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Short communication

Purification of bovine hemoglobin via fast performance liquid chromatography

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Abstract

Bovine hemoglobin (bHb) was purified from bovine red blood cells (bRBCs) via anion exchange chromatography preceded by dialysis. This is a fast and effective way to obtain bHb from bRBCs using Q Sepharose XL, a strong anion exchange resin. This resin had double the binding capacity for bHb compared to three other anion exchange resins that were studied in this work. Methemoglobin levels remained below 2% with bHb concentrations between 0.7 and 1.7 mM. The high purity of bHb was confirmed via SDS-PAGE and size exclusion chromatography (SEC).

Keywords: Hemoglobin; Purification; Anion exchange chromatography; Oxygen carrier; Blood substitute

1. Introduction

The purification of hemoglobin (Hb) is a necessary first step in the synthesis of Hb-based oxygen carriers (HBOCs) that are being developed for application in transfusion medicine and tissue engineering [1,2]. There are many different types of HBOCs that are currently in development, including polymerized acellular Hbs [3–7], liposome encapsulated Hbs [8–10], polymersome encapsulated Hbs [11], and hydrogel-based O_2 carriers [12–14]. Since each of these HBOCs require purified stroma-free Hb as the starting material, it is essential to develop an efficient and streamlined purification method of Hb to ensure that the starting product is of high purity and high yield.

There have been many studies that have focused on the purification of Hb from red blood cells (RBCs). Initial studies focused on the use of centrifugation to first wash the RBCs and remove plasma proteins followed by the addition of a hypotonic buffer to extract Hb from the RBCs [15]. This was sometimes followed by the addition of toluene or another organic solvent to remove cell debris; however, it was found that toluene could remain within a hydrophobic pocket in Hb [16]. To avoid the use of organic solvents, some studies have focused on the use of microfiltration, ultrafiltration, and hemolysis [17] to purify Hb. However, excess cell debris tends to block the membrane pores in these methods, which ultimately compromises the integrity of the membrane. Another technique that has been utilized in bHb purification has been ion exchange chromatography. While there has been much success with conventional ion exchange resins, new resins are continually being manufactured and should be evaluated to determine if they can more effectively purify Hb.

Within this study, four anion exchange resins (Q Sepharose Fast Flow, Q Sepharose XL, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow) were purchased from GE Healthcare (Piscataway, NJ) to compare their binding affinity for bHb. Q Sepharose XL was found to have the highest binding affinity for bHb, which included DEAE Sepharose, the follow-up to DEAE Sephadex that was used by Riggs [16] and Dozy et al. [18] to purify bHb. Additionally, Q Sepharose XL's binding affinity for bHb was much higher than the 26.1 mg of bHb/mL resin that was measured for QMA-Accell by Christensen et al. [19]. Shorr et al. [20] utilized QMA-Spherosil to purify Hb via ion exchange chromatography. QMA-Spherosil does not capture bHb; instead, DNA and endotoxins bind to the resin while allowing the bHb product to freely elute [20]. In this case, there is concern that other proteins and lipids could also elute into the

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final bHb product. Additionally, this purification was performed at 3–8 °C and yielded methemoglobin (metHb) levels less than 3% compared to the purification described in this study which was performed at room temperature and yielded metHb levels less than 2%. Hb purification has also been performed with Q Sepharose Big Beads by Lu et al. [21]. However, this method was performed at a pH 6.8 [21], which is of concern since metHb forms rapidly at a pH less than 7 [15]. Additionally, the Hb was not absorbed by the resin, which increases the amount of lipid in the final bHb product.

This study describes the purification of bHb that entails the extraction of bHb from bRBCs with a hypotonic phosphate buffer solution, the removal of cell debris via filtration and dialysis, and the capture of bHb with Q Sepharose XL resin at a pH of 7.9 and at room temperature. The final product was determined to be pure by SDS-PAGE and size exclusion chromatography (SEC).

2. Materials and methods

2.1. Selection of resin

A HiTrap IEX Selection Kit was purchased from GE Healthcare (Piscataway, NJ) to evaluate the binding affinity of bHb to four 1 mL columns of anion exchange resins: Q Sepharose Fast Flow, Q Sepharose XL, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow. Each column was overloaded with bHb, before being equilibrated with start buffer (20 mM triethanolamine, pH: 7.9). The bHb was subsequently removed from the column with 1 M sodium chloride. The total mass of the bHb that was bound to each column was determined from the concentration of Hb and the total volume of fractions containing bHb. The concentration of bHb was determined via the cyanomethemoglobin technique (see below).

2.2. Scale up

Q Sepharose XL had the highest binding capacity for bHb, and was chosen as the anion exchange resin for scale-up studies. A XK50 column (I.D. = 5 cm) and 300 mL of Q Sepharose XL media stored in 20% ethanol were purchased from GE Healthcare in order to scale up the process and obtain the required yield of bHb per purification. The column was packed at a flow rate of 16.3 mL/min, and the final bed height was measured to be 9 cm for a total column volume of 177 mL. The column was loaded with bHb at 30% of its binding capacity and operated at flow rates no greater than 75% of the packing flow rate.

2.3. Initial purification

Sterile bRBCs in citrate buffer were purchased from Quad 5 (Ryegate, MT). bRBCs were washed three times with isotonic saline (0.9%, w/v) solution to remove acellular Hb, and any remaining plasma proteins in a centrifuge for 15 min at a speed of 4500 rpm at 4 °C. bHb was subsequently extracted with three equivalents of 15 mOsM phosphate buffer (pH 7.2) for 1 h in an ice–water bath. The extract was then passed through glass wool three times and qualitative filter paper to remove excess cell debris. The filtrate was placed into a dialysis bag with a molecular weight cut-off of 10 kDa and dialyzed overnight at 4 °C with start buffer (20 mM triethanolamine, pH 7.90) at a dialysis buffer to filtrate concentration of 35,000 to 1. This process removed small particles from the filtrate and placed the bHb solution into the proper start buffer.

2.4. FPLC purification

The FPLC system utilizing the scaled up column, which was packed with Q Sepharose XL resin, was equilibrated with 10 column volumes (CVs) of start buffer (20 mM triethanolamine, pH 7.9) at flow rates of 6, 9, and 12 mL/min. One hundred milliliters of 1 mM bHb solution in 20 start buffers was loaded onto the column where it was captured by the resin. A five CV linear gradient, consisting of equal volumes of start buffer and running buffer (20 mM triethanolamine + 0.5 M NaCl, pH 7.9) connected by a U-tube, was developed and run through the column at room temperature. The column was subsequently washed with five CVs of 1 M NaCl, and then stored with five CVs of 20% ethanol. All buffers were filtered through a 0.22 μm filter. Fractions were collected with a fraction collector, and the absorbance and conductivity of the samples were measured. The absorbance of each fraction was measured at a wavelength of 280 nm with a Synergy HT 96-well plate reader (Biotek; Winooski, VT) on Costar UV transparent flat bottom plates (Corning; Corning, NY). The fractions that constituted the peak of the bHb product were pooled together as the final purified product. The conductivity of the fractions was measured with a Cole-Palmer (Chicago, IL) conductivity meter.

2.5. Bovine hemoglobin and methemoglobin concentrations

The concentration of bHb was determined using the cyanomethemoglobin method [22]. In this method, Hb is converted into cyanomethemoglobin, which has a known ε of 11/mM/cm at 540 nm [23,24]. The ε of metHb at 630 nm is 3.7/mM/cm [25]. The absorbance of both cyanomethemoglobin and metHb were measured in triplicate for each bHb solution to determine the metHb level, the percent of bHb in the metHb state.

2.6. SDS-PAGE

The purity of the bHb was determined via gel electorphoresis in a Mini-PROTEAN 3 Cell (Bio-Rad; Hercules, CA). Samples were collected post-lysis, post-filter, post-dialysis, and from the two fractions closest to the peak of the bHb chromatogram at flow rates of 6, 9, and 12 mL/min. Additionally, for the 12 mL/min purification, two fractions that were not captured by the Q Sepharose XL resin and eluted prior to the bHb peak were run on the gel. The samples were run along with a prestained broad range molecular weight marker (Bio-Rad), which consisted of proteins having molecular weights between 7.1 and 209 kDa, in a Laemmli buffer system [26] with a 6% acrylamide stacking gel and a 16% acrylamide resolving gel. bHb

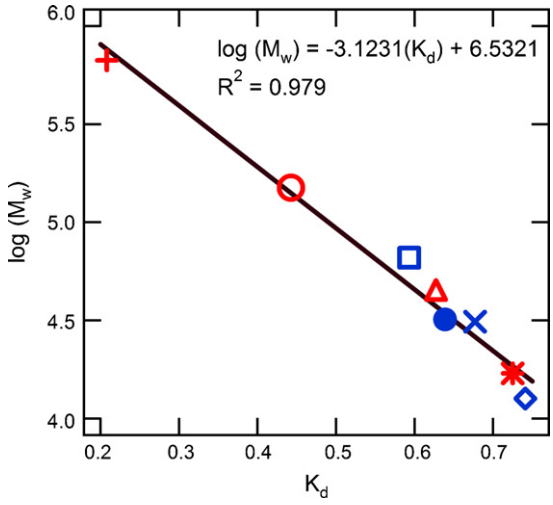

Fig. 1. The column was injected with the following protein standards: (◊) cytochrome *c* (12.3 kDa); (*) myoglobin (17 kDa); (×) superoxide dismutase (31.2 kDa); (△) ovalbumin (44 kDa); (□) BSA (66 kDa); (◯) IgA (150 kDa); and (+) Bovine thyroglobulin (670 kDa). bHb (●) was found to elute as an αβ dimer (32 kDa).

was found to migrate as a monomeric band due to the denaturant. The gels were then stained with EZBlue (Sigma) and imaged on a Kodak EDAS 290 (Rochester, NY) to determine relative band intensities.

2.7. Size exclusion chromatography

The purity of the bHb was also tested via SEC. The final product was dialyzed into 0.1 M phosphate buffer (pH 6.8). The purified bHb sample (800 μg) was injected into a Waters' HPLC (Milford, MA) system that was connected to a Phenomenex (Torrance, CA) SEC column (Biosep-SEC-S 3000; 60 cm × 7.8 mm). The column was run at a flow rate of 1 mL/min, and the chromatogram was evaluated for purity. The column was calibrated with proteins having molecular weights between 12 and 670 kDa to determine the size of the bHb species by plotting the log of the molecular weight versus K_d (Fig. 1). Proteins that are labeled red were injected into the SEC column by Phenomenex, while the blue proteins were injected into the SEC column in this study. The K_d is defined by Eq. (1) [27] where Vol_{unk} is the elution volume of the species, Vol_o is the void volume, and Vol_{inc} is the included elution volume. The Vol_o was determined with a high molecular marker to be 10.978 mL, while the Vol_{inc} was determined to be 22.91 mL via the injection of uridine (244.2 Da) into the column.

$$K_d = \frac{Vol_{unk} - Vol_o}{Vol_{inc} - Vol_o} \quad (1)$$

2.8. Column cleaning

After each run, the resin was cleaned with 1 M NaCl and then stored in 20% ethanol to prevent bacterial growth. Additionally, thorough cleanings of the resin were performed with 10 mM EDTA in a 0.1% TritonX solution to remove free heme groups that bound to the column. This step can be avoided with careful preparation in the washing and filtering stages of the bHb purification procedure.

3. Results and discussion

3.1. Selection of resin

Q Sepharose XL had more than twice the binding capacity for bHb compared to Q Sepharose Fast Flow, ANX Sepharose 4 Fast Flow, and DEAE Sepharose Fast Flow (Fig. 2). The binding capacity is reported in milligrams of bound bHb per milliliter of packed resin. The binding capacities reported here are artificially high, since the columns were overloaded with bHb before equilibrating with start buffer and eluting the bHb from the column with 1 M NaCl. Additionally, the Q Sepharose XL purification was able to yield metHb levels less than 2% while operating at a pH 7.9 and room temperature.

3.2. FPLC Purification

Fig. 3 shows the FPLC elution profiles at each of the flow rates used in the 300 mL column packed with Q Sepharose XL, where the absorbance is in red and the conductivity is in blue. Fig. 3A also shows the different stages of the FPLC purification, starting with the equilibration of the column with start buffer to the reestablishment of the baseline with 1 M NaCl. Lipids and metHb have previously been found to elute earlier in the purification process [19], and this peak can be seen best in Fig. 3A which utilized bRBCs that were 4 weeks old compared to Fig. 3C which utilized bRBCs that were 2 weeks old. Significant cell debris remained in the product even after filtering the lysate through glass wool and dialyzing this product overnight at 4 °C. This was evident by an increase in cloudiness in the initial fractions of the purification fractions post-injection, and suggests that ion exchange chromatography should be able to capture the bHb product to ensure that all cell debris is removed

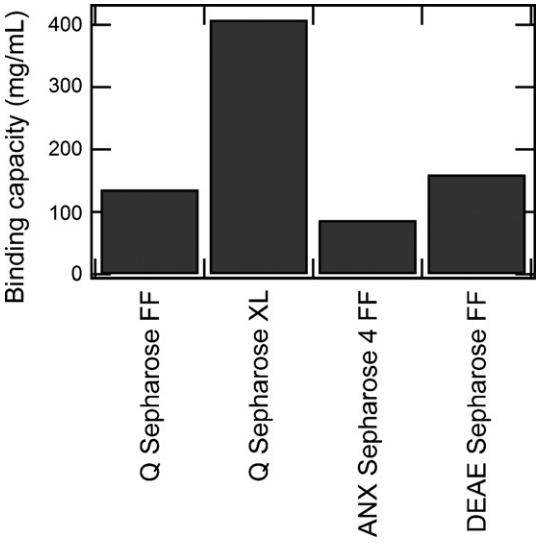

Fig. 2. The binding capacity of the four anion exchange resins within 1 mL columns at room temperature and a pH of 7.9.

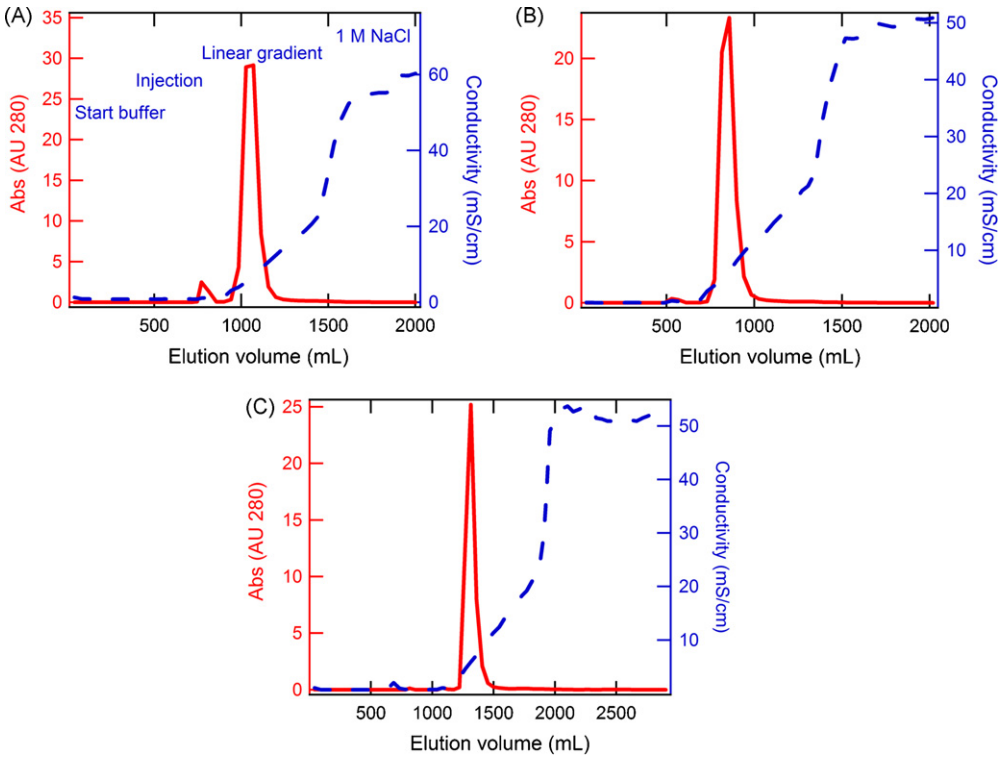

Fig. 3. Typical FPLC elution profiles for the XK50 column packed with 300 mL of Q Sepharose XL and operated at flow rates of (A) 6 mL/min, (B) 9 mL/min, and (C) 12 mL/min.

from the final product. The final bHb product was collected from the fractions that represented the bHb peak. Typical concentrations of the final bHb product ranged between 0.7 and 1.7 mM with metHb levels between 0.5 and 2% as determined by the cyanomethemoglobin technique. It is important to emphasize that the FPLC purification was performed at room temperature and was still able to achieve these low metHb levels.

Fig. 4 shows the SDS-PAGE results for each step of the bHb purification procedure described above, at flow rates of 6, 9, and 12 mL/min. Bovine hemoglobin represents 90% of the protein content within bRBCs with the remaining protein content mainly consisting of carbonic anhydrase and superoxide dismutase [21]. Additionally, albumin, a serum protein, has been found as an impurity in previous bHb purification methods. In order to assess

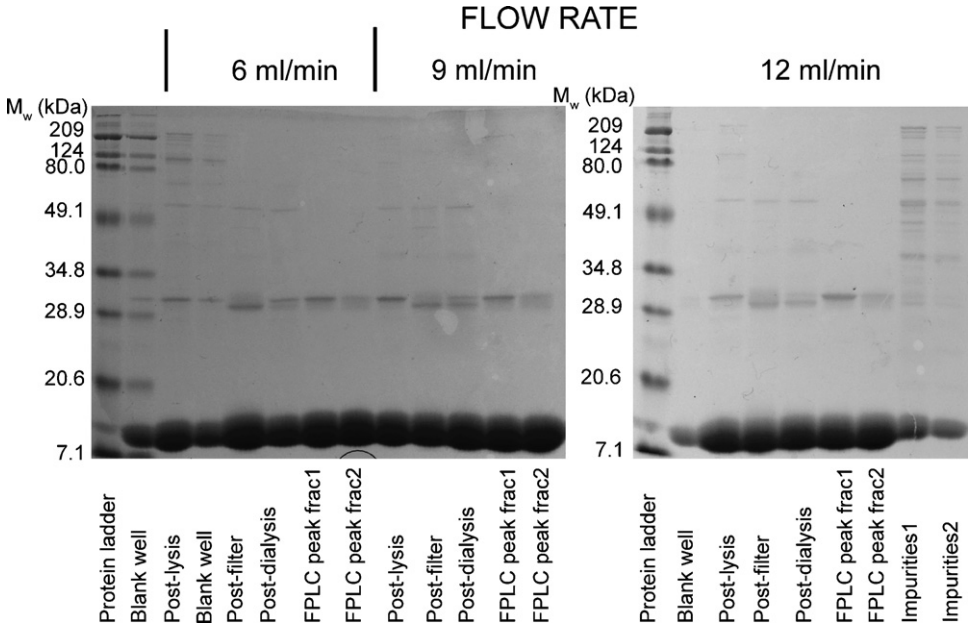

Fig. 4. SDS-PAGE results after each step of the bHb purification procedure at flow rates of 6, 9, and 12 mL/min.

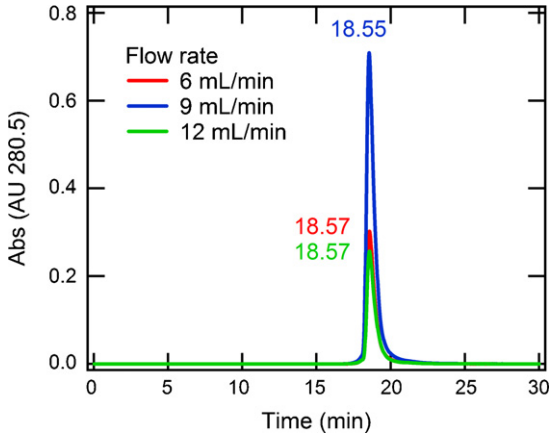

Fig. 5. HPLC size exclusion chromatogram of the final purified bHb product at flow rates of 6, 9, and 12 mL/min at an absorbance of 280.5 nm.

the level of impurities, each lane was overloaded with 75 μg of bHb, with the exception of the last two lanes in the 12 mL/min gel which were loaded directly from two fractions that were not captured by the resin prior to the bHb peak in Fig. 3C. The bHb travels through the gel as a monomer (α and β globin chains) and is present as a thick band at the bottom of the gel. The post-lysate product is mostly bHb for each flow rate studied; however, a decrease in impurities can be seen throughout the purification process, with the only impurity that remains in the final product occurring at around 32 kDa. This impurity was thought to be either bHb dimers that do not fully denature or remnant superoxide dismutase. There is no significant difference in the purity of the bHb product at any of the three flow rates within this study.

On the 12 mL/min gel, two lanes were loaded with FPLC fractions containing impurities not captured by the resin. Within these lanes, the bHb concentration is smaller compared to the other lanes as evidenced by the smaller area of the bHb band. These lanes show a significant amount of impurities that were removed from the final bHb product during the FPLC purification with Q Sepharose XL resin. The high purity of the final bHb product was confirmed via SEC on a HPLC system (Fig. 5), where only one peak was observed. The bHb product was determined to elute from the SEC column as αβ dimers rather than as tetrameric bHb, since its K_d is between the K_d of superoxide dismutase (31.2 kDa) and ovalbumin (44 kDa) (Fig. 1). Since bHb αβ dimers are in equilibrium with the bHb tetramer, a higher available pore volume is available to bHb resulting in the elution of bHb as an αβ dimer. Additionally, superoxide dismutase was observed to have a retention time of 19.056 min within the SEC column. However, no peak was observed at this retention time for the final bHb product, suggesting that the impurities seen in the SDS-PAGE gel at 32 kDa (Fig. 4) were αβ dimers rather than superoxide dismutase.

4. Conclusions

The purification of bHb is an essential first step in the synthesis of HBOCs for use as artificial blood substitutes and in tissue engineering applications. This study determined that Q Sepharose XL had a superior binding capacity for bHb compared to the other resins within this study. It was determined that it is essential to have the ion exchange resin capture the bHb, in order to ensure the removal of all cell debris. MetHb levels were kept below 2% at room temperature which reduces energy costs compared to purifications that operate in a cold room.

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