CHINESE JOURNAL OF BIOTECHNOLOGY
Volume 23, Issue 4, July 2007
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Biotech, 2007, 23(4), 692−699.

RESEARCH PAPER

Comparison of Adsorbent with Varying Spacer Arm Length and Ligand Density for the Purification of Recombinant Hepatitis B Virus Surface Antigen

LI Rui-Hong[1,2], LI Yan[2], BI Jing-Xiu[2], ZHAO Lan[2], ZHOU Wei-Bin[2], HUANG Yong-Dong[2], ZHANG Yan[2], SUN Li-Jing[2], WANG Hua-Jun[1], SU Zhi-Guo[2*]

1 Civil & Environmental Engineering School, University of Science and Technology of Beijing, Beijing 100083, China

2 National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China

Abstract: Novel hydrophobic adsorbents were synthesized by immobilizing butyl derivative onto the highly cross-linked agarose beads manufactured in China, which are used as matrix. The effects of the spacer arm length (3C, 8C, and 10C) and ligand density (from 13 to 45 μmol/mL) on the hydrophobicity were investigated using purified Hepatitis B surface antigen (HBsAg) expressed by CHO cell lines. Also, considering the effects of salt concentration and pH on HBsAg recovery and purification factor, orthogonal experiment design method was used to evaluate the adsorbents. The results showed the butyl-S adsorbent with the spacer arm length of 8C and the ligand density of 22 μmol/mL gel showed the best performance for the separation of HBsAg. Approximately 100% HBsAg recovery and 60 as purification fold were achieved by this media under the operating conditions of pH 7.0 and 9% salt concentration.

Key Words: hydrophobic adsorbent; spacer arm; ligand density; recombinant hepatitis B surface antigen; purification; orthogonal experiment design

Hydrophobic interaction chromatography (HIC) technique is being widely used for protein purification, both in the industrial and in the laboratory scale. But proteins may undergo structural alteration. In hydrophobic interaction chromatography (HIC), the β-lactoglobulin is fractionated into three peaks, which correspond to tetramer, octamer, and dodecamer of β-lactoglobulin, respectively[1]. In case of HIC for multimeric proteins, the the dissociation of subunits is also identified . Tumor necrosis factor (TNF) splits into more than two peaks, which corresponded to the subunit of TNF in HIC[2]. The chromatographic behavior of protein was related to the ligand density of the adsorbent. Hearn *et al* found that the protein adsorption capacity and the affinity of media of hydrophobic chromatography varied with the ligand density[3]. Hepatitis B virus surface antigen (HBsAg) isolated from Chinese hamster ovary (CHO) cell is composed of more than 100 subunits, and the molecular weight of the protein, which contains saccharide and lipid[4], is more than 2 000 000 dalton[5]. During the course of purification, the CHO–HBsAg assembly may be adsorbed irreversibly or may undergo structural change on the surface of highly hydrophobic adsorbent. So, it is important to choose the adsorbent with appropriate ligand density for the purification of CHO-HBsAg.

This study investigated the alternative of using HIC adsorbent, which could be successfully applied to the separation of CHO–HBsAg in a cost effective manner. The purification process consists of hydrophobic interaction chromatography (HIC), ion-exchange chromatography (IEC),

Received: November 24, 2006; Accepted: December 18, 2006.
* Corresponding author. Tel: +86-10-62561817; Fax: +86-10-62562813; E-mail: zgsu@home.ipe.ac.cn
This work was supported by grants from the Hi-tech Research and development Program of China (No.2002AA217031) and National Natural Science Fund (No. 20536050, 20576136 and 20636010).

and gel filtration chromatography (GFC). Approximately 95% impurity protein, more than 99% saccharide and lipid, and more than 99% DNA and pigment were removed when HIC was performed[6–10]. At present, the media used in HIC was Bytul-S Sepharose FF, a product of GE Healthcare, the price of which was about 10 times that of other media. As the first step of the process, the media of HIC was contaminated so heavily that the working life was limited. This study investigated the alternative of using HIC adsorbents, which could be successfully applied to the separation of CHO-HBsAg in a cost effective manner. Wangyan *et al*[11]synthesized the HIC media, with the spacer arm length of 3C applied to the separation of HBsAg, but the matrix was Sepharose 6FF, a product of Healthcare. In our previous study, we synthesized a novel hydrophobic adsorbent based on highly cross-linked agarose beads manufactured in China by immobilizing butyl derivative onto the matrix linkage[12]. This study further investigated the use of the media with varying spacer arm length based on highly cross-linked agarose beads manufactured in China to improve the HBsAg recovery during the purification process.

The effects of the ligand density and the length of spacer arm on the HBsAg recovery were investigated first in this article. And then the optimal adsorbent and operation condition were screened out by orthogonal experiment of ligand density, spacer arm length, pH, and salt concentration.

1 Materials and methods

1.1 Materials and chromatographic equipments

The highly cross-linked agarose beads manufactured in China were purchased from Zhengguang Gel Company (Hangzhou, China). The chemicals used were analytical grade reagents. All the solutions used were prepared using Mill-Q grade water (Millipore, USA).

The type of peristaltic pump was P-1 (Pharmacia, Sweden). UV detector was Single Path Monitor UV-1 (Pharmacia, Sweden). The interface was Agilent Interface 95000E (Agilent, USA). The detector was connected with the interface, and the data were recorded by computation. The columns were purchased from Jinhua, Shanghai, China.

1.2 Synthesis and evaluation of hydrophobic interaction chromatography adsorbent

On the basis of the Maisson's method[13], HIC media was prepared by a two-step process, matrix activation and ligand cross-linking. The dehydrated QZT beads (Zhengguang Gel Company, Hangzhou, China) were applied as the matrix. The ligand density of the butyl group was controlled by controlling the reaction concentration of butanethiol (from 25 to 45, from 13 to 25, and from 13 to 40 mmol butyl groups/(g wet gel), respectively, for the arm lengths of 3C, 8C, and 10C).

1.3 Hydrophobic interaction chromatography

The adsorbents were packed into a chromatographic column (50 mm × 10 mm I.D. CV=4 mL) and then washed by 0.5mol/L NaOH for five bed volumes. Buffer A (20 mmol/L PB containing certain concentration of ammonium sulfate) and buffer B (20 mmol/L PB) were prepared according to the required pH and conductivity.

For the chromatography of purified HBsAg, about 40 μg purified HBsAg was loaded onto the column (50 mm×10 mm I.D., CV=4 mL) pre-equilibrated with buffer A for 10 CV And then the column was washed with buffer A for 2 CV to collect fraction A (P0). Elution was achieved with 100% buffer B to collect fraction B (P1). The flow rate was 0.8 mL/min, and the detection wavelength was set at 280nm.

For purification of CCS, approximately 20 mL CCS of the same conductivity to buffer A was loaded onto the column (50 mm×10 mm I.D., CV=4 mL) pre-equilibrated with buffer A for 10 CV. And then the column was washed with buffer A for 1 CV to collect fraction A (P0). Elution was achieved with 100% buffer B to collect fraction B (P1) and with 30% (*V/V*) isopropanol to collect fraction C (P2) in sequence. The flow rate was 0.8 mL/min, and the detection wavelength was set at 280 nm.

1.4 Assay for biological activity of r-HBsAg

The r-HBsAg was quantitatively assayed using a commercially available HBsAg ELISA kit, which was purchased from Shanghai Kehua Bio-Engineering Co., Ltd. (Shanghai, China), and the protocol was followed based on the instructions: (1) warm up the 96 well plate pre-coated by the monoclonal antibody of HBsAg at room temperature for 30 min before use; (2) add 50 μL of standard or samples and 50 μL of polyclonal antibody-HBs-HRP, and then incubate for 30 min at 37°C; (3) wash the plates 30 minutes later, and then add 50 μL of tetramethylbenzidine (TMB)–based substrate and 50 μL of H_2O_2, and then incubate for 15 minutes at 37°C; (4) finally, add 50 μL of 20% H_2SO_4 to terminate the reaction. The plates were then read by the ELISA plate reader 550 (Bio-lab, USA) at 450 nm as the measured wavelength and against 630nm as the reference wavelength.

1.5 Assay for protein concentration

Protein concentration was determined according to Bradford procedure[14] using bovine serum albumin (BSA) as a reference standard.

1.6 High-performance size-exclusion chromatography (HPSEC)

High-performance size-exclusion chromatography (HPSEC) was performed using an Agilent 1100 HPLC system, equipped with a degasser and a variable wavelength detector with UV monitoring at 280 nm. The column, TSK G5000 PW (300 mm×7.5 mm, I.D.) with TSK GPW guardcolumn (75 mm×7.5 mm, I.D.), was purchased from Tosohaas (Stuttgart, Germany). Elution buffer was 100 mmol/L sodium phosphate, pH 6.8, containing 100 mmol/L sodium sulfate, and the flow rate was 0.5 mL/min. The injection volume was 100 μL. The multiangle laser light scattering photometer, a DAWN DSP