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A highly efficient hydrophobic interaction chromatographic absorbent improved the purification of hepatitis B surface antigen (HBsAg) derived from *Hansenula polymorpha* cell

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Abstract

To improve the purification efficiency of recombinant hepatitis B surface antigen derived from *Hansenula polymorpha* (Hans-HBsAg), a serial of absorbents for hydrophobic interaction chromatography with the controllable ligand density and spacer arm were synthesized, then developed and further applied to purify Hans-HBsAg. The absorbent, Butyl-S QZT with the ligand density of 25 μmol/(g wet gel) and spacer arm of 3C, was screened out and its physical and chemical properties were evaluated. High rigidity and low backpressure (<0.06 MPa) were obtained at the flow rate up to 20 ml/min. Moreover, it has the stable chemical characteristics of subjecting to high concentrations of acid, alkali and detergents. This HIC absorbent was further applied to purify Hans-HBsAg with the recovery 94% and purification-fold 9 under the optimized operation condition at pH 6.5 and concentration of ammonium sulfate 7.5%. Finally, the HIC adsorbent of Butyl-S QZT was applied in the integrated three-step chromatographic purification process to purify Hans-HBsAg. About 140 mg of purified Hans-HBsAg was obtained from 1 l cell disruption supernatant at the total recovery of 27% and the purification-fold of 151.8. Based on the assay of SDS-PAGE and SEC-HPLC, the purity of the purified HBsAg was over 99% to meet the requirement for the further inoculation use.

Keywords: Recombinant HBsAg; Purification; Hydrophobic interaction chromatography; *Hansenula polymorpha*; Ligand density; Absorbent

1. Introduction

Hepatitis B virus (HBV) infection, the primary cause of cirrhosis and hepatocellular cancer, is one of the major causes of death globally [1]. Immunization with hepatitis B vaccine is the most effective way to prevent HBV infection, so more than one billion doses of hepatitis B vaccine are being administered annually around the world [2]. Plasma-derived hepatitis B surface antigen (P-HBsAg) has been dissuaded by WHO for the limitation of plasma supply and the risk of plasma components (e.g. HIV infection) [3]. Hepatitis B surface antigen (HBsAg) has been successfully expressed in several expression systems such as prokaryotic organisms, yeast, mammalian cells, insect cells and plants, etc. [3–7]. Among these, HBV vaccines derived from yeast cells (*Saccharomyces cerevisiae* or *Pichia pastoris*) and Chinese hamster ovary (CHO) were commercially available [3]. However, the recovery of purification for both CHO- and yeast-derived HBsAg is less than 20%, which will increase the manufactory cost of this vaccine [8,9].

Hydrophobic interaction chromatography (HIC) technique is being widely used both in the industrial and laboratory scale for protein purification [10–12]. Several purification processes have been reported to capture r-HBsAg by HIC from either yeast or CHO cultures [8,13]. It was reported that two commercially available absorbents, Butyl Sepharose 6B and Butyl-S Sepharose 6FF from GE Healthcare were used to purify HBsAg from *S. cerevisiae* yeast cell [13] and CHO cells, respectively [8]. Although the higher rHBsAg expression obtained from *Hansenula polymorpha* yeast cell (Han-HBsAg) made it low cost when applied to produce HBsAg vaccine, it was reported that molecular characterization of Han-HBsAg was different from CHO-HBsAg [14]. No detailed investigation

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about the purification of Hans-HBsAg by HIC has been reported so far. This paper explored the alternative of HIC absorbent, which could be successfully applied to separate Han-HBsAg in cost effective. Meanwhile, to maximize the recovery and the purity of the final product by the integrated chromatographic purification process, the operating condition of HIC was further optimized.

2. Materials and methods

2.1. Materials and chromatographic equipments

All chemicals were analytical grade reagents. The chromatography systems were ÄKTA explorer 100 and Bio-Pilot (GE Healthcare, Uppsala, Sweden). The columns were purchased from Jinhua, Shanghai, China. All solutions were prepared using Mill-Q grade water (Millipore, USA).

2.2. Pretreatment of H. polymorpha cell disruption supernatant (CDS)

The methylotrophic *H. polymorpha* yeast cell, containing the coding region of S protein of HBsAg, was used to express rHBsAg particles. The expression level of HBsAg was about 500–700 μg HBsAg/ml cell disruption supernatant. After fermentation, the cells were harvested by centrifugation followed by washing and cell disruption by a high-pressure homogenizer. The cell debris was removed by centrifugation (4000 × *g*, 4 °C, 30 min), repeated twice. The crude Hans-HBsAg was captured from 600 ml supernatants by anion exchange chromatography with a stepwise elution and was ready for the further use in HIC.

2.3. Synthesis and evaluation of hydrophobic interaction chromatography absorbent

Based on the Maisson's method [15], HIC media was prepared by two-step process, matrix activation and ligand cross-linking. The scheme of the synthesis was shown in Fig. 1. The dehydrated QZT beads (Zhengguang Gel Company, China) were applied as the matrix. The ligand density of Butyl group was controlled by the concentration of reaction concentration of butanethiol (from 15 to 35 μmol butyl groups/(g wet gel), Table 1). A serial of HIC absorbents (Nos. 1–5) were obtained with different ligand densities detected by ICP method [15]. The absorbents were packed into a chromatographic column (50 mm × 26 mm i.d.) and then washed by 8% ammonium sulfate, pH 7.0, with a flow rate of 5.0 ml/min. The absorbent with the best performance was screened out, named as Butyl-S QZT, for the further use.

2.4. Determination of physical and chemical properties of Butyl-S QZT

2.4.1. Electron microscopy

The particle size was detected by electron microscopy following the method described in reference [16]. Briefly, the absorbent, Butyl-S QZT, was applied to copper grid, stained by 2% uranyl acetate, and then detected by scanning electron microscope (SEM, JSM-6700F, JEOL, Japan).

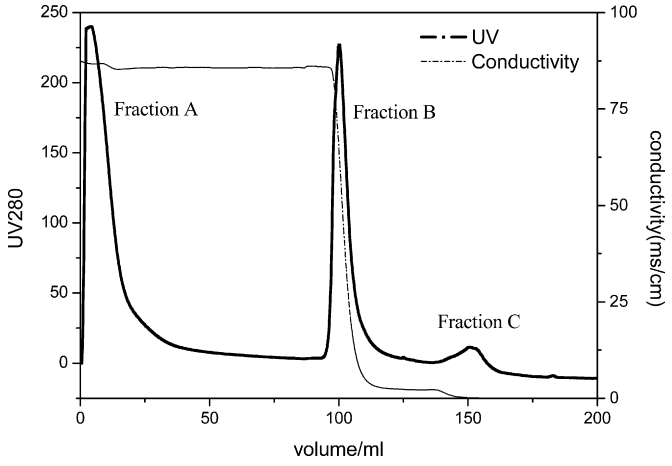

Fig. 1. Profile of HIC. Column: 50 mm × 26 mm i.d., CV = 27 ml; buffer: PB; flow: 5.0 ml/min; UV_{280}: (—); conductivity: (- - -). Fraction A: unbound impurities; fraction B: elution of Hans-HBsAg by 20 mM PB; fraction C: tightly bound impurities eluted by 30% isopropanol.

2.4.2. Rigidity and fluidity

The absorbent was packed into the chromatographic column (50 mm × 16 mm i.d.). Under the flow rate range of 0–20 ml/min, the corresponding backpressure between the inlet and outlet of the column was detected by manometer, respectively.

2.4.3. Chemical stability

The absorbent was submerged individually into the harsh solution of 1.0 M HCl, 1.0 M NaCl, 20% ethanol, 30% isopropanol, 15% ammonium sulfate, 50 mM PB, 6 M GuHCl and 8 M urea, or at the temperature of 4 °C. After the treatment for 50 days, the absorbents were washed by water and then the ligand density was detected with ICP method [15].

2.5. Optimization of hydrophobic interaction chromatography operation

The pretreated Hans-HBsAg from the CDS was purified by HIC column (50 mm × 16 mm i.d., CV = 10 ml) packed with synthesized absorbent of Butyl-S QZT. The ammonium sulphate powder was added to CDS and loading buffer until reach the desired conductivity. The pH value was regulated by 20 mM PB and 1.0 M HCl or NaOH, and then the resulted CDS was loaded onto the HIC column which has been pre-equilibrated by buffer A (20 mM sodium phosphate, containing desired concentration of ammonium sulphate). HBsAg fraction was eluted by 100% buffer B (20 mM sodium phosphate) and the impurity protein was washed out by 30% (v/v) isopropanol in sequence. The

Table 1
Separation performance of HIC absorbents with different ligand density

Absorbent number	Butyl density (μmol/(g wet gel))	Recovery of HBsAg (%)			PF[a] ($n = 3$)
		Fraction A ($n = 3$)	Fraction B ($n = 3$)	Fraction C ($n = 3$)	
1	15	6.6 ± 0.3	82.8 ± 1.5	1.9 ± 0.2	3.3 ± 0.3
2	20	5.2 ± 0.1	86.9 ± 5.7	2.6 ± 0.5	3.5 ± 0.1
3	25	3.9 ± 0.2	92.1 ± 3.6	4.5 ± 2.0	3.8 ± 0.2
4	30	2.4 ± 0.3	84.3 ± 4.7	6.3 ± 1.7	3.1 ± 0.1
5	35	0 ± 0.0	82.5 ± 2.8	8.4 ± 0.8	2.7 ± 0.3

[a] Purification-fold was defined as the ratio of specific bioactivity of HBsAg after and before purification. The specific bioactivity of HBsAg is defined as the ratio of HBsAg based on ELISA assay to the total protein based on Bradford assay.