic charge-induction adsorbents for porcine immunoglobulin purification☆

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ARTICLE INFO

Article history:
Received 13 October 2014
Received in revised form 6 May 2015
Accepted 6 May 2015
Available online 2 July 2015

Keywords:
Hydrophobic charge-induction chromatography
Porcine immunoglobulin
Step elution
Molecular simulation

ABSTRACT

Three hydrophobic charge-induction adsorbents with functional ligands of 4-mercapto-ethyl-pyridine, 2-mercapto-methyl-imidazole or 2-mercapto-benzimidazole were evaluated in the purification of porcine immunoglobulin from porcine blood. Adsorption isotherms were studied under different pH conditions. The adsorbent with 2-mercapto-methyl-imidazole as the ligand showed reasonable adsorption capacity (43.60 $mg \cdot g^{-1}$ gel) with great selectivity and it also showed the best elution performance in chromatographic studies. A multi-pH step elution process was proposed for the 2-mercapto-methyl-imidazole adsorbent, and the results showed that high immunoglobulin purity (94.3%) and a yield of 9.8 $mg \cdot (ml\ plasma)^{-1}$ could be achieved under the optimal condition of loading (pH 5.0)–pre-elution (pH 7.0)–elution (pH 3.8). Moreover, molecular simulation was employed to help in analyzing the binding mechanism between the ligands and immunoglobulin, and the results showed that both 2-mercapto-benzimidazole and 2-mercapto-methyl-imidazole ligands were docked on the same pocket (around TYR319 and LEU309) of the Fc fragment of immunoglobulin, with 2-mercapto-benzimidazole showing stronger binding interactions.

1. Introduction

Immunoglobulins known as antibodies are glycoprotein molecules produced by plasma cells in response to the presence of foreign antigens [1]. These proteins have important biological functions and have been found useful in diagnostic and therapeutic applications [2,3]. Immunoglobulin (Ig) can be found and purified from various natural resources, such as animal blood [4–7], milk [8] and poultry eggs [9–10]. For example, porcine blood contains up to 2% Ig which shows potential applications as antibacterial and antiviral agents, and it would be valuable if porcine Ig can be purified rather than wasted in the pork industry. Therefore, cost-effective and efficient purification processes are needed for large-scale production of these antibodies, which can benefit human health in addition to economic profits.

Currently, affinity chromatography is still the most widely used technique for antibody purification [11], and Protein A affinity chromatography is the most successful method for monoclonal antibody (mAb) purification due to its excellent selectivity. High degree of purity and recovery can be achieved in a single operation with Protein A resins [12]. However, high cost of Protein A-based resins definitely restricts their application in large-scale antibody production. Moreover, Protein A has other limitations such as low reusability, ligand leaching and low tolerance in clean-in-place (CIP) procedures [13]. Consequently, a variety of ligands and chromatographic techniques have been developed to purify antibodies over the years [14]. Mixed-mode chromatography (MMC) is one type of new techniques which has received great attention in recent years [15]. There are multiple interactions between functional ligands of MMC and target biomolecules during purification processes, such as hydrophobic, electrostatic and hydrogen bonding interactions [16]. Compared with single-mode chromatography, MMC usually has higher selectivity and loading capacity [17]. For example, Wang and co-workers [18] evaluated four mixed-mode resins for the purification of IgG from serum albumin containing feedstock, and high purity (92.3%) and high recovery (95.6%) were achieved after an optimized purification process.

Burton and Harding [19] introduced a form of MMC known as hydrophobic charge-induction chromatography (HCIC) in 1998, which is based on the pH dependent behavior of ligands [20]. HCIC is salt-independent and the adsorption and elution processes can be performed under physiological conditions [21,22]. For example, the MEP HyperCel resin with 4-mercaptoethyl-pyridine (MEP) as the ligand is the first commercial HCIC adsorbent developed by Pall Corporation. This ligand contains a pyridine ring and a sulfur atom in the hydrophobic chain. Target proteins can be adsorbed on uncharged ligands at neutral pH *via* hydrophobic forces and eluted due to electrostatic repulsion under acidic conditions. The dynamic binding capacity of MEP HyperCel for murine IgG can reach up to 25–35 $mg \cdot ml^{-1}$ [20]. HCIC has been considered to be superficially similar to Protein A

☆ Supported by the National Natural Science Foundation of China (21276228 and 21476198), the Natural Science Foundation of Zhejiang Province (LR12B06003), and the Fundamental Research Funds for the Central Universities (2013QNA4032).
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http://dx.doi.org/10.1016/j.cjche.2015.06.019
1004-9541/

chromatography which also involves hydrophobic interactions for adsorption and elution because of ionization of histidine residues [23]. In addition to the hydrophobic and electrostatic interactions, the thiophilic sites on ligands also play an important role in antibody binding. Research has shown that replacing the sulfur atom with either nitrogen or oxygen leads to much lower IgG binding capacity [20]. The mechanism behind the thiophilic effects is not clear and it was postulated that the presence of an electron donor–acceptor pair on both the ligand and the antibody is responsible for this interaction [23–25]. Molecular simulation indicates that the molecular structure of ligands is the main factor affecting the binding process, and a sulfonic group on the spacer arm might form additional hydrogen bonds to enhance the binding selectivity [26–28].

A series of HCIC cellulose adsorbents with thiophilic sites and mercaptoheterocyclic ligands were developed in our previous work [29], and the applications of these new resins for the purification of immunoglobulin from egg yolk were studied in both packed and expanded beds [10,30]. In this study, new HCIC adsorbents will be evaluated for the purification of porcine Ig from porcine blood. Due to multi-interactions involved in HCIC, the operational conditions will be optimized for better separation/purification performance. The adsorption behaviors of the three adsorbents will be compared under different pH conditions and an optimized multi-pH elution process will be proposed. Moreover, the binding mechanism will be explored with the help of molecular simulation.

2. Materials and Methods

2.1. Materials

Fresh porcine blood was kindly provided by a local slaughterhouse. Porcine Ig was supplied by Sonac Co., Ltd. (Wuhan, China). Buffer solutions with pH 3.0–3.6 were prepared with citric acid/phosphate buffer solutions, and buffer solutions with pH = 3.8–5.0 were prepared with acetate buffer solutions. Phosphate buffer solutions were used for preparing buffer solutions with a pH value of 6–8. All of the buffer solutions were pre-filtered with 0.22 μm polymer membranes. The chemicals used were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Feedstock preparation

Fresh porcine blood was first mixed with 0.1 $mol \cdot L^{-1}$ sodium citrate buffer solution (pH 8.7) with a ratio of 1:9, and then porcine plasma was obtained *via* centrifugation (3000 $r \cdot min^{-1}$, 10 min) (Thermo Scientific, Waltham, MA, USA). The plasma was treated with ammonium sulfate to remove fibrinogen using an optimized process reported earlier [31]. The supernatant was used as the feedstock for further purification of porcine Ig.

2.3. HCIC adsorbent preparation

Details of the preparation method for the HCIC adsorbents have been reported previously [29] and the structures are shown in Fig. 1. Briefly, macroporous cellulose particles were first prepared and used as matrices. A water-in-oil suspension thermal regeneration method was used for the preparation, which was similar to the method published earlier [32]. Some 5wt% gelatinized cassava starch solution was mixed with cellulose viscose under agitation, and then the mixture was dispersed in 300 g oil phase (vacuum oil:chlorobenzene, 5:1) for 20 min. The suspension was heated to 95 °C and kept at this temperature for 1.5 h before cooled down to 20 °C and filtered. The particles formed were washed with boiling water and then ethanol, and treated with 1% amylase solution for 30 min. The final particles were washed with water and screened to obtain particles with a size range of 50–250 μm. These particles were then activated by divinylsulfone (DVS). The activated particles (Cell-DVS) were coupled with 4-mercapto-ethyl-pyridine (MEP), 2-mercapto-methyl-imidazole (MMI) and 2-mercapto-benzimidazole (MBI), respectively. The corresponding adsorbents were denoted as Cell-DVS–MEP, Cell-DVS–MMI and Cell-DVS–MBI. These adsorbents have a size range of 50–250 μm and porosity of 85%. The wet density and the ligand density are approximately 1.8 $g \cdot ml^{-1}$ and 60 $\mu mol \cdot ml^{-1}$, respectively.

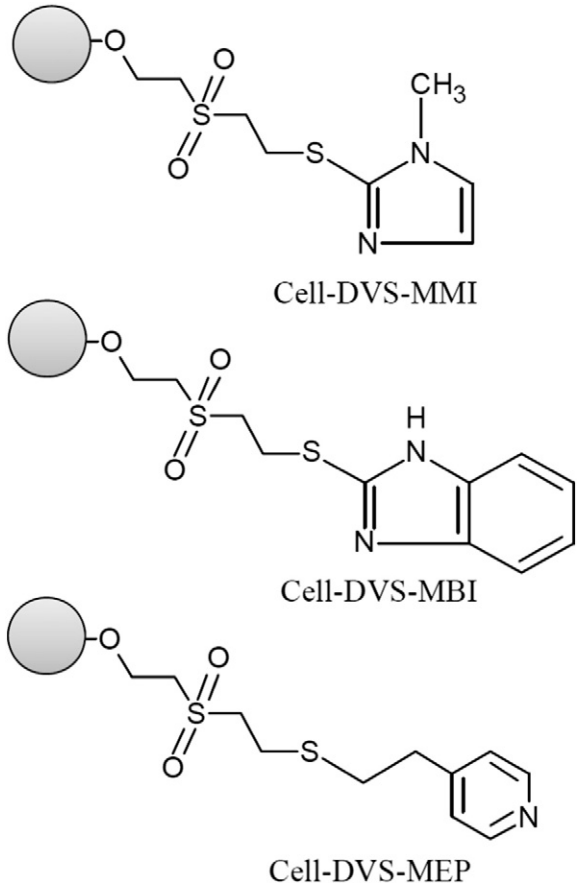

Fig. 1. Schematic diagrams of the chemical structures of the three HCIC adsorbents.

2.4. Adsorption isotherms

The adsorbents were equilibrated with related buffer solutions for 15 min, and then drained and added into five flasks (0.1 g adsorbent in each flask). Each of the flask contained 7 ml related buffer solutions with different initial porcine Ig concentrations of 0.1–0.5 $mg \cdot ml^{-1}$. The mixture was kept at 25 °C for 10 h in a shaking incubator (200 $r \cdot min^{-1}$). The solutions were then filtered and the supernatant was analyzed *via* UV spectroscopy at 280 nm (Ultrospec 3300 Pro, Amersham Biosciences, Uppsala) to measure the final protein concentration in the liquids. The weights of protein absorbed onto the adsorbents were obtained by mass balance calculation, and the adsorption equilibrium was fitted by the Langmuir equation,

$$Q^* = \frac{Q_m \cdot C^*}{K_d + C^*} \quad (1)$$

where Q^* ($mg \cdot g^{-1}$) and C^* ($mg \cdot ml^{-1}$) are the equilibrium adsorption capacity and protein concentration in liquid phase, respectively, Q_m ($mg \cdot g^{-1}$) is the saturated adsorption capacity and K_d ($mg \cdot g^{-1}$) is the dissociation constant.