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Purification of *Lumbricus terrestris* erythrocruorin (LtEc) with anion exchange chromatography

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ABSTRACT

The naturally extracellular hemoglobin (erythrocruorin) of the Canadian nightcrawler, *Lumbricus terrestris* (LtEc), is a unique oxygen transport protein that may be an effective substitute for donated human blood. Indeed, this ultra-high molecular weight (~3.6 MDa) hemoglobin has already been shown to avoid the side effects associated with previous hemoglobin-based oxygen carriers and its high thermal stability (T_m = 56°C) and resistance to heme oxidation (k_{ox} = 0.04 hr^{-1} × 10^3 at 20°C) allow it to be stored for long periods of time without refrigeration. However, before it can be tested in human clinical trials, an effective and scalable purification process for LtEc must be developed. We have previously purified LtEc for animal studies with tangential flow filtration (TFF), which allows rapid and scalable purification of LtEc based on its relatively large size, but that type of size-based purification may not be able to specifically remove some impurities and high MW (> 500 kDa) contaminants like endotoxin (MW = ~1–4 MDa). Anion exchange (AEX) and immobilized metal affinity chromatography (IMAC) are two purification methods that have been previously used to purify mammalian hemoglobins, but they have not yet been used to purify large invertebrate hemoglobins like LtEc. Therefore, the goal of this study was to determine if AEX and IMAC resins could successfully purify LtEc from crude earthworm homogenate, while also preserving its macromolecular structure and function. Both processes were able to produce purified LtEc with low levels of endotoxin, but IMAC purification induced significantly higher levels of heme oxidation and subunit dissociation than AEX. In addition, the IMAC process required an additional desalting step to enable LtEc binding. In contrast, AEX produced highly pure LtEc that was not dissociated. LtEc purified by AEX also exhibits similar oxygen binding characteristics (P_{50} = 27.33 ± 1.82 mm Hg, n = 1.58 ± 0.17) to TFF-purified LtEc (P_{50} = 28.84 ± 0.40 mm Hg, n = 1.93 ± 0.02). Therefore, AEX appears to be the optimal method for LtEc purification.

1. Introduction

Despite being the safest and most effective treatment for severe blood loss, donated human blood must be continuously refrigerated and it expires after 42 days [1]. Seasonal shortages of rare blood types (e.g., AB = 3.75%, B = 13.45%, and Rh^- = 9.66% of the population) can also occur, further restricting access to life-saving transfusions [2]. To address these issues, researchers have been working towards the development of a safe, effective, and acellular alternative to donated human blood that is readily available and can be stored for long periods of time without refrigeration [3].

Naturally, the first blood substitute candidates were human hemoglobin (HbA) and bovine hemoglobin (bHb) purified from red blood cell (RBC) lysate [4]. These products boasted the advantage of being "universal donors" because they lacked red blood cell antigens, but they ultimately failed clinical trials due to dissociation of the Hb tetramer into dimers that extravasated into tissues (e.g., kidneys), leading to oxidative stress and renal failure. These issues were addressed by the next generation of blood substitutes, which utilized glutaraldehyde to cross-link the subunits into polymerized Hbs (PolyHbs). However, new issues emerged with PolyHbs, including scavenging of the gaseous hormone NO and heme oxidation, which were linked to a significant increase in the occurrence of myocardial infarctions and stroke among patients [3,5,6].

An interesting alternative to mammalian hemoglobins like HbA and bHb is the high molecular weight and naturally extracellular hemoglobin (erythrocruorin) of some invertebrate species. For example, the erythrocruorin of the Canadian nightcrawler, *Lumbricus terrestris*

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(LtEc), has a molecular weight of ~3.6 MDa and an oxygen affinity (P_{50} = 26.25 ± 0.63 mm Hg) similar to whole human blood (P_{50} = 26 mm Hg) [7,8]. The structure of this massive protein consists of 180 subunits, including 144 globin monomers that assemble into dodecamers and 36 linker subunits that connect the dodecamers into a hexagonal bilayer (HBL) structure. Furthermore, the subunits are held together with both intra- and inter-subunit disulfide linkages and a network of calcium binding sites that prevent dissociation of the globin subunits [9,10].

Our lab and others have previously demonstrated that LtEc is an attractive blood substitute. It can be purified using tangential flow filtration (TFF) due to its relatively large size and then transfused into mice and hamsters with no signs of oxidation or NO scavenging *in vivo* [10–12]. In addition, LtEc can be stored at high temperatures for extended periods of time (e.g., 37°C for at least 7 days) by deoxygenating its storage buffer to prevent oxidation or cross-linking it with glutaraldehyde to prevent aggregation and denaturation [13,14]. However, before LtEc can advance to human clinical trials, additional measures must be taken to ensure that impurities are completely removed from the final product. For example, lipopolysaccharides (LPS, endotoxins) have been shown to induce an acute inflammatory response in humans, but not in mice [15]. These molecules are heterogeneous, with a variety of molecular weights, from monomers in the 10–20 kDa range to polymers as large as 4 MDa [16,17]. Additionally, other large earthworm proteins like catalase (MW = 231 kDa) may not be entirely removed via TFF [18].

Other blood substitutes that have entered clinical trials have relied mostly upon anion exchange chromatography (AEX) to purify hemoglobin [20–23]. For example, a displacement strategy is commonly used in which the relatively high affinity of HbA for AEX resin allows the user to remove impurities from the column by saturating the resin with HbA [22,23]. Alternatively, HbA and other hemoglobins also have an unusually high affinity for divalent cations (e.g., Zn^{2+}) that allows them to be purified with immobilized metal affinity chromatography (IMAC) [24,27]. Both of these processes are high-throughput, and can be easily scaled for commercial production. Customizable columns are also readily available for these applications from a variety of vendors.

However, while both AEX and IMAC can purify large amounts of HbA and bHb, the relatively large size of LtEc (~30 nm) prevents it from entering the internal pores of most resins. For example, Tosoh Toyopearl HW-40 has a pore diameter (M) of 5 nm, which precludes binding of LtEc within the pores and significantly limits the overall LtEc binding capacity of the resin. In addition to selecting a resin with a large pore size, an ideal LtEc purification process must also provide a high recovery while minimizing dissociation of the HBL structure and oxidation of the heme iron (Fe^{2+}). Some groups have accomplished these goals by purifying LtEc with size exclusion chromatography (SEC), but that approach cannot be scaled up to meet the large global demand for blood substitutes [19,25]. Therefore, the goal of this study was to determine if LtEc could be purified with IMAC and AEX resins with relatively large pore sizes (Tosoh AF-Chelate 650 M = 100 nm, Tosoh Toyopearl DEAE-650 M = 100 nm). The properties (e.g., purity, oxidation, subunit dissociation, oxygen affinity, and cooperativity) of the LtEc samples purified with these resins were then compared to determine the most promising chromatographic method for LtEc purification.

2. Materials and methods

2.1. Preparation of crude LtEc

LtEc was extracted from a batch of 1,000 *Lumbricus terrestris* specimens (Wholesale Bait, Cincinnati, OH). After isolating worms from soil, they were homogenized in a SKG 2081 Juicer and the liquid fraction was centrifuged at 20,000 g for 20 min at 4°C to remove solid debris. Crude LtEc samples were then stored at −72°C until needed. Samples were then thawed and centrifuged again at 10,000 g for 5 min to remove any precipitates that formed during the freeze/thaw process.

2.2. IMAC

A Pharmacia Biotech XK 16 Column (Pfizer, New York, NY) was packed with 65 μm particle size (1000 Å pore size) Tosoh AF-Chelate 650 M resin (Part No. 0014475, Tosoh Biosciences, Tokyo, Japan) and then used for the purification process shown in Fig. 1A. Prior to each run, the resin was charged with Zn^{2+} ions using 10 mM $ZnCl_2$ (pH = 7.0) and excess zinc was flushed from the column using 20 mM Tris (pH = 7.4). Crude LtEc samples were prepared for IMAC by treating them with 100 mM EDTA, pH 7.4, then removing the EDTA with a GE PD-10 desalting column (GE Healthcare, Chicago, IL) using 20 mM Tris (pH = 7.4) as the mobile phase. Samples were then loaded onto the IMAC column and washed with 20 mM Tris (pH = 7.4) to remove non-binding impurities. The column was then washed with 20 mM Tris/500 mM NaCl (pH 7.0) to remove impurities that were non-specifically bound to the resin via electrostatic interactions. An additional wash with 200 mM Tris (pH = 7.4) was then used to remove weakly bound impurities (since Tris is a weak chelating agent), followed by a final wash with 20 mM Tris (pH 7.4) to remove additional impurities. LtEc was then eluted by stripping the column with 20 mM EDTA. Fractions from all wash and elution steps were collected and stored at −72°C while the column was cleaned and stored in 0.1 M NaOH.

2.3. AEX

A Pharmacia Biotech XK 16 Column (Pfizer, New York, NY) was packed with 65 μm particle size (1000 Å pore size) Tosoh DEAE 650 M resin (Part No. 43201, Tosoh Biosciences, Tokyo, Japan) and then used for the purification process shown in Fig. 1B. The column was first equilibrated with 20 mM Tris (pH = 7.4), then crude LtEc samples were directly loaded onto the column and washed with 20 mM Tris (pH = 7.4) to remove non-binding impurities. Weakly bound impurities were then removed by washing the column with 20 mM Tris/100 mM NaCl (pH = 7.4). Finally, LtEc was eluted with 20 mM Tris/300 mM NaCl (pH = 7.4). The column was cleaned with 50 mM EDTA in 0.1% Triton X-100, then rinsed and stored in 2 M NaCl. Fractions from all wash and elution steps were collected and stored at −72°C.

2.4. UV–Vis spectroscopy

The concentration of LtEc was determined with UV–Vis spectroscopy using the Beer-Lambert law and the absorbance of the sample at 540 nm, using a path length of 0.6 cm (for 200 μL in a 96 well plate), and an extinction coefficient of 13.8 cm^{-1} mM $heme^{-1}$. Oxidation levels (%Fe^{2+}) were determined using the cyanmethemoglobin method, as previously described [26].

2.5. HPLC analysis of subunit dissociation

Dissociation of the LtEc HBL into smaller oligomers was detected and quantified using size-exclusion HPLC on a Shimadzu UFLC system (Shimadzu, Kyoto, Japan) with a 3 μm particle size/300 Å pore size TSKgel Ultra-SW Aggregate column (Part No. 22856, Tosoh Biosciences. Tokyo, Japan). Samples were run at a flow rate of 0.75 mL/min for 40 min at ambient temperature, using a mobile phase of 20 mM Tris (pH = 7.4). The absorbance of the column eluent was measured at 280 nm and LabSolutions software was used to analyze chromatograms and calculate peak areas.

2.6. SDS-PAGE

The purity of the IMAC and AEX-purified LtEc samples was

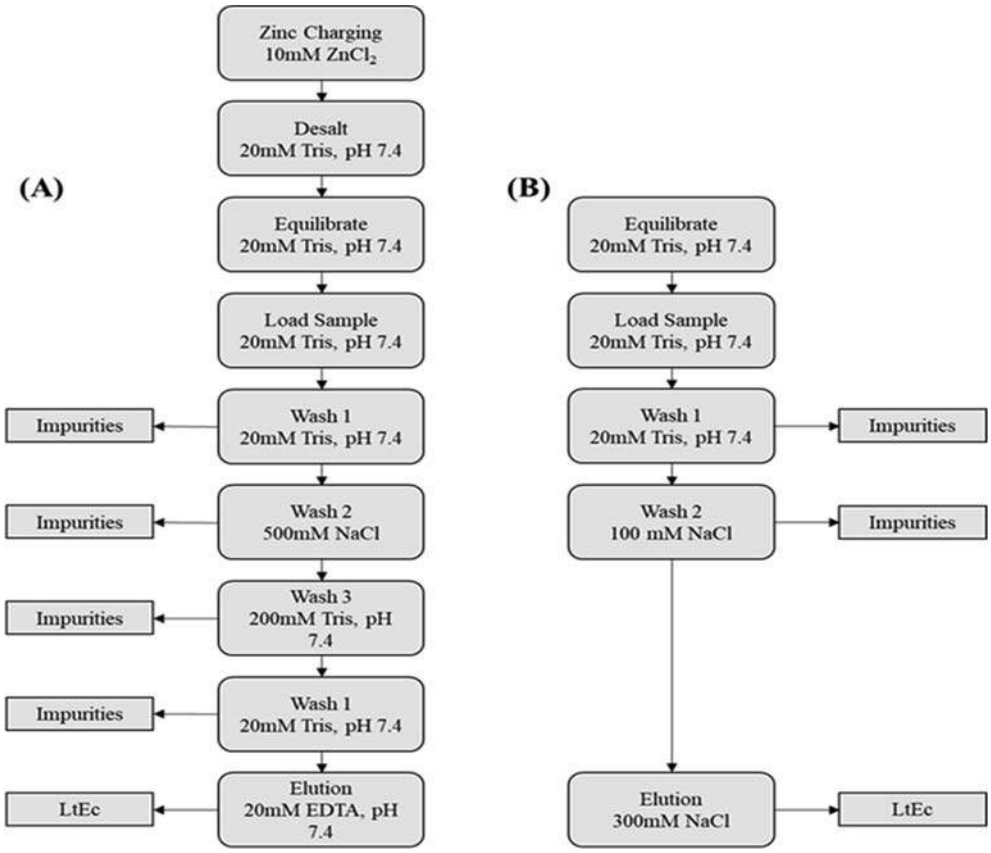

Fig. 1. Process diagrams for IMAC (A) and AEX (B) purification of LtEc.

determined by loading the samples onto an Any-kDa Precast Gel (BioRad – Laboratories, Hercules, CA) submerged in electrophoresis buffer (0.05 M Tris, 0.38 M glycine, 0.2% SDS, pH 9). A Blue Protein Standard-Broad Range (New England BioLabs, Ipswich, MA) was run as a reference for protein molecular weights. All samples were prepared for PAGE analysis by mixing them in a 1:1 ratio with Laemmli buffer containing β-mercaptoethanol, followed by an incubation at 95°C for 5 min. Gels were run at 50 V for 2 h, then stained with EZ Blue Gel Staining Reagent (Sigma Aldrich, B0149) and destained for 1 h with a solution of 10% acetic acid, 20% ethanol, and 70% water.

2.7. Endotoxin ELISA

Endotoxin concentrations were measured using an Aviva Systems Biology Endotoxin ELISA Kit (Aviva Systems Biology, San Diego, CA). A standard curve was generated using endotoxin concentrations in the range of 0.031–2 EU/mL. Final endotoxin concentrations were normalized per mM heme.

2.8. Hemox analysis

Oxygen equilibrium curves for IMAC and AEX-purified LtEc were obtained with a HEMOX Analyzer (TCS Scientific, New Hope, PA). Samples were diluted 5-fold in Hemox buffer (135 mM NaCl, 30 mM TES, 5 mM KCl, antifoam, pH = 7.4) prior to each run, then flushed with air until the partial pressure of O_2 (pO_2) reached 150 mm Hg. The samples were then flushed with pure N_2 until the pO_2 decreased to ~ 2 mm Hg while the absorbance of the sample was simultaneously measured to calculate the fraction of globin subunits with bound oxygen (Y). The oxygen affinity (P_{50}) of each sample was estimated as the pO_2 at which 50% of the LtEc hemes were bound to O_2 (Y = 0.5). Eq. (1) was then used to calculate the Hill coefficient (n).

$$n\log\left(\frac{pO_2}{P_{50}}\right) = \log\left(\frac{Y}{1-Y}\right) \quad (1)$$

2.9. Statistical analysis

All statistical analyses were performed using a multiple comparison of means ANOVA in Matlab. Statistical significance was defined as $p < 0.05$. All data values shown are the mean of 3 replicate samples, while the error bars represent standard deviations.

3. Results and Discussion

3.1. IMAC purification

LtEc was initially purified by loading it directly onto IMAC resin charged with Zn^{2+} as previously described for mammalian hemoglobins [24,27]. However, this approach resulted in most of the LtEc washing through the column in the initial wash step (data not shown). Since this problem may have been caused by an excess of metal ions in the crude LtEc, which may have saturated the metal binding sites on the LtEc surface and precluded resin binding, subsequent samples were treated with 100 mM EDTA (pH = 7.4) and then desalted. As expected, this pre-treatment process significantly increased the retention of LtEc in the initial flow through step, leading to significantly higher recovery of LtEc (62.7 ± 8.7%), as shown in Table 1.

After the LtEc sample was loaded onto the column, impurities were removed with 3 washes, including an initial rinse with 20 mM Tris (pH = 7.4) to remove unbound impurities, a subsequent rinse with 20 mM Tris/500 mM NaCl (pH = 7.4) to remove negatively charged impurities that electrostatically bound to the Zn^{2+}-charged resin, and a final rinse with 200 mM Tris (pH = 7.4) to remove impurities with a weak affinity for the immobilized Zn^{2+} cation. An overview of this

Table 1
Percentage of the original LtEc sample that eluted during each step during IMAC and AEX, along with the extent of dissociation (%HBL) and oxidation level (%Fe^{3+}). Asterisks (*) indicate significant differences between IMAC- and AEX-purified LtEc ($p < 0.05$).

Method	Wash 1	Wash 2	Wash 3	Wash 4	Recovery	HBL (%)	Fe^{3+} (%)
	Percentage (%) of total LtEc that eluted from the column in all steps						
IMAC	28.3 ± 12.2	3.0 ± 0.6	6.0 ± 8.5	0.0 ± 0.0	62.7 ± 8.7	20.5 ± 7.1*	36.3 ± 2.6*
AEX	6.1 ± 1.4	3.4 ± 4.0	N/A	N/A	90.5 ± 5.2	81.2 ± 9.5*	12.1 ± 3.3*

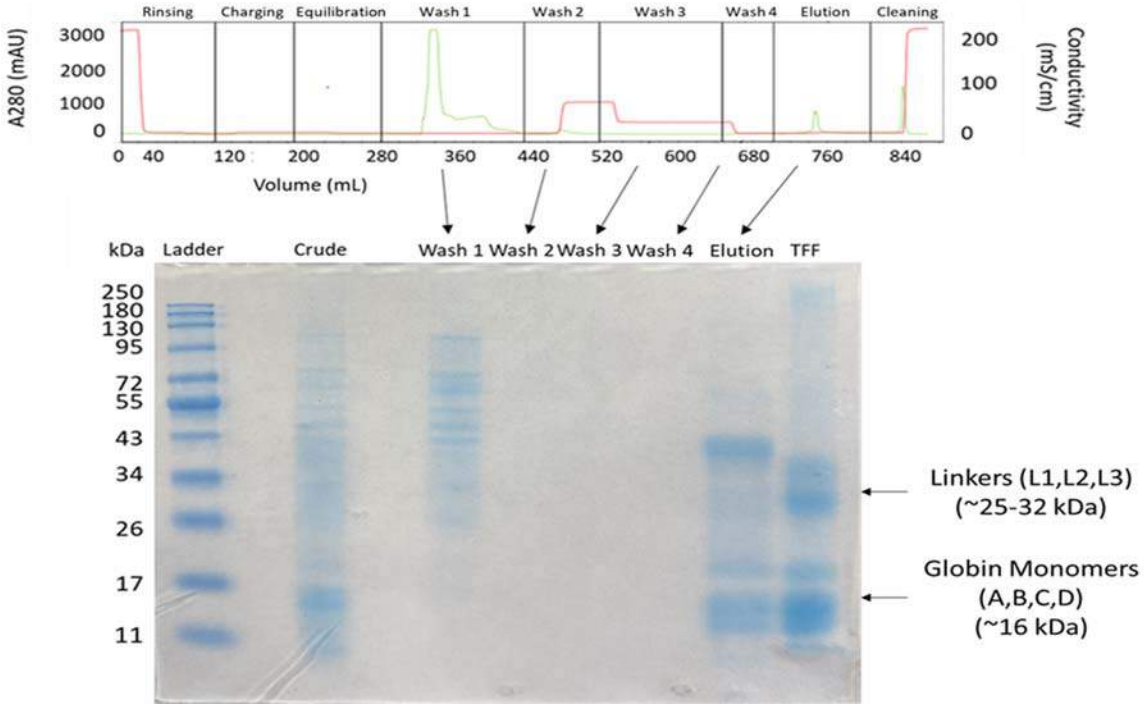

Fig. 2. IMAC Purification of LtEc. Top: Chromatogram of LtEc purification on IMAC resin, with eluate absorbance (280 nm) and conductivity (mS/cm) on the y-axes and volume (mL) on the x-axis. Bottom: SDS-PAGE analysis of samples collected from the column during each was step, with a MW ladder (Lane 1) and TFF-purified LtEc (Lane 10) included for reference.

process is shown in Fig. 1A, while Fig. 2 shows a representative chromatogram for the process. As shown in the chromatogram and PAGE gel, the initial wash step with 20 mM Tris (pH = 7.4) removed a large quantity of impurities (including 28.3 ± 12.2% of the total amount of LtEc recovered) while fewer impurities were removed in the subsequent wash steps. The LtEc was finally eluted by stripping the column with 20 mM EDTA, pH 7.0. Alternatively, elution of the LtEc was also achieved with 10–50 mM imidazole (pH = 7.4), but that method caused extensive dissociation of the LtEc HBL (data not shown).

The SDS-PAGE gel in Fig. 2 shows the impurities removed in each step and the purity of the IMAC-purified LtEc. As expected, both the TFF-purified and IMAC-purified LtEc samples exhibited the typical banding pattern, with globin subunits (A, B, C, D1′, D2) around 10–20 kDa and linker subunits (L1, L2, L3, L4) around 25–32 kDa. Most of the impurities seem to have been removed during the initial 20 mM Tris wash step (as indicated by a tall peak in the chromatogram and several bands on the PAGE gel), while the final eluted sample consists primarily of purified LtEc.

3.2. AEX purification

The additional desalting step that was necessary for effective purification of LtEc on the IMAC resin motivated us to evaluate AEX as an alternative and potentially simpler purification method. AEX is an especially attractive purification method for LtEc, since LtEc has a relatively low isoelectric point (pI = 5.28) [28] that could enable the removal of other impurities with higher pI values through isoelectric focusing. Initial AEX experiments were performed with the 45 µm Tosoh NH_2-750F strong salt tolerant anion exchanger resin (Tosoh Biosciences, Tokyo, Japan), but this resin bound the LtEc too tightly, making it difficult to elute (even with 2 M NaCl). Elution from the NH_2-750F resin was achieved at pH 2.5, but these acidic conditions also induced complete dissociation of the purified LtEc (data not shown). Therefore, a weaker anion exchange resin, Tosoh DEAE 650 M (Tosoh Biosciences, Tokyo, Japan), was evaluated next. This resin also tightly bound the LtEc, but it could be eluted with either a slightly higher pH (3.0) or 300 mM NaCl. However, elution of LtEc at pH 3.0 completely dissociated the LtEc HBL, so isoelectric focusing was deemed infeasible for this process. Instead, 300 mM NaCl was used to elute LtEc in subsequent experiments.

After the LtEc sample was loaded onto the column, impurities were removed using 2 washes – an initial rinse with 20 mM Tris (pH = 7.4) to remove unbound impurities and a subsequent rinse with 100 mM NaCl (pH = 7.4) to remove weakly bound impurities (see Fig. 1B). Fig. 3 shows a representative chromatogram that illustrates removal of impurities during each of these steps and a PAGE gel that shows the proteins that eluted during each step. A large amount of impurities flowed through during the first wash step with 20 mM Tris (pH = 7.4), while fewer impurities were removed during the second wash step with 100 mM NaCl. LtEc was then eluted with a buffer containing 20 mM

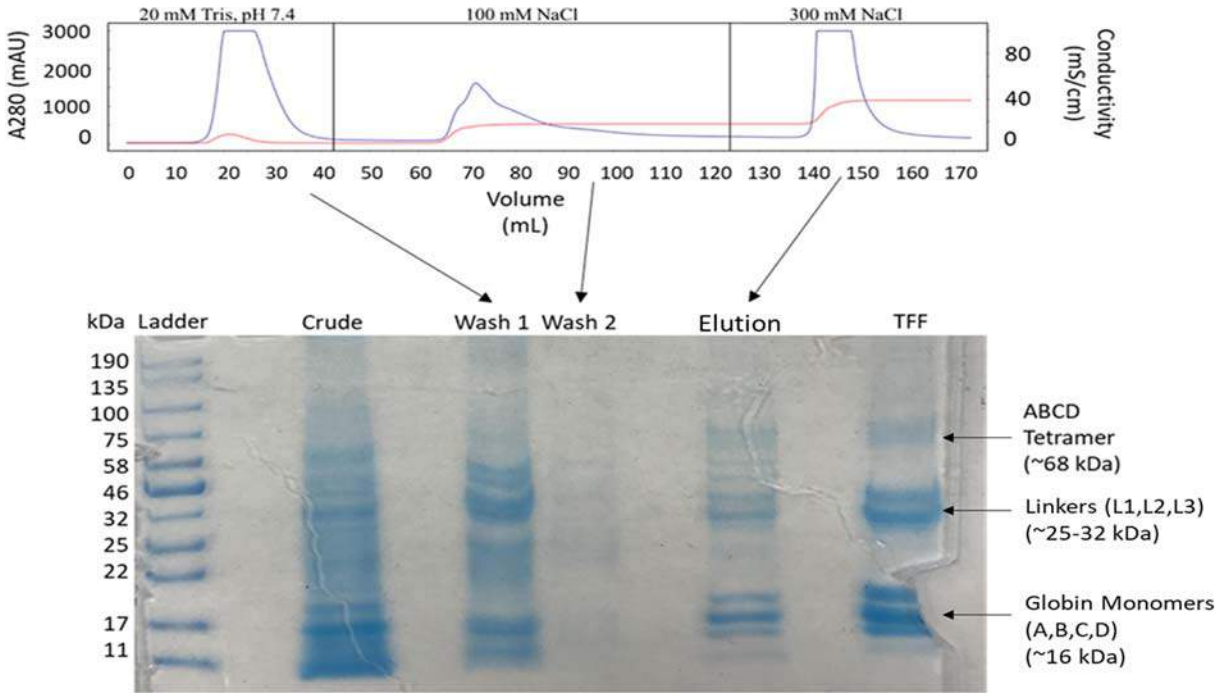

Fig. 3. AEX Purification of LtEc. Top: Chromatogram of LtEc purification on AEX resin, with eluate absorbance (280 nm) and conductivity (mS/cm) on the y-axes and volume (mL) on the x-axis. Bottom: SDS-PAGE analysis of samples collected from the column during each was step, with a ladder (Lane 1) and TFF-purified LtEc (Lane 10) included for reference.

Tris and 300 mM NaCl (pH = 7.4). The LtEc product obtained with AEX appeared to be highly pure and comparable to TFF-purified LtEc, with only a few additional impurity bands between 40 and 60 kDa. All the expected globin and linker bands were observed in the AEX-purified and TFF-purified LtEc samples.

3.3. Comparison of IMAC and AEX products

The properties of the LtEc samples purified with IMAC and AEX are shown in Table 1. In terms of recovery, IMAC provided a modest recovery of LtEc (62.7 ± 8.7%) when LtEc samples were pre-treated with EDTA and desalted. In contrast, AEX provided a superior recovery of LtEc (90.5 ± 5.2%), which is comparable to yields previously reported for AEX-purified HbA (~95%) [21].

In addition to maximizing recovery, it is also important to maintain the structure of LtEc during purification. Indeed, dissociation of the HBL could lead to a significant decrease in clearance time *in vivo* or induce renal toxicity, as observed with mammalian Hbs [29]. The effects of each purification method of the structural stability of LtEc are shown in Fig. 4. For example, LtEc purified via TFF exists as a high-molecular weight HBL that quickly eluted from the SEC column at 7.5 min (Fig. 4A). In contrast, dissociation of LtEc at alkaline pH (e.g., pH 10) creates a mixture of smaller oligomers and monomers that eluted as separate peaks at later times (10–20 min, Fig. 4A). Fig. 4B shows that IMAC purification induced the dissociation of LtEc, since IMAC-purified LtEc samples exhibited several peaks, including one sharp peak corresponding to the HBL that eluted around 7.5 min and several other peaks that could represent dissociation products or other impurities. Comparing the area under the HBL peak to the combined area of the other peaks indicated that a large fraction (79.5% ± 7.1%) of the LtEc dissociated from the HBL during the IMAC process.

In contrast, the AEX-purified LtEc (Fig. 4C) exhibited a single predominant peak that eluted around 7.5 min and accounted for 81.2 ± 9.5% of the total area under the curve (AUC). This measurement suggests that approximately 20% of the LtEc may have dissociated during the AEX process, but it is also possible that some of the area under the other peaks could be due to other protein impurities. A slight decrease in the retention time of the HBL was also observed in AEX-purified LtEc compared to TFF-purified LtEc, but this may be attributed the presence of high salt concentrations in AEX-purified LtEc that could limit ionic interactions between LtEc and the column, thereby hastening its elution.

The extensive dissociation observed in IMAC-purified LtEc may have been caused by the EDTA that was used to prepare the LtEc for IMAC and subsequently used to elute LtEc from the IMAC column. Indeed, the EDTA may have chelated the Ca^{2+} and Zn^{2+} ions that are known to bind to the surface of LtEc and contribute to its structural stability [9]. Previous studies with invertebrate Ecs have also shown that EDTA can be used to enhance dissociation of the HBL [30].

Another disadvantage of the IMAC process is that the IMAC-purified LtEc samples had a significantly higher level of heme oxidation (%Fe^{3+} = 36.3 ± 2.6%) than both the AEX-purified LtEc (%Fe^{3+} = 12.1 ± 3.3%), and TFF-purified LtEc (%Fe^{3+} = 9.5 ± 1.9%). This can likely be explained by the significantly higher level of dissociation seen in IMAC-purified LtEc, which is known to increase the exposure of heme pockets to oxygen and consequently increase the oxidation rate of the sample. This phenomenon has been previously reported in other studies which intentionally dissociated HbA into dimers [31,32].

3.4. Endotoxin analysis

The crude earthworm homogenate from which LtEc is purified may contain endotoxin from the bacteria in the worms' gut. This may pose a serious risk for patients, since bacterial endotoxins have been shown to provoke acute inflammatory responses in humans [15]. For that reason, we assessed the presence of endotoxin in our purified samples. These molecules have a net positive charge at neutral pH, suggesting that they may be removed during AEX or IMAC purification [17]. We employed a competitive ELISA to quantify the amount of endotoxin (EU) in the purified LtEc samples (relative to the heme concentration, in mM heme), which is shown Table 2.

Both IMAC and AEX-purified LtEc showed significantly higher levels

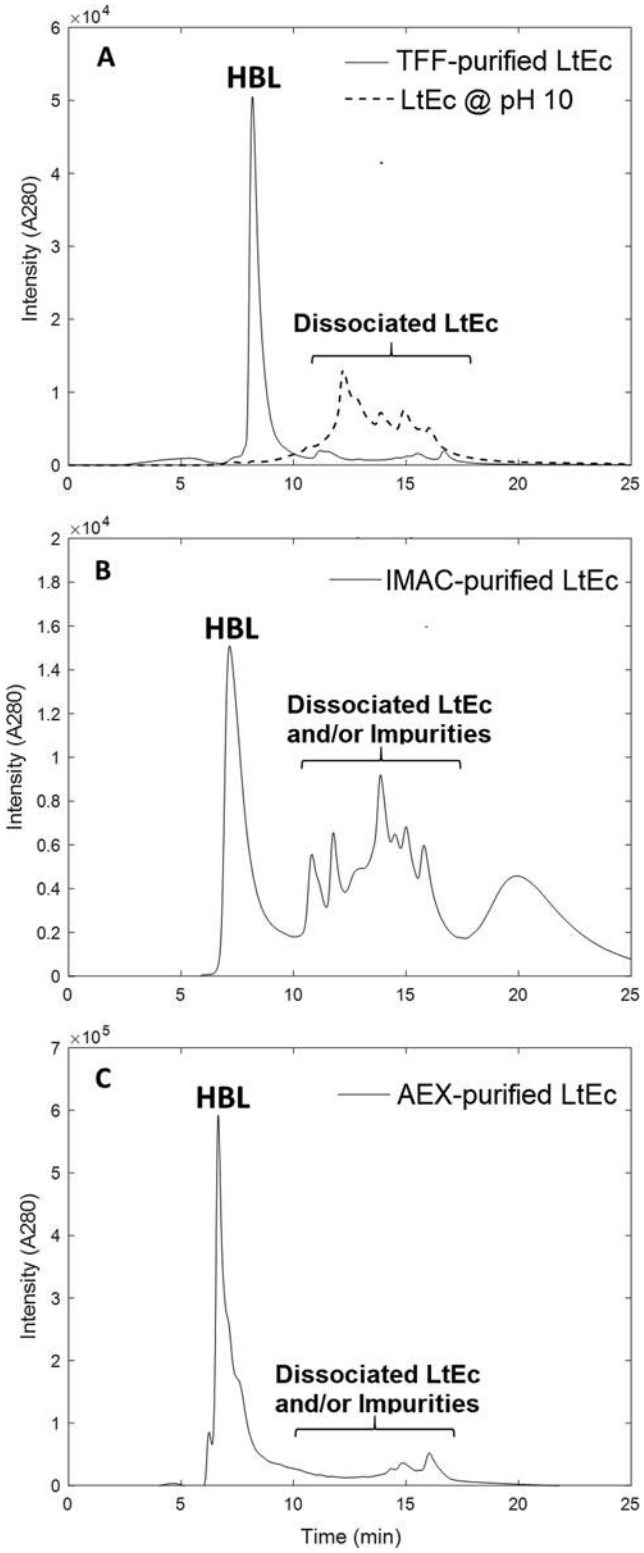

Fig. 4. SEC-HPLC chromatograms of (A) TFF-purified LtEc (black solid line) and TFF-purified LtEc that was dissociated at pH 10 (black dashed line), (B) IMAC-purified LtEc, and (C) AEX-purified LtEc. The y-axis shows the absorbance of the eluate at 280 nm.

of endotoxin than TFF-purified LtEc, but there was not a significant difference between IMAC and AEX-purified LtEc. Subsequent tests on buffers used in both IMAC and AEX indicated the presence of endotoxin (data not shown), so it is possible that either TFF removes more endotoxin than AEX and IMAC or the higher levels of endotoxin in these

Table 2
Endotoxin concentrations (EU/mM heme) in IMAC-, AEX-, and TFF-purified LtEc samples determined by competitive ELISA. Asterisks (*) indicate values significantly different from TFF ($p < 0.05$).

	IMAC	AEX	TFF
Endotoxin Concentration (EU/mM heme)	19.51 ± 13.44*	6.26 ± 0.89*	0.06 ± 0.02

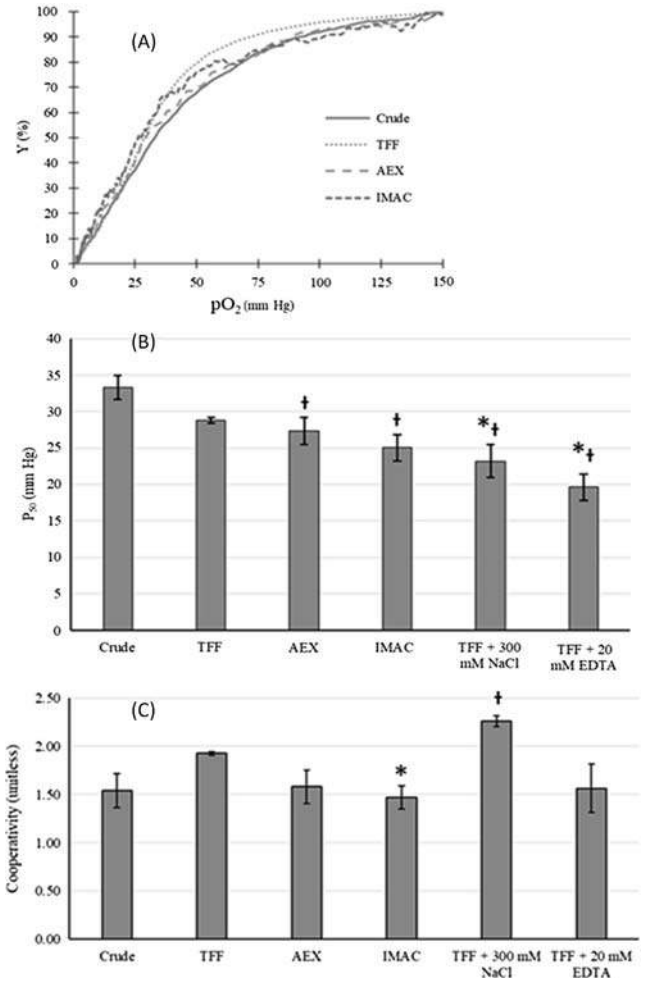

Fig. 5. Oxygen transport properties of purified LtEc samples. (A) Oxygen equilibrium curves, (B) P_{50} values, and (C) Hill coefficients for crude LtEc and LtEc purified via TFF, AEX, and IMAC. P_{50} and n values are also shown in B and C for TFF-purified LtEc supplemented with either 300 mM NaCl (elution buffer used in AEX) or 20 mM EDTA (elution buffer used in IMAC). Asterisks (*) indicate samples with statistically significant differences from TFF-purified LtEc. Crosses (ǂ) indicate samples with statistically significant ($p < 0.05$) differences from crude LtEc.

samples may be due to endotoxin contamination from the buffers used during chromatography.

The FDA has set maximum limits for endotoxin concentrations in drug products, specifically 5 EU/(kg body weight) for parenteral drug products [33]. This results in a maximum endotoxin dose for a patient of 150 lb (~68 kg) of 340 EU. In a unit of blood (~525 mL), the standard concentration of Hb is 2.71 mM (10.86 mM heme) [34]. If the values in Table 2 are scaled to match this heme concentration of 10.86 mM, the resulting amount of endotoxin in one unit of each purified LtEc product would be roughly: 212 EU for IMAC, 68 EU for AEX, and ≤1 EU for TFF. Therefore, safe doses of each sample would be ~1 unit of IMAC-purified LtEc, ~5 units of AEX-purified LtEc, and > 500

Table 3
Costs and characteristics of the resins used in this study, along with TFF membranes [37–40].

Method	Supplier	Part #	Binding Capacity	Cost	Autoclavable?	pH Range
AEX	Tosoh	07974	25–35 g/L	$4,403/5L	Yes	2–13
IMAC	Tosoh	14908	60 g/L	$17,878/5L	Yes	2–12
TFF	Repligen	K06-E500-05-S	N/A	$4,500	No	2–13

units of TFF-purified LtEc (per the FDA limit) [33]. Consequently, IMAC-purified LtEc could not be used for a large transfusion.

3.5. Hemox analysis

Finally, the LtEc samples purified by IMAC and AEX were evaluated with a Hemox Analyzer to determine their oxygen transport properties (P_{50} and n). As shown in Fig. 5A, the oxygen equilibrium curves (OECs) for crude LtEc and the purified samples are all highly similar. The oxygen affinity (P_{50}) and cooperativity (n) values calculated from the OECs are shown in Fig. 5B and 5C, respectively. First of all, it is interesting to note that the oxygen affinity of all the purified LtEc samples are significantly higher (i.e., lower P_{50}) than the crude LtEc sample. Furthermore, a slight (but statistically insignificant) decrease in P_{50} was observed in both the IMAC- and AEX-purified LtEc samples relative to the TFF-purified LtEc. Since this difference could be due to the 20 mM EDTA used to elute the LtEc from the IMAC resin or the 300 mM NaCl used to elute the LtEc from the AEX resin, we also measured the oxygen affinity of TFF-purified LtEc samples that were supplemented with 20 mM EDTA and 300 mM NaCl (Fig. 5B). Indeed, both EDTA and NaCl significantly decreased the P_{50} of the TFF-purified LtEc. Overall, however, the P_{50} values for all of the samples were similar to the P_{50} of whole human blood (26 mm Hg), suggesting that the products would perform similarly to donated human blood in transporting O_2 [35].

The cooperativity of the purified LtEc samples (represented by the Hill coefficient, n) is shown in Fig. 5C. There was no significant difference in cooperativity between TFF and AEX-purified LtEc, however, the cooperativity of IMAC-purified LtEc was significantly lower than that of TFF LtEc. There was not a significant difference in Hill coefficient following addition of 300 mM NaCl or 20 mM EDTA to TFF-purified LtEc, so it is unlikely that EDTA caused the decreased cooperativity. Instead, this decrease in cooperativity may be attributed to the higher level of oxidation that occurred during IMAC purification, since oxidation of human hemoglobin has also been shown to significantly decrease its cooperativity [36].

3.6. Practical considerations

In addition to the effects of each resin on the biophysical properties of LtEc, there are several other properties that are also important to consider when selecting a resin for a manufacturing process. First of all, the cost of the process is directly dependent upon the unit cost of the resin and its binding capacity, which are both shown in Table 3 for the resins used in this study and the TFF filters that we have previously used to purify LtEc.

Overall, the cost of the AEX resin and TFF cartridges are similar, while the IMAC resin is the most expensive (although it does have double the binding capacity of the AEX resin). An additional benefit of the AEX and IMAC resins is that they can be autoclaved to maintain sterility, while the mPES ultrafiltration membranes used in TFF cannot be autoclaved. The IMAC and AEX resins can also be exposed to a wide range of pH conditions for extended periods without damage (> 4 weeks for AEX resin) [37]. These properties allow for both resins to be easily cleaned in place and reused several times before fresh resin is needed. However, one important difference between the AEX and IMAC processes it that AEX uses relatively inert buffers containing only Tris buffer and NaCl, while the IMAC process would require the disposal of buffers containing EDTA and $ZnCl_2$ that are considered environmentally hazardous [37–40].

4. Conclusions

Overall, AEX appears to be a more suitable chromatographic purification method for LtEc than IMAC, since IMAC induces a significant amount of oxidation and dissociation in the LtEc sample. AEX is also a simpler and faster process with fewer steps. However, the two-step AEX protocol described in this study provides an LtEc product that is not completely pure (as indicated by PAGE and SEC analysis). Therefore, AEX is still not able to produce LtEc that is as pure as TFF-purified LtEc, but it may still be useful as a pre-treatment or final polishing step that could further increase the purity of TFF-purified LtEc. This hypothesis was not evaluated in this study, but will be the focus of future scale-up experiments.

CRediT authorship contribution statement

Brandon Timm: Project administration, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing - original draft, Visualization. **Osheiza Abdulmalik:** Funding acquisition, Resources, Writing - review & editing. **Atis Chakrabarti:** Resources, Writing - review & editing. **Jacob Elmer:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing - review & editing, Visualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests [The authors declare that a contributing author (Atis Chakrabarti) is an employee of Tosoh Biosciences].

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