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Short communication

Polyethylene glycol increases purification and recovery, alters retention behavior in flow-through chromatography of hemoglobin

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Abstract

Flow-through chromatography was a method for purification of hemoglobin (Hb) from red cell lysate. The presence of 0–5% polyethylene glycol (PEG) increased the retention time of Hb peak from 15 min to 20 min in flow-through ion-exchange chromatography (IEC) but decreased the retention time from 88 min to 62 min in the hydrophobic interaction chromatography (HIC). However, the purification and the recovery were both increased. For IEC the recovery of hemoglobin increased from 75% to more than 90%, and the purified Hb showed single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and one peak in size-exclusion HPLC. For HIC, the recovery of hemoglobin was improved significantly from 20% to 85% and the removal of lipids was 100%. The bioactivity of hemoglobin was well preserved in these two chromatographic processes. The mechanism for the effect of PEG in these two flow-through chromatographic processes was discussed.

Keywords: Ion-exchange chromatography; Hemoglobin; Hydrophobic interaction chromatography; Polyethylene glycol; Purification

1. Introduction

Chromatographic process is a key step for purification of protein products. In most cases, the column is used to adsorb the product and to let the impurity pass-through. However, there are some circumstances where impurities can be adsorbed by the column and the product can pass through without being retained. The later can be termed as flow-through chromatography. From industrial point of view, "flow-through" has certain advantages. It is a fast process enabling the product stream to go directly to the next step without waiting for washing and elution of the product from the column. There is a minimal product loss due to the effect of adsorption and desorption. If the process is further optimized with respect to the chromatographic medium and condition, it is possible to achieve high purity and high recovery.

We therefore developed a flow-through mode of chromatography for purification of hemoglobin (Hb), which was used largely as a starting material for preparation of blood substitute [1,2]. The process involved two chromatographic steps. The first is an ion-exchange chromatography (IEC) to remove other proteins which are co-released with hemoglobin from the red cell. The second is a hydrophobic interaction chromatography (HIC) to remove lipids from the cell membrane. Both chromatographic columns were run in flow-through mode, i.e., Hb going through the column without being adsorbed, and impurities being retained. However, the product recovery was not satisfactory, especially for the HIC process, which was as less as 20%. Process optimization was carried out on the chromatographic media used, the flow conditions, and the solution environment. We found that the presence of polyethylene glycol (PEG) improved the product recovery and purification.

Several years ago, we reported that PEG could improve the purification of recombinant tumor necrosis factor in an

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IEC process [3]. In that case, the product was adsorbed by the ion-exchange medium and eluted with a buffer containing PEG. In a previous study, we found that the presence of 0.75% PEG600 could improve the recovery of hemoglobin in ion-exchange chromatography [4]. Now we report our new results on flow-through IEC and HIC in the presence of polyethylene glycol.

2. Experimental

2.1. Preparation of hemolysate

Methods of collection, treatment of bovine blood and breaking of erythrocyte were described in detail in a previous publication [4]. Hemolysate after cell lysis was pretreated with a 0.45 μm microfiltration membrane and a M_r 30 000 ultrafiltration membrane before the IEC and HIC processes. The equipments for microfiltration and ultrafiltration were Pellicon Cassettes and M12 system from Millipore Corp., USA. The final buffer used for Hb solution was 10 mmol/L phosphate solution, pH 6.8.

2.2. Ion-exchange chromatography

The chromatography column was a self-packed one with dimension of 100 mm × 16 mm i.d. The packing medium was Q Sepharose Big Beads from Amersham Bioscience, Sweden (now GE Healthcare). The chromatographic controller was AKTA Purifier 10, also from Amersham Bioscience.

Polyethylene glycol 4000 and 600 was purchased from Beijing Chemical Reagents Co. All other reagents used in the experiments were analytically pure.

Before feed loading, the IEC column was equilibrated with 10 mmol/L phosphate buffer at pH 6.8. In the case of PEG addition, the buffer was prepared with the required PEG concentration and the column was equilibrated with the buffer containing PEG. The pump speed was set at 1 mL/min, and the detection wavelength was set at 280 nm. The retention time was defined as the chromatographic time corresponding to the tip of the retention peak. The retention fraction was collected for analysis of Hb concentration, purity and oxygen carrying ability. The concentration of Hb in the feed was kept at 10 mg/mL, and the feed volume was 5 mL throughout the experiment.

2.3. Hydrophobic interaction chromatography

The chromatography column was a self-packed one with dimension of 130 mm × 16 mm i.d. The packing medium was Phenyl Sepharose 6FF (low sub) from Amersham Bioscience. The chromatographic controller was AKTA Purifier 10 from the same manufacturer.

Polyethylene glycol 4000 was the same as that used in ion-exchange chromatography. All other reagents used in the experiments were analytically pure.

For the HIC experiment, the column was equilibrated with 10 mmol/L phosphate buffer at pH 6.4 with the required PEG concentration. The flow rate was set at 0.5 mL/min, controlled by AKTA purifier 10. The wavelength for detection was set at 280 nm. The retention time was recorded at the peak point when the absorbance reached the maximum. The flow-through fraction was collected for analysis of Hb concentration, purity and oxygen carrying ability. The concentration of Hb in the feed was kept at 10 mg/mL, and the feed volume was 6 mL throughout the experiment.

2.4. IEC and HIC in sequence

The preparation of feed solution was the same as the above two sections except that the pH of the buffer was 6.8 for both IEC and HIC. The PEG concentration in IEC was 1% of PEG600. After the IEC, the PEG concentration was adjusted to 5% of PEG4000 before the HIC process. The flow rate was 0.5 mL/min for both IEC and HIC. The starting concentration of Hb in the feed was 10 mg/mL, and the feed volume was 5 mL.

2.5. Analytical methods

The concentration of protein was determined according to the Bradford method [5]. The activity of hemoglobin, in terms of P_{50} and Hill coefficient, was measured by Hemox Analyzer (TCS Scientific Corp., USA). Lipid was detected by high-performance thin-layer chromatography (HPTLC) [6]. The purity of hemoglobin was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion HPLC.

3. Results

3.1. PEG in ion-exchange chromatography of hemoglobin

Fig. 1 is a comparison of the flow-through peaks in the absence and in the presence of PEG for ion-exchange chromatography of Hb. The experiment was carried out in flow-through mode where hemoglobin flows through the column without being adsorbed. Impurity such as serum albumin which has a lower p*I* than Hb was captured by the column. Fig. 1 shows that the presence of 2% PEG4000 in the feed solution resulted in a delayed the retention of Hb.

Table 1 demonstrates the relationship between the retention time of Hb peak and the PEG concentration. As the concentration of PEG4000 in the feed increased from 0% to 5%, the retention time of Hb peak increased from 15 min to 20 min. However, PEG600 had little effected on the retention of Hb. The addition of both PEG4000 and PEG600 improved the IEC recovery of Hb. As shown in Table 1, the presence of 1%, 2% and 5% PEG in the feed increased the recovery from about 75% to more than 90%. Adding 1% PEG600 resulted

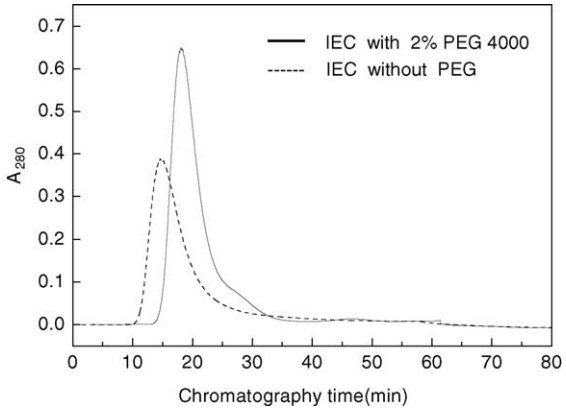

Fig. 1. Comparison of hemoglobin flow-through curves of ion-exchange chromatography (IEC) with and without 2% PEG4000 (Hb injected was 50 mg).

Table 1
Retention time and recovery of hemoglobin in ion-exchange chromatography with different concentration of PEG (Hb injection was 50 mg)

	Concentration of PEG (%)			
	0	1	2	5
PEG4000				
Retention time of hemoglobin (min)	14.9	17.8	18.3	20.2
Recovery (%)	76.2	92.5	91.8	90.8
PEG600				
Retention time of hemoglobin (min)	14.9	15.0	15.1	15.3
Recovery (%)	76.2	95.0	93.2	92.8

in the recovery of Hb as high as 95% and could be the best choice. The bioactivity of Hb was not changed significantly. Table 2 shows two parameter's values of Hb. The P_{50} denotes its oxygen affinity and the Hill coefficient is an indication of the cooperation of its tetrameric structure. Both before and after chromatography with addition of 1% PEG600, the P_{50} value and Hill coefficient of Hb were about the same, indicating that the activity of hemoglobin was well preserved during chromatography. Without addition of PEG, the P_{50} value of Hb decreased slightly.

3.2. PEG in hydrophobic interaction chromatography of hemoglobin

Hydrophobic interaction chromatography was used to remove lipids from the hemoglobin preparation. Similar to the IEC process, the HIC process was also performed in flow-through mode. Fig. 2 is a comparison of the flow-through peaks with and without 2% PEG4000. Contrarily to the IEC, the retention time of Hb was decreased in the presence of PEG. We also compared presence of PEG4000 and PEG10000 with the concentration from 0% to 5%. As shown in Table 3, the increase in concentration and molecular weight of PEG decreased the retention time of Hb.

Table 2
P_{50} and Hill coefficient of Hb in ion-exchange chromatography (IEC)

Parameters	P_{50} (mmHg)	Hill coefficient
Before IEC	25.2	2.42
Elution from IEC without PEG	23.8	2.36
Elution from HIC with 1%PEG600	24.8	2.40

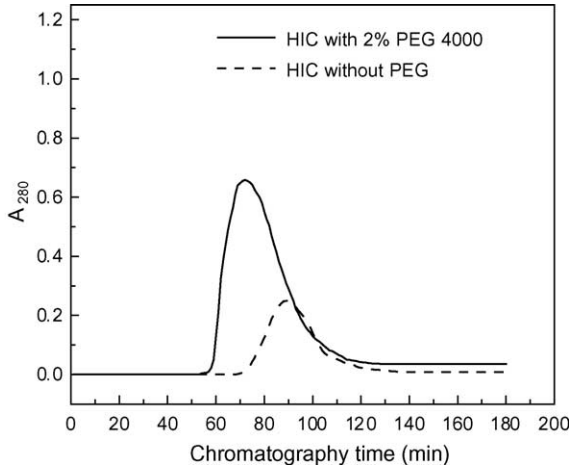

Fig. 2. Comparison of hemoglobin flow-through curves of hydrophobic interaction chromatography (HIC) with and without 2% PEG4000 (Hb injected was 60 mg).

Furthermore, there was a considerable difference in peak areas. As shown in Fig. 2, the flow-through peak area of HIC without addition of PEG is much smaller than that of HIC with 2% PEG4000, which reflects the recovery of Hb. Table 3 also shows the recovery of Hb in different HIC processes. For all the processes, no lipids were detected in the final product. However, the recovery of Hb varied considerably. The HIC without PEG addition had only 20% of recovery while 5% PEG4000 brought about 85% of recovery. The recovery was increased for PEG10000 addition, but was lower than that adding PEG4000. Therefore, adding 5% PEG4000 was the best for HIC.

Table 4 shows the P_{50} and Hill coefficient of HIC processes. Similar to IEC, the P_{50} of HIC with PEG4000 was even better than without, decreasing only slightly from the original. Its Hill coefficient was also better than the comparison.

Table 3
Retention time and recovery of hemoglobin in hydrophobic interaction chromatography with different concentration of PEG (Hb injection was 60 mg)

	Concentration of PEG (%)			
	0	1	2	5
PEG4000				
Retention time of hemoglobin (min)	88.5	80.6	73.2	62.1
Recovery (%)	20.0	53.2	66.9	85.0
PEG10000				
Retention time of hemoglobin (min)	88.5	76.8	65.2	58.3
Recovery (%)	20	63.6	61.8	53.5

Table 4
P_{50} and Hill coefficient of Hb in hydrophobic interaction chromatography (HIC)

Parameters	P_{50} (mmHg)	Hill coefficient
Before HIC	24.8	2.40
Elution from HIC without PEG	22.6	2.25
Elution from HIC with 5%PEG4000	23.8	2.34

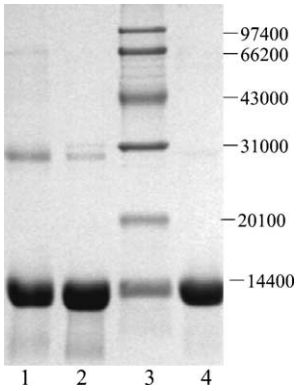

Fig. 3. SDS-PAGE of prepared hemoglobin by ion-exchange chromatography and hydrophobic interaction chromatography in the presence of PEG4000. A 4% stacking gel with a 15% running gel was used with 10 μl of hemoglobin solution on a minivertical slab gel apparatus (Bio-Rad, USA) at 200 V for approximately 1 h. A discontinuous Tris–glycine–SDS buffer system was used. After electrophoresis, the gel was stained with Coomassie blue R250. From left to right: lane 1, standard bovine hemoglobin (Sigma); lane 2, hemoglobin before chromatography; lane 3, marker; lane 4, purified hemoglobin after ion-exchange chromatography and hydrophobic interaction chromatography.

3.3. Flow-through of IEC and HIC in sequence

The two chromatographic processes, i.e., ion-exchange chromatography with 1% PEG600 and hydrophobic interaction chromatography with 5% PEG4000 were connected in series to purify hemoglobin from red cell lysate. The IEC is for removal of other proteins and the HIC is for clearance of lipids. Fig. 3 presents the purification result of the process. The purified Hb showed single band in SDS-PAGE. Only one peak was eluted from gel filtration HPLC as shown in Fig. 4. The total recovery of Hb was 77%. The final P_{50} and Hill coefficient were 23.8 mmHg and 2.34, respectively, which were comparable to other Hb purification reported in the literature [7].

4. Discussion

Flow-through chromatography can be used in such a situation that the target product is in high concentration and impurities present in a small amount. This is the case for hemoglobin purification where the red cell lysate contains mainly Hb. Other proteins such as albumin, carbonic anhydrase, superoxide dismutase (SOD) are less than 10% of the total protein content. By using ion-exchange chromatography, it is possible to adsorb other proteins like albumin, whose p*I* is 4.9, and to allow Hb, whose p*I* is 6.9, pass through the column when the pH of the fluid phase is controlled at 6.5. However, partial adsorption of Hb was found, which decreased the recovery of Hb in the flow-through fraction. The addition of PEG changed the solution environment, increasing the hydrophobicity of the system.

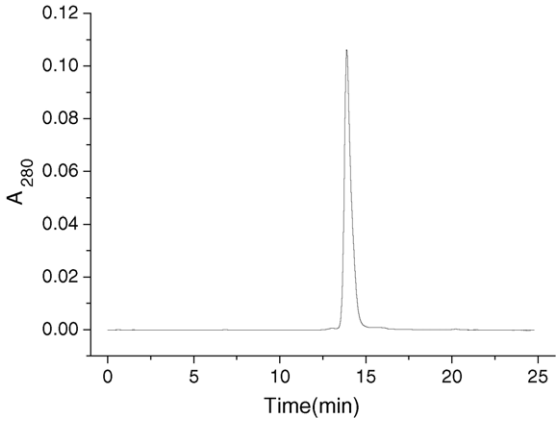

Fig. 4. Size-exclusion HPLC analysis of purified hemoglobin with ion-exchange chromatography and hydrophobic interaction chromatography. It was conducted with Agilent 1100, USA on TSK 3000^{SW} column at 280 nm. The mobile phase was 100 mM phosphate buffer containing 0.1 mol/L Na_2SO_4, pH 6.4, and the flow rate was 0.5 mL/min. Hb injected was 20 μg.

Polyethylene glycol has been widely used in protein fractionation, crystallization, two-aqueous-phase extraction, and chemical modification. Effects of adding PEG in gel filtration chromatography (GFC) has been studied [8,9]. Timasheff [10] studied the thermodynamic property of protein solution in the presence of glycerol, PEG and other cosolvents. He proposed the mechanism of preferential exclusion in which the presence of PEG increased the free energy of the system, leading to a thermodynamically unfavorable environment for the protein to change its structure. Thus, denaturation of hemoglobin was minimized, and the recovery was increased.

Gagnon et al. [11] proved that the addition of PEG to the mobile phase altered the adsorption and retention behavior of proteins in ion-exchange chromatography and increase the selectivity. An explanation with cosolvent exclusion model was that PEG response was influenced by protein surface chemistry, to the extent that it determined hydratability. For the flow-through mode of IEC, the delayed retention was supposed for the weak interaction between Hb and the ion-exchange medium when the thermodynamic property of the system was altered in the presence of polyethylence glycol.

Lipid removal is very important in preparation of blood substitutes. Although the content of lipids can be minimized by careful hemolysis process, it is essential to develop a safe and repeatable process to get rid of all the lipids in the production. Flow-through HIC is an efficient way for lipid removal. However, the co-adsorption of Hb with lipids by the HIC medium is a serious problem. Here, we were successful

in suppressing the Hb adsorption with addition of PEG. The possible reason, apart from the preferential exclusion mechanism for stabilization of the protein structure, could be the hydrophobic interaction between PEG and the chromatographic medium. PEG is known to have certain hydrophobicity and would be adsorbed by the hydrophobic ligand of the medium. The effect could be the shielding of the interaction between Hb and the ligand. When PEG is present, Hb molecule moves faster in the HIC column due to minimal interaction with the medium. Its recovery was increased considerably. However, PEG10000 produced lower recovery than PEG4000 at increased concentration, possibly due to the effect of precipitation. PEG may precipitate protein when the molecular weight and the concentration are increased. The effect can also be explained by preferential exclusion model. Protein molecules tend to aggregate to minimize the surface area at high PEG concentration.

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