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Journal of Chromatography A, 1095 (2005) 119–125
JOURNAL OF CHROMATOGRAPHY A
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Ion-exchange chromatography of hepatitis B virus surface antigen from a recombinant Chinese hamster ovary cell line

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Received 20 April 2005; received in revised form 13 July 2005; accepted 2 August 2005
Available online 31 August 2005

Abstract

About 10% of the Chinese population are chronic carriers of hepatitis B virus (HBV). Thus, the development of a highly efficient process for the preparation of a vaccine based on a recombinant hepatitis B surface antigen (HBsAg) is very important to the Chinese national immunization program. To this end, the ion exchange chromatography recovery of CHO-HBsAg from a recombinant Chinese hamster ovary cell line was shown to increase from about 55 to 80% by the addition of 1% poly(ethylene glycol) (PEG 10,000) to the mobile phase. Furthermore, based on analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), the intact glycoprotein form of CHO-HBsAg was completely preserved by the addition of PEG. In the absence of PEG the glycoprotein form of CHO-HBsAg was also spread out into the high salt elution fraction. High-performance size-exclusion chromatography with on-line multiangle-laser-light scattering (HPSEC-MALLS) analysis was performed to monitor the status of the CHO-HBsAg aggregate structure assembly, particle size and molecular weight distribution after each purification step, and the results showed further that the presence of PEG facilitated the separation and recovery of intact glycoprotein form of CHO-HBsAg and promoted their assembly to proper virus-like particles, which are both important features and prerequisites of their immunogenicity.

Keywords: Hepatitis B surface antigen; Ion-exchange chromatography; Poly(ethylene glycol); Retention behaviour

1. Introduction

Hepatitis B is a serious illness caused by a virus that attacks the liver. The virus, called hepatitis B virus (HBV), can cause lifelong infection, cirrhosis of the liver, liver cancer, and death [1,2]. Hepatitis B virus is one of the world's most widespread infectious agents. Immunization with hepatitis B vaccine is the most effective means of preventing HBV infection and its consequences. More than one billion doses are administered annually [3–5]. The first generation vaccine against HBV to be applied to humans was purified from the plasma of chronic carriers [6]. However, this procedure was dissuaded by WHO because of plasma limitation and the risk of HIV infection [7]. By the introduction of the DNA recombinant technique it became possible to express the S gene of the hepatitis B surface antigen (HBsAg) in a number of different hosts such as procaryotic organisms [8], yeast [9,10], mammalian cells [11,12], insect cells following infection with recombinant baculoviruses [13] and plants [14]. Comparative studies have shown that glycosylated forms of CHO-HBsAg which produced by Chinese hamster ovary mammalian cell lines give a more favourable cellular immune response than y-HBsAg which obtained from yeast [15,16].

Process scale purification of CHO-HBsAg from transformed mammalian cell line cultures has been achieved by ultracentrifugation [17], by chromatography and ultracentrifugation [18,19] or by a combination of chromatography methods [20]. A process based on the integration of several chromatography steps has the advantage of being easily automated and scaleable. However, the relatively low recovery obtained when ion-exchange chromatog-

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doi:10.1016/j.chroma.2005.08.006

raphy (IEC) was used for large-scale production of CHO-HBsAg was not properly addressed. The low expression level obtained in mammalian expression systems and the relatively low total purification process recovery (less 20% from CHO cell culture supernatants), had led to a high vaccine production cost and can be regarded the main challenge in comparison with y-HBsAg.

Ion-exchange chromatography (IEC) is an efficient protein purification method, widely used not only in the laboratory but also in production processes [21–23]. It is known that the protein adsorption capacity of many ion-exchange media decreases drastically when the molecular mass exceeds 100 kDa [22,24]. IEC can also be used for the separation of other large biomolecules such as plasmid DNA and also particles such as viruses. Virus separation by chromatography has become more important recently with the advent of gene therapy [25] and their use for the manufacturing of vaccines [26].

Poly(ethylene glycol) (PEG) has been widely used in biochemical engineering, such as protein precipitation, two-aqueous-phase extraction, protein chemical modification, etc. The addition of PEG reportedly increases protein partition coefficients in size-exclusion chromatography (SEC), IEC and protein A affinity chromatography (AC) [27–29]. Study on effects of adding PEG in SEC displayed the partition coefficient increases in proportion to the PEG concentration, with large molecular weight proteins generally being more affected than low molecular weight proteins. Timasheff studied the thermodynamic property of protein solution in the presence of PEG and proposed the theory of preferential exclusion [30]. The preferential exclusion of PEG from the protein surface creates an energetically favourable sharing of the co-solvent exclusion (hydration) shells surrounding the protein molecules as well as the chromatography media, hence increases the partition coefficients [31,32]. Gagnon proved that the addition of PEG into the mobile phase during chromatographic process altered the adsorption and retention behaviour of proteins in IEC and increased the selectivity [33]. Previous work has demonstrated that the purification of recombinant human tumour necrosis factor by IEC could be improved by the addition of PEG to the equilibration and elution buffers [28]. In the present study, the effect of PEG addition on peak broadening and adsorption behaviour of the 22 nm diameter CHO-HBsAg during IEC has been investigated with the purpose of improving the purification level as well as the recovery.

2. Experimental

2.1. Materials and chromatographic equipments

All chemicals were analytical grade reagents. Purified CHO-HBsAg reference used as standard was a kindly gift from North China Pharmaceutical Group Corporation (NCPC, Shijiazhuang, China). The chromatography systems used were ÄKTAexplorer and Bio-Pilot from GE Healthcare, Uppsala, Sweden. Columns with 26 and 55 mm I.D. for laboratory and pilot scale operations were from Jinhua, Shanghai, China. All the gels for chromatography were from GE Healthcare, Uppsala, Sweden. All solutions were made using Mill-Q grade water (Millipore, USA).

2.2. Harvest and concentration of cell culture supernatant (CCS)

The CHO-HBsAg particles were produced by Chinese hamster ovary (CHO) cells, containing the coding region for the S protein of HBsAg. CHO cell cultures producing CHO-HBsAg were grown in roller bottles containing Dubelco's Modified Eagle's Medium (DMEM) and 5% (v/v) of calf serum. Every 2 days after the cells reached confluence, the cell culture supernatants (CCS) were harvested and the same volume new medium was added to each roller bottle. The collected CCS was centrifuged ($4000 \times g$, 4 °C, 30 min) to remove cell debris and concentrated by ultrafiltration or precipitated by 45% of saturated ammonium sulphate, and then stored in the presence of 0.05% Merthiolate at 4 °C until further use.

2.3. Purification of CHO-HBsAg

The rHBsAg was purified as described previously [20] with some adjustments. To the CCS (3000 ml sample containing, ca. 6.0 g protein) was added 8% (w/v) ammonium sulphate followed by loading onto a HIC column (Butyl-S Sepharose 6 FF, 70 mm × 55 mm I.D., CV = 180 ml) pre-equilibrated in buffer A [20 mM sodium phosphate, pH 7.0, containing 8% ammonium sulphate, w/v]. Elution was achieved with 100% buffer B (20 mM sodium phosphate, pH 7.0) and 30% (v/v) isopropanol in sequence. The flow rate was 58 cm/h controlled by ÄKTAexplorer and the detection wavelength was set at 280 and 260 nm.

The eluted fractions containing the CHO-HBsAg, as determined by enzyme-linked immunosorbent assay (ELISA), were pooled and desalted by hollow-fibre ultrafiltration using a 100 kDa molecular weight cut-off membrane (Millipore, USA). Finally, the desalted sample was loaded onto an IEC column (DEAE Sepharose FF column, 75 mm × 26 mm I.D., CV = 40 ml) pre-equilibrated in buffer B (20 mM sodium phosphate pH 7.0) and eluted step-wise with the 12 and 100% buffer C (20 mM sodium phosphate pH 7.0, added 1.0 M NaCl) in sequence. The flow rate was 50 cm/h. In the IEC procedure of addition of PEG, PEG with molecular weights 600, 2000, 6000 and 10,000, respectively, at different concentrations (0, 1, 4, or, even up to 16%), were added to both buffers B and C during the ion-exchange chromatography.

2.4. Change the retention time by addition of PEG

4.0 mg standard CHO-HBsAg from NCPC and 8.0 mg BSA as applied as model proteins and loaded in the DEAE-Sepharose FF column (56 mm × 26 mm, CV = 30 ml, with flow rate of 50 cm/h). Sixteen percent PEG 600 or 1% PEG 10000 were added to both buffers B and C during the ion-exchange chromatography. The retention time was recorded at the peak point when the absorbance reached the maximum. Here the absence of PEG was taken as the control.

2.5. Assay for biological activity of CHO-HBsAg

The CHO-HBsAg was quantitatively assayed by a commercially available HBsAg ELISA kit from Kehua, Shanghai, China. Briefly, 50 μl of diluted sample containing HBsAg and 50 μl poly-HBs anibodies-HPR were added in sequence in the 96-well microplate pre-coated with mono-HBs antibody. The microplate was then incubated at 37 °C for 30 min, washed three times before adding 100 μl substrates and further incubated at 37 °C for 15 min. The reaction was then stopped and the absorbance was measured by microplate reader 550 (Bio-Rad) by 450 nm as measurement wavelength and 630 nm as the reference wavelength. The amount of CHO-HBsAg was calibrated against a reference standard obtained from NCPC.

2.6. Determination of protein concentration

Protein concentration was determined according to Bradford's method with slight modification. Briefly, 96-wells microplate was applied as the reaction well and the absorbance was measured by microplate reader 550 (Bio-Rad) controlled by the microplate manager 4.0PC data analysis software (Bio-Rad). The amount of protein was calibrated against bovine serum albumin (BSA) as reference standard. Multi-samples were measured in the same microplate and the results were reliable (the relative coefficient (R) of calibration curve of reference protein was above 0.99).

2.7. SDS-PAGE

Samples containing about 0.5–2 μg protein were heated to 100 °C for 10 min in sample buffer containing 2% 2-mercaptoethanol, and applied to 15% homogeneous polyacrylamide gels containing SDS as described by Laemmli [34]. Afterwards, the gels were stained by silver.

2.8. HPSEC-RI-MALLS

The HPSEC-MALLS system used was composed of a DAWN EOS multi-angle laser light scattering detector (MALLS, Wyatt Technology Inc., Santa Barbara, USA), an Optilab DSP refractive index monitor (RI, Wyatt Technology, Santa Barbara, USA) and an Agilent 1100 HPLC system with a SEC column.

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 columns, TSK G3000 SW (300 mm × 7.5 mm, I.D.) and TSK G5000 PW (300 mm × 7.5 mm, I.D.) with TSK GPW guard column (75 mm × 7.5 mm, I.D.), were from Tosohaas, Stuttgart, Germany. Elution buffer was 100 mM sodium phosphate pH 6.8 containing 100 mM sodium sulphate with the flow rate of 0.5 ml/min. The injection volume was 100 μl at a protein concentration of 100–500 mg/l.

After HPSEC, each eluted fraction was measured simultaneously at 18 angles by MALLS detection and by RI detection. The MALLS detector was fitted with a K10 cell and a He–Ne laser (λ = 690 nm). The Astra v-4.9 software was used for calculating the molecular weight (M_w) and the radius of gyration (r_g) by extrapolating the scattered light to zero-angle at each slice. The MALLS and RI detectors were calibrated using filtered toluene and NaCl, respectively. The MALLS instrument was normalized using standard monomer BSA (Sigma, USA) as reference.

3. Results and discussion

3.1. Optimization of the CHO-HBsAg recovery in the ion-exchange chromatography step

In a previous report on the chromatographic purification of CHO-HBsAg from CHO cell culture supernatants [20], composed of a combination of hydrophobic interaction chromatography (HIC), IEC and SEC, the total recovery was in the range of 30–40%. The major loss (60–70%) was in the IEC step on DEAE-Sepharose FF. So, the aim of the present study is to optimize the IEC step by introducing a stabilizing environment during the chromatography.

The purification efficiency of HIC step was evaluated by measuring the compositions of the CHO cell culture supernatant (CCS) and the eluted fraction from the HIC by SEC-HPLC and is shown in Fig. 1 (Fig. 1A: before HIC, Fig. 1B: after HIC). As can be seen, HIC was an efficient step by removing around 99% of the culture media protein (primarily bovine serum albumin, Fig. 1A and B). The CHO-HBsAg containing fractions from HIC were pooled and desalted by hollow-fibre ultrafiltration membrane before application to the IEC column.

Fig. 2 shows the result of the step-wise elution of the CHO-HBsAg from the DEAE Sepharose FF column. All the applied protein was bound and then displaced as two fractions (fractions A and B) by step-wise elution at 0.12 and 1.0 M NaCl in

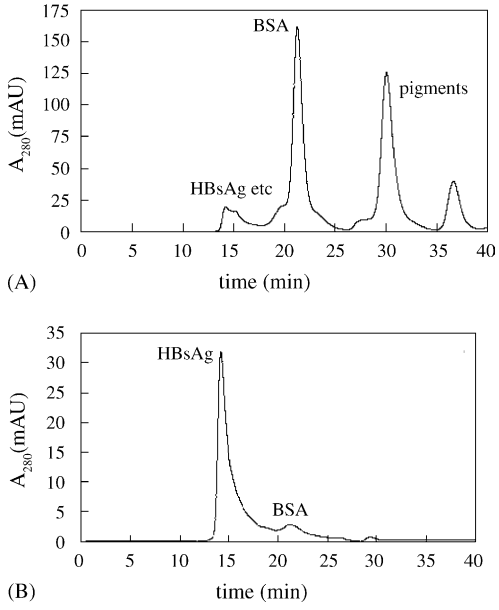

Fig. 1. HPSEC assay of CCS (A) and eluted fraction from HIC (B). *Conditions*: TSK G3000 SW (300 mm × 7.5 mm); eluted by PBS (0.1 M PB added with 0.1 M Na_2SO_4, pH 6.8) at room temperature (20–25 °C) with 0.5 ml/min flow rate; 100 μl of injection volume; the applied total protein concentration for CCS and HIC active fraction was, ca. 2.0 and 0.4 mg/ml, respectively.

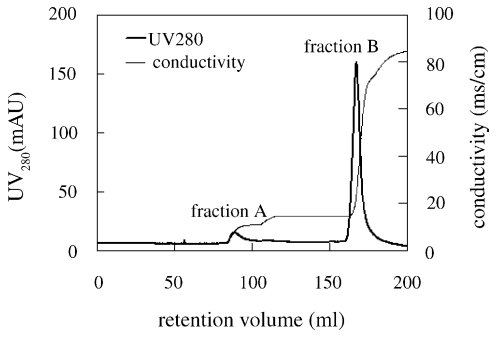

Fig. 2. Elution profile in ion-exchange chromatography. *Conditions*: DEAE-Sepharose FF column (75 mm × 26 mm I.D., CV = 40 ml) was applied and 30 ml of desalted HIC active fraction (protein concentration: 0.3 mg/ml) was applied to ÄKTAexplorer 100 and eluted by step-wise elution [12% followed by 100% buffer B (20 mM PB added 1.0 M NaCl, pH 7.0)].

sequence. No protein, including BSA and CHO-HBsAg, could be detected in the break-through fraction, whereas fraction A contained a small amount of less tightly bound protein and fraction B the more tightly bound protein. Five repetitive IEC batch experiments were performed with the ÄKTAexplorer using identical conditions. Assay for CHO-HBsAg by ELISA and protein concentration by modified Bradford assay were performed for the estimation of recovery and specific activity. Data are shown in Table 1. It is obvious that the CHO-HBsAg recoveries in the IEC step were not very reproducible with about 48–70% of the applied CHO-HBsAg associated with the less tightly bound fraction A. Approximately 4–10% of the applied total protein co-eluted with CHO-HBsAg in fraction A with a purification factor around 7–18. Fraction A was regarded as the major active fraction and was applied in the next purification step. Based on the result of the CHO-HBsAg and protein concentration assays, most of the applied protein and other relatively high-molecular weight protein, such as IgGs seem to be eluted in fraction B. In addition, 27–50% of the applied CHO-HBsAg was eluted in fraction B. The relatively low recovery of CHO-HBsAg in fraction A and the low degree of reproducibility in the IEC step, even under identical conditions, was the most problematic issue in the CHO-HBsAg purification process.

3.2. Retention behaviour of CHO-HBsAg in IEC in the presence of PEG

The low CHO-HBsAg recovery in the IEC step most likely is the result of sub-optimal retention conditions for the very large CHO-HBsAg virus-like particles on the DEAE-Sepharose FF column. The CHO-HBsAg particle has a diameter of 22 nm with a reported total molecular weight of more than 2000 kDa, and it is composed of about 100 subunits stabilized by multiple disulfide bonds [35]. This fact makes the CHO-HBsAg adsorption-desorption process very complex resulting in low elution recoveries. This is why it is important to improve the conditions in order to increase the recovery of active CHO-HBsAg in the IEC step. Reportedly, poly(ethylene glycol) (PEG) functions as a purification chaperone in IEC [28]. The addition of PEG to the mobile phase has also been shown to affect the IEC protein retention behaviour [24]. In the present work the addition of PEG is shown to change the retention time (RT) for both pure CHO-HBsAg and BSA as model proteins. To this end, the DEAE Sepharose FF column was equilibrated in binding buffer and the protein desorbed using desorption buffer both containing varying concentrations of PEG 600 (0, 4, 8 and 16%,) and PEG 10,000 (0.5, 1.0 and 4%), respectively. The result is shown in Fig. 3. Both CHO-HBsAg and BSA were delayed on the col-

Table 1
Evaluation of IEC[a] by step-wise gradient NaCl elution

Series number	Total HBsAg by Elisa (μg)	Total protein (μg)	HBsAg recovery (%)	Specific activity[b] (μg/μg)	Purification fold[c]
HIC[d]-sample	416.2	10804.28	100.0	0.039	1
No. 1					
Fraction A	257.2	566.72	61.8	0.454	11.8
Fraction B	132.4	8445.42	31.8	0.016	0.4
No. 2					
Fraction A	240.2	537.85	55.3	0.697	18.1
Fraction B	137.2	11557.75	33	0.012	0.3
No. 3					
Fraction A	290.8	904.85	69.9	0.321	8.3
Fraction B	128.2	9425.18	30.8	0.014	0.4
No. 4					
Fraction A	257.5	1055.70	61.9	0.244	6.3
Fraction B	110.1	13718.46	26.4	0.008	0.2
No. 5					
Fraction A	203.7	460.99	48.9	0.442	11.5
Fraction B	274.1	10783.83	65.9	0.025	0.7

[a] After being desalted by UF(100KD MWCO hollow fiber membrane), HIC samples contained, ca. 10.0 mg protein was applied in IEC column (DEAE Sepharose FF, 75 mm × 26 mm, I.D., CV = 40 ml at the flow rate of 50 cm/h).
[b] Specific activity was represented by the ratio of total amount of HBsAg measured by ELISA vs. total protein measure by Bradford's method.
[c] Purification fold was defined as the ratio of specific activity of fraction A or B after IEC to the specific activity before IEC.
[d] 2800–3250 ml CCS (ca. 6.0 g protein) was applied in HIC column (Butyl-S Sepharose 6 FF, 7.0 cm × 5.5 cm, I.D., CV = 166 ml at the flow rate of 58 cm/h).

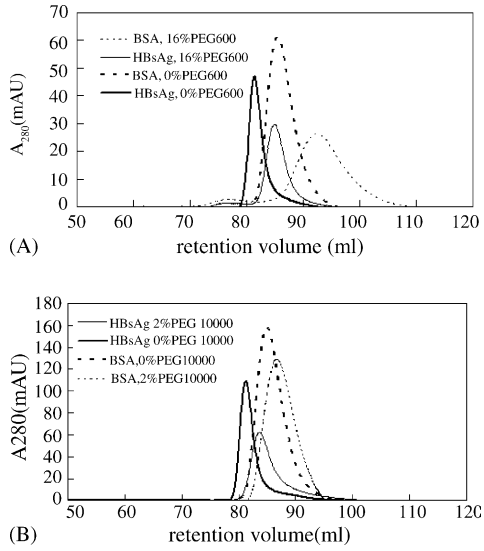

Fig. 3. Retention behavior of pure CHO-HBsAg and BSA in IEC. 4.0 mg of HBSAg and 8.0 mg of BSA was applied respectively on DEAE-Sepharose FF column (56 mm × 26 mm, CV = 30 ml) by 50 cm/h of flow rate. PEG 600 (A) and PEG 10,000 (B) were added in the mobile phase of IEC. CHO-HBsAg (solid line) and BSA (dot line) were eluted by the same linear gradient (20 mM PB with 0–1.0 M NaCl, 15 CV).

umn in the presence of PEG. The magnitude of the delay is proportional to both the molecular weight and the concentration of the added PEG. The retention time difference between CHO-HBsAg and BSA under identical conditions was defined as ΔRT. The calculated ΔRT as a function of PEG concentration and the molecular weight of PEG is shown in Fig. 4.

For both CHO-HBsAg and BSA the retention time increases by PEG addition, with fair correlation also with protein molecular weight. A maximum ΔRT of 1.43 min was obtained at 16% PEG 600, whereas a maximum ΔRT of 1.02 min was obtained at 1% PEG 10,000. This can be interpreted as if the addition of PEG with low molecular weight at high concentration to the mobile phase creates increased selectivity, whereas the addition of PEG with high molecular weight causes increased peak width.

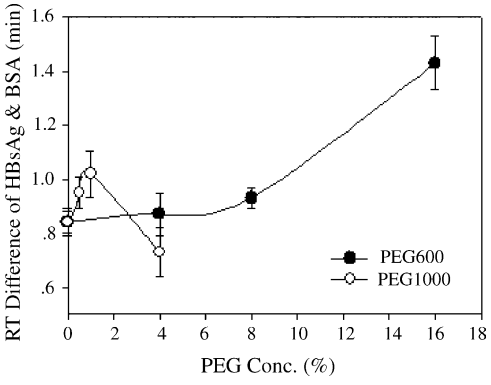

Fig. 4. ΔRT profile of purified CHO-HBsAg and BSA in IEC as a function of PEG concentration. PEG600 (close circle) and PEG 10,000 (open circle).

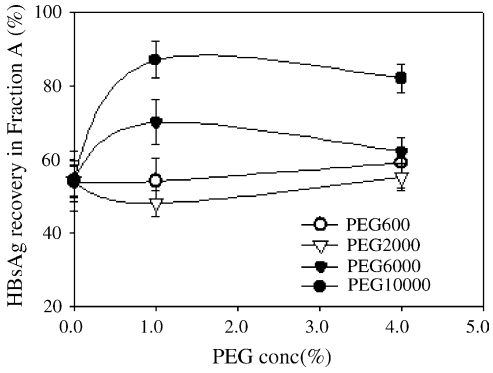

Fig. 5. Effect of PEG to CHO-HBsAg recovery in fraction A in IEC. 5 ml (concentration: 5 mg/l) of desalted HIC active fractions was loaded in DEAE-Sepharose FF column (56 mm × 26 mm, CV = 30 ml) at the flow rate of 50 cm/h.

3.3. Improvement of CHO-HBsAg recovery in IEC by the addition of PEG

PEG with molecular weights 600, 2000, 6000 and 10,000, respectively, at concentrations of 0, 1 and 4%, were added to the mobile phase during the separation of CHO-HBsAg by IEC after the HIC step. Taking into consideration the relatively high toxicity of low molecular weight PEG [36], the highest concentration of PEG 600 (16%) was not investigated in spite of an expected increased selectivity. Linear gradient elution was first applied followed by step-wise elution using the conditions that gave the highest selectivity. Fig. 5 shows the CHO-HBsAg recoveries obtained in fraction A at different PEG additions. High molecular weight PEG (6000 and 10,000) increased the CHO-HBsAg recovery from DEAE Sepharose FF column, with PEG 10,000 somewhat better than PEG 6000. Thus, the CHO-HBsAg recovery increased from 55% to around 80% by the addition of PEG 10,000, with small differences at 1 and 4% added polymer. Taking into consideration that the addition of PEG also increases the mobile phase viscosity, with concomitant effects such as reduced flow-rate and dynamic binding capacity, respectively, 1% PEG 10,000 was chosen for further investigations.

As shown in Fig. 5, the CHO-HBsAg recovery in fraction A was surprisingly not affected by low PEG molecular weight, whereas PEG 600 had a unique selectivity when separating pure CHO-HBsAg from BSA. With the knowledge that there is a risk with toxicity at high concentrations of low molecular weight PEG (PEG 600) and that it had no effect to the CHO-HBsAg recovery in IEC, the high molecular weigh (PEG 10,000) at low concentrations was chosen in IEC to change the retention behaviour and to improve the recovery of CHO-HBsAg.

Table 2 shows a comparison of CHO-HBsAg recovery and degree of purification in the presence or absence of 1% PEG 10,000. Step-wise elution using 0.12 M NaCl or 0.15 M NaCl was chosen for further investigation. In the absence of PEG an CHO-HBsAg recovery of 57 % and eight-fold purification were obtained by elution at 0.15 M NaCl comparing to around 41% recovery and 13-fold purification at 0.12 M NaCl. In the presence of 1% PEG 10,000, the CHO-HBsAg recovery at 0.15 M NaCl was 80% with and 11-fold purification.

Table 2
Comparison of HBsAg recovery and purification fold in the presence and absence of PEG

Elution gradient	HBsAg recovery (assay by Elisa %)[a]	Average purification fold[b]
12% buffer B	41.4 ± 4.2	13.6 ± 2.1
15% buffer B	57.3 ± 3.5	7.8 ± 1.3
15% buffer B + 1% PEG 10,000	74.1 ± 5.5	10.5 ± 2.5

[a] The average HBsAg recovery was calculated by tri-repeated experiments.
[b] Purification fold was calculated by the ratio of the specific activities after and before the operation.

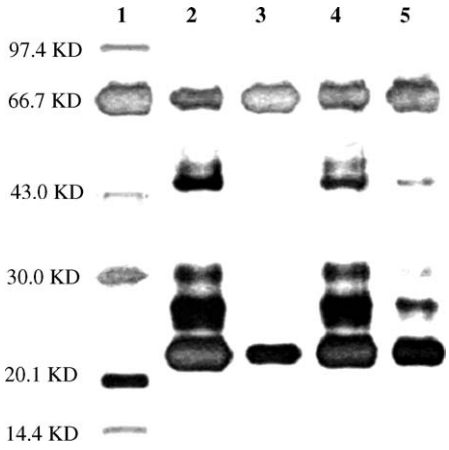

Fig. 6. Silvered stained SDS-PAGE of CHO-HBsAg elution in the absence and presence of PEG in IEC mobile phase. Around 5 μg of total protein was loaded separately. Lanes: 1, marker; 2, fraction A from IEC in the presence of PEG; 3, fraction B from IEC in the presence of PEG; 4, fraction A from IEC in the absence of PEG; 5, fraction B from IEC in the absence of PEG.

3.4. SDS-PAGE and HPSEC-MALLS analysis

SDS-PAGE analysis of the CHO-HBsAg fraction eluted in the absence and presence of PEG in the IEC mobile phase is shown in Fig. 6. No significant difference in the composition of fraction A was observed in the presence or absence of PEG (Fig. 6, lanes 2 and 4), protein bands identified as 24, 27 and 30 kDa proteins, as well as the CHO-HBsAg dimmer band (about 45 kDa), were detected in fraction A as well as the BSA band (about 67 kDa). However, in fraction B, in the absence of PEG, four bands (24, 27, 30 and 45 kDa) were observed (Fig. 6, lane 5), comparing only the 24 and 67 kDa (BSA) bands were detected (Fig. 6, lane 3) and no CHO-HBsAg glycoprotein forms (GP27 and GP30 kDa) were detected in fraction B when PEG was added to the mobile phase. The tentative interpretation of these results is that in the presence of PEG the CHO-HBsAg glycoprotein forms can be completely recovered in fraction A and that the non-glycoprotein form (24 kDa) is found only in fraction B.

No significant difference was detected in the laser light scattering signal when HPSEC-MALLS analysis was applied to fraction A proteins obtained in the absence and presence of PEG. (Fig. 7B and C). However, for the RI signal a significant elution time difference was observed. In the presence of PEG (Fig. 7C), the RI main peak had a retention time of 17.4 min, nearly the same retention time as that of purified CHO-HBsAg (Fig. 7A). However, for the protein obtained in the absence of PEG, the RI main peak was eluted with a retention time of 12.8 min (Fig. 7B). Since a high molecular weight protein was eluted earlier in a SEC column and the light scattering signal is proportional to the molar mass times mass concentration, the fraction A protein separated in the presence of PEG should represent the proper form of the CHO-HBsAg particle. This is supported by the fact that the CHO-HBsAg main peak eluting close to that of purified CHO-HBsAg has higher specific activity [37]

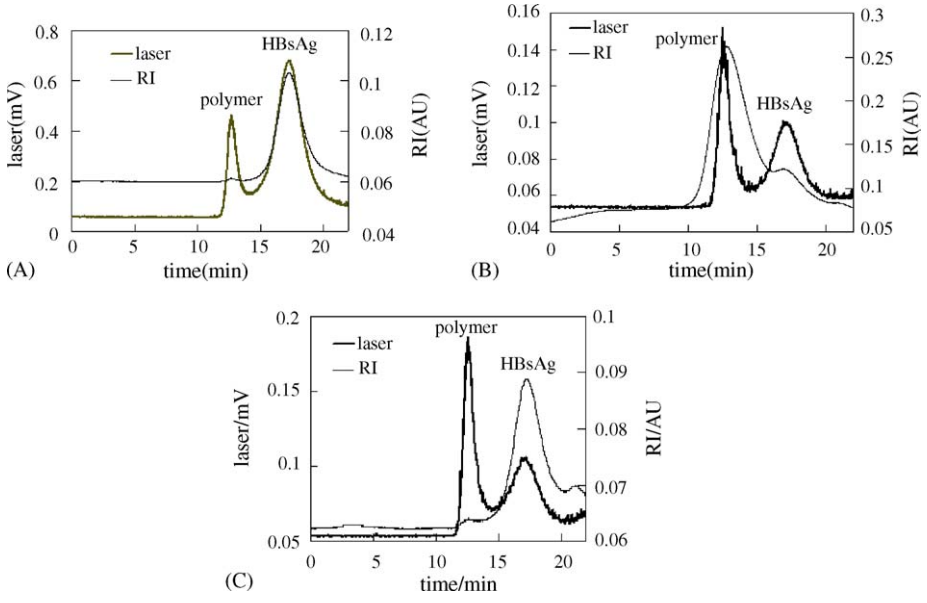

Fig. 7. HPSEC-MALLS analysis of CHO-HBsAg elution fraction from IEC in the absence (B) and presence (C) of PEG in IEC mobile phase compared with pure CHO-HBsAg (A). One hundred microliters samples (about 10 times concentrated fraction A from IEC by ultrafiltration using 10 kDa MWCO UF membrane) were loaded onto the TSK G5000 PW (300 mm × 7.5 mm, I.D.) with a flow of 0.5 ml/min in PBS.

than the other peaks. This is why we tentatively conclude that fraction A separated by IEC in the presence of PEG had higher activity and higher immunogenicity than that in the absence of PEG.

Theoretically, addition of PEG into mobile phase of IEC increased the free energy of solution system, therefore leading to a thermodynamically unfavourable environment for the protein to change its structure [30]. Thus, denaturation of HBsAg was minimized and multimeric structure of CHO-HBsAg was stabilized, the specific recovery of CHO-HBsAg was increased accordingly as well. There is a possibility of disadvantage in the present of PEG which PEG can enhance interaction with IEC gel by desolvating the ionic sites or driving hydrophobic interactions, but the disadvantage is neglectable in the presence of low concentration PEG.

4. Conclusions

The retention times of CHO-HBsAg and albumin was both increased as a consequence of the addition of PEG to the mobile phase in IEC. However, even if their isoelectric points are very close, i.e., 4.7 for BSA [38] and 4.8 for HBsAg [15], which may result in overlapping elution peaks in IEC, the addition of 1% PEG 10,000 to the mobile phase, caused both the recovery and the degree of purification of CHO-HBsAg to be improved significantly. The presence of PEG also facilitated the separation and recovery of intact glycoprotein form of CHO-HBsAg and promoted their assembly to proper virus like particles, which both are important features and prerequisites of their immunogenicity.

Acknowledgement

We are grateful for the support by Chinese “863”-Hi-Tech, 2002AA217031.

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