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# A facile method for isolation of recombinant human apolipoprotein A-I from *E. coli*

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ABSTRACT

Apolipoprotein (apo) A-I is the major protein component of high-density lipoprotein (HDL) and plays key roles in the Reverse Cholesterol Transport pathway. In the past decade, reconstituted HDL (rHDL) has been employed as a therapeutic agent for treatment of atherosclerosis. The ability of rHDL to promote cholesterol efflux from peripheral cells has been documented to reduce the size of atherosclerotic plaque lesions. However, development of apoA-I rHDL-based therapeutics for human use requires a cost effective process to generate an apoA-I product that meets "Good Manufacturing Practice" standards. Methods available for production and isolation of unmodified recombinant human apoA-I at scale are cumbersome, laborious and complex. To overcome this obstacle, a streamlined two-step procedure has been devised for isolation of recombinant untagged human apoA-I from *E. coli* that takes advantage of its ability to re-fold to a native conformation following denaturation. Heat treatment of a sonicated *E. coli* supernatant fraction induced precipitation of a large proportion of host cell proteins (HCP), yielding apoA-I as the major soluble protein. Reversed-phase HPLC of this material permitted recovery of apoA-I largely free of HCP and endotoxin. Purified apoA-I possessed α-helix secondary structure, formed rHDL upon incubation with phospholipid and efficiently promoted cholesterol efflux from cholesterol loaded J774 macrophages.



## 1. Introduction

Apolipoprotein (apo) A-I is the major protein component of plasma high-density lipoprotein (HDL). The ability of isolated apoA-I to induce self-assembly of discoidal reconstituted HDL (rHDL) *in vitro* has led to numerous applications. For example, apoA-I rHDL have been administered to patients as therapy for cardiovascular disease [1,2]. Others have employed apoA-I mimetics as a stabilizing scaffold for miniature membranes that allow assimilation of transmembrane proteins in a native-like conformation [3]. In yet other applications, apoA-I serves as an integral component of "nanodisk" delivery particles [4,5] or as a structural component of rHDL harboring contrast agents for magnetic resonance imaging [6].

Further development and broader use of this rapidly advancing technology, as well as potential commercialization of rHDL based therapeutics, requires a robust supply of high purity apoA-I. Whereas research scale production and downstream processing of recombinant apoA-I is simplified by appending cleavable tags onto the protein [7–9], these extraneous sequences must be removed prior to use in therapeutic agents intended for humans. At the same time, production and isolation of untagged recombinant proteins in *Escherichia coli* is oftentimes plagued by contamination from host cell protein (HCP) and endotoxin [10]. Contaminating HCP in a biopharmaceutical drug can act as adjuvants or be immunogenic [11]. Thus, *E. coli* HCP present a safety concern such that, typically, their levels are reduced to the ng/mg range in recombinant therapeutic proteins. Current methods to achieve this require multiple chromatography steps, often conducted in the presence of chaotropic salts. A complementary, but painstaking, strategy involves the use of genetic engineering to delete genes encoding HCP contaminants [10].

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One of the key intrinsic properties of apoA-I is structural resilience. Indeed, it is well known that apoA-I refolds to a native state following thermal denaturation [12,13]. In the present study, this property is exploited to isolate mature recombinant WT human apoA-I from *E. coli* without the use of affinity tags or cumbersome and laborious chromatographic steps. The method described is a facile two-step purification scheme that yields highly purified apoA-I that retains native structure-function properties with minimal contamination by HCP or endotoxin.

## 2. Material and methods

### 2.1. ApoA-I cDNA cloning and bacterial transformation

A cDNA encoding mature human apoA-I (243 amino acids) was cloned into a pET-22b(+) vector at *Nde*I and *Not*I restriction sites. The insert contained a stop codon at the final position to prevent expression of a vector encoded downstream His-tag. The assembled plasmid was transformed into *E. coli* DH5α cells and cultured on ampicillin-infused agar plates to select transformed clones. Colonies were picked and cultured for plasmid preparation. DNA sequencing was performed on the plasmid vector to confirm the desired insertion was achieved.

### 2.2. Expression of recombinant human apoA-I

The apoA-I pET-22b plasmid vector was used to transform BLR(DE3) competent *E. coli* cells (Novagen Inc) by heat shock (BL21 and BLR cells were also tested but resulted in lower yield). Transformed cells were grown overnight in NZCYM media (Sigma-Aldrich) supplemented with 50 μg/mL ampicillin (Sigma-Aldrich) at 37 °C with shaking (200 rpm). Overnight cultures were used to inoculate 1 L of NZCYM/ampicillin media followed by culturing at 37 °C and 200 rpm until the $OD_{600}$ = 0.7. Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added (0.5 mM final concentration) to induce apoA-I expression and bacteria were cultured for a further 5 h at 25 °C with shaking.

### 2.3. Purification procedure

Bacteria were pelleted by centrifugation, re-suspended in 10 mL phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) per liter bacterial culture and disrupted by sonication on a Branson 450 sonicator fitted with a 1 cm tip probe. Ten ml suspensions were subjected to three rounds of sonication for 30 s each at 50% duty cycle (output level 5). The resulting cell lysate was centrifuged at 20,000 × g for 30 min at 4 °C to separate insoluble material. The supernatant fraction was collected and heated for 10 min at specified temperatures (range: 65 °C–95 °C). Subsequently, the sample was cooled and centrifuged at 20,000 × g for 30 min at 4 °C. The recovered supernatant was subjected to reversed-phase high performance liquid chromatography (HPLC) on a Perkin-Elmer Series 200 instrument. The sample was applied to a semi-preparative RXC-8 Zorbax 300SB column and eluted with a linear A/B gradient of 2% solvent B per min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Absorbance was monitored at 230 nm and the peak corresponding to apoA-I collected, lyophilized and stored at −20 °C until use. As a control for comparison, His-tag apoA-I was expressed in *E. coli* and isolated by nickel chelation affinity chromatography according to Ryan et al. [8].

### 2.4. Analytical procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using either a 10–20% acrylamide gradient tricine gel or a fixed 18% acrylamide slab. For Western blotting, proteins were electrophoretically transferred to a PVDF membrane, probed with anti-apoA-I (Meridian Life Science Inc., K45252G, 1:10,000 dilution) and visualized via enhanced chemiluminescence (ECL) reagent. Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin (BSA) as standard. Endotoxin (i.e. lipopolysaccharide; LPS) was quantified using the Limulus Amebocyte Lysate Kinetic-QCL assay (Lonza).

### 2.5. Far UV circular dichroism spectroscopy

Far UV circular dichroism (CD) spectroscopy was performed on a Jasco 810 spectropolarimeter. Scans were recorded between 185 and 260 nm in 20 mM sodium phosphate, pH 7.2, at a protein concentration of 0.15 mg/mL in a 1 mm path length circular cuvette. Secondary structure content was calculated as previously described [14]. For guanidine HCl denaturation experiments, protein samples (0.2 mg/mL) were incubated overnight at a given denaturant concentration to attain equilibrium, and ellipticity measured at 222 nm in a 1 mm path length cuvette.

### 2.6. Cholesterol efflux assays

J774 macrophages were simultaneously cholesterol-loaded and radiolabeled by incubation with 25 μg/mL acetylated LDL containing 3 μCi/mL [$^3$H]cholesterol. Cells were washed with media containing 2 mg/mL BSA to remove unincorporated radioactivity and equilibrated overnight in media supplemented with BSA. After equilibration, cholesterol efflux assays were conducted by introducing 10 μg of a specified apoA-I preparation to the cells and incubation for 1–24 h. At indicated times, the media was collected and radioactivity measured by liquid scintillation spectrometry (PerkinElmer). Subsequently, cells were treated with 0.5 mol/L NaOH for 12 h and residual cell-associated radioactivity determined. Percent efflux was computed from the [$^3$H]cholesterol in media over total [$^3$H]cholesterol (media + cell).

### 2.7. Statistical analysis

Statistical significance between groups was calculated using the two-tailed Student's t-test. Data are presented as mean ± standard deviation (n = 3). P-values < 0.05 were considered significant.

### 2.8. rHDL formation

Five mg dimyristoylphosphatidylcholine (DMPC) was dissolved in chloroform/methanol (3:1 v/v) and dried under a stream of $N_2$ gas, forming a thin film on the vessel wall. Residual organic solvent was removed under vacuum. The prepared lipid was then dispersed in 1 mL PBS followed by the addition of 2 mg apoA-I (4 mg/mL stock solution in PBS). The sample was bath sonicated at 22 °C for approximately 5 min until the turbid sample clarified.

### 2.9. Electron microscopy (EM)

A freshly prepared untagged apoA-I rHDL sample was processed for negative staining as previously described [15–17]. In brief, the sample was diluted to 5 μg/mL protein with Dulbecco's PBS. An aliquot (~4 μL) of the sample was placed on a glow-discharged thin-carbon-coated 200 mesh copper grid (CF200-Cu, Electron Microscopy Sciences, Hatfield, PA and CU-200CN, Pacific grid-tech, San Francisco, CA). After ~1 min, excess solution was blotted with filter paper, the grid was washed with water, stained with 1% (w/v)

uranyl formate and dried under a stream of $N_2$ gas.

### 2.10. EM data acquisition and image processing

The negatively stained specimen was examined on a Zeiss Libra 120 Plus TEM (Carl Zeiss NTS, Oberkochen, Germany) operating at 120 kV with 20 eV in-column energy filtering at room temperature. Micrographs were acquired by a Gatan UltraScan 4Kx4K CCD at 80,000× magnification (each pixel corresponding to 1.48 Å) under near Scherzer focus (0.1 μm) and defocus of 0.4 μm. Micrographs were processed with EMAN, SPIDER, and FREALIGN software packages as described elsewhere [17–20]. The contrast transfer function parameters of each micrograph were determined and corrected. A total of 2986 rHDL were used for reference-free class averaging with the EMAN software package.

## 3. Results

### 3.1. Overview of strategy

Seeking an improved method for isolation of recombinant untagged human apoA-I from *E. coli*, we took advantage of the unique structural resilience of this protein. Naturally devoid of cysteine, biophysical studies [12,13] have documented that apoA-I re-folds to its native conformation following denaturation (*i.e.* exposure to chaotropic salts or heat treatment). To incorporate this intrinsic property into an isolation scheme for recombinant apoA-I, the process described below was devised.

### 3.2. The effect of culture temperature on apoA-I expression

When recombinant WT human apoA-I was expressed in *E. coli* under standard conditions the bulk of apoA-I produced remained in the bacteria. Therefore, following expression, *E. coli* were pelleted by centrifugation, re-suspended in PBS, lysed by probe sonication and centrifuged. The corresponding supernatant and pellet fractions were collected and analyzed by SDS PAGE (Fig. 1). When *E. coli* was transformed with empty vector no apoA-I was detected. When apoA-I expression was examined as a function of culture temperature, increased apoA-I was detected as the culture temperature was lowered from 37 °C to 30 °C to 25 °C. However, at culture temperatures of 20 °C or 15 °C, apoA-I protein production was lower than that observed at 25 °C (see Supplementary Fig. S1). Fig. 1 also revealed that the bulk of apoA-I is recovered in the supernatant fraction obtained after probe sonication of the bacterial cell lysate. Based on this data, all subsequent apoA-I expression studies were performed at 25 °C.

### 3.3. Step 1: heat treatment

Despite the large amount of apoA-I present in the supernatant fraction, numerous HCP were also present. In an effort to selectively remove HCP, the supernatant was heated to various temperatures ranging from 65 °C – 95 °C for 10 min. After cooling and centrifugation to pellet insoluble material, SDS-PAGE was performed to assess the relative distribution of apoA-I between the supernatant and pellet fractions (Fig. 2). At every temperature tested, large amounts of HCP, but little apoA-I, were recovered in the pellet fraction. Conversely, the supernatant fractions were greatly enriched in apoA-I. Heat exposure for longer time periods (30 min–1 h) resulted in significantly less apoA-I recovery in the supernatant fraction with only modest further reductions in HCP (Supplementary Fig. S2). On the other hand, when the time of heat exposure was limited to 10 min, increased temperature resulted in less HCP contamination in the supernatant with little change in apoA-I distribution. Thus, 10 min heat exposure at 95 °C was adopted as the optimal procedure. Based on the relative enrichment of apoA-I following heat treatment, it was hypothesized that reversed-phase HPLC could achieve two goals: a) isolation of apoA-I and b) removal of bacteria-derived endotoxin from the preparation.

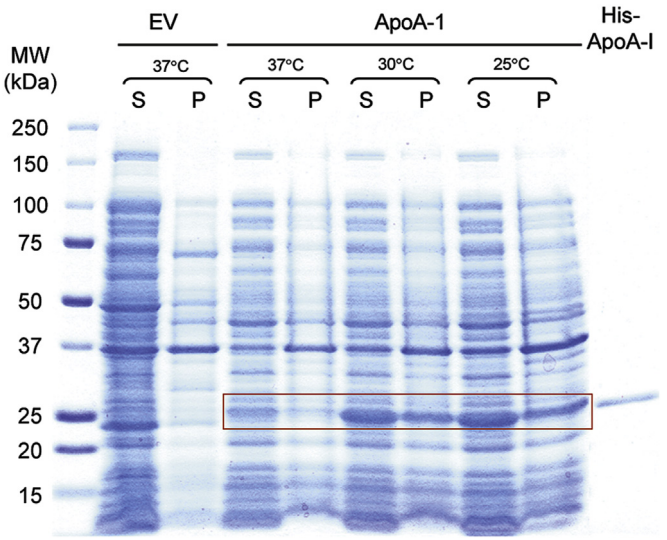


**Fig. 1. Effect of culture temperature on apoA-I production**. *E. coli* were transformed with a vector harboring the coding sequence of human apoA-I and cultured at 37 °C, 30 °C and 25 °C. Control bacteria were transformed with empty vector (EV) and cultured at 37 °C. Five h after induction with IPTG, bacteria were pelleted by centrifugation, re-suspended in PBS, lysed by probe sonication and centrifuged to separate insoluble material. The supernatant (S) and pellet (P) fractions from each culture condition were then subjected to SDS PAGE analysis. Far left lane: molecular weight standards; far right lane: apoA-I standard. A red box highlights the position of apoA-I in bacterial culture samples. The results shown are representative of 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.4. Step 2: reversed-phase HPLC

The soluble fraction obtained following heat treatment of an *E. coli* cell lysate (*Step 1*) was subjected to reversed-phase HPLC. ApoA-I eluted as a single major peak at approximately 50% acetonitrile (solvent B). The peak corresponding to apoA-I was collected, dialyzed into PBS and analyzed by SDS-PAGE (Fig. 3, **panel A**). The stained gel indicates this chromatographic step removes the majority of HCP, resulting in a sample with higher apparent purity than a control His-tag apoA-I purified by nickel chelation chromatography. The yield of untagged apoA-I was ~6 mg/liter bacterial culture. A corresponding anti-apoA-I Western blot (**panel B**) revealed a single immune-reactive band at 28 kDa.

### 3.5. Endotoxin assay

Given the importance of minimizing endotoxin contamination for therapeutic and biotechnology related applications, bacteria-derived endotoxin levels were determined at various stages of the purification process (Fig. 4). The initial heat treatment step led to a ~10-fold reduction in endotoxin levels relative to the starting material. This was similar to the degree of reduction observed following nickel chelation chromatography of control His-tag apoA-I. Following the reversed phase HPLC step, however, a further 100,000-fold decrease in endotoxin was achieved for an overall six orders of magnitude reduction, relative to starting material ($p < 0.05$). Endotoxin levels in the final preparation were 60.8 ± 36.3 EU/mL ($n = 3$). Based on an average protein

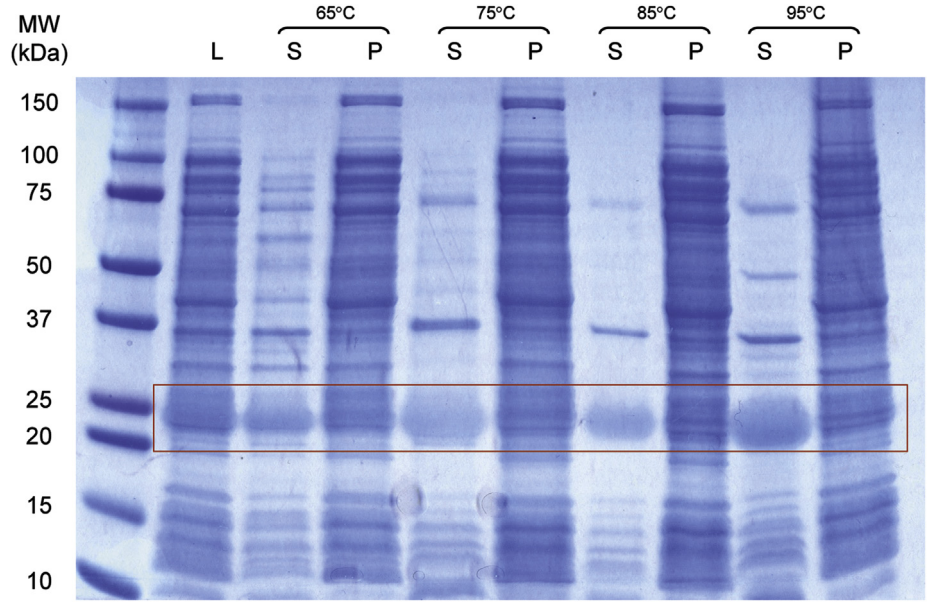


**Fig. 2. Effect of heat treatment on bacterial lysate protein solubility**. *E. coli* were transformed with a vector harboring the coding sequence of human apoA-I and cultured at 25 °C. The processed bacterial lysate supernatant was heated to the indicated temperatures for 10 min and cooled. Following this, samples were centrifuged to separate insoluble material and the resulting supernatant (S) and pellet (P) fractions analyzed by SDS PAGE. Far right lane: molecular weight standards; lane L: unprocessed bacterial lysate. The major band in each supernatant lane corresponds to apoA-I (28 kDa). A red box highlights the migration position of apoA-I on the gel. The results shown are representative of 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

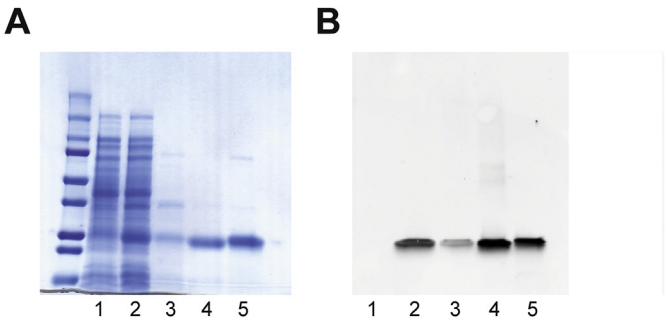


**Fig. 3. Step-wise purification of recombinant untagged apoA-I**. *E. coli* were transformed with an empty vector or a vector harboring the coding sequence of human apoA-I and cultured at 25 °C. The processed bacterial lysate supernatant was heated to 95 °C for 10 min. Following this, the sample was centrifuged to remove insoluble material and the supernatant applied to a reversed phase HPLC column. Aliquots were removed at various stages of the purification protocol for analysis. A) SDS PAGE; Lane 1: control *E. coli* lysate, Lane 2: apoA-I vector transformed *E. coli* lysate, Lane 3: 95 °C exposure supernatant; Lane 4: apoA-I peak from HPLC; Lane 5: His-tag apoA-I standard. Molecular weight standards were applied to the far left lane. B) Anti human apoA-I Western blot; lane assignments same as panel A. The results shown are representative of 4 independent experiments.

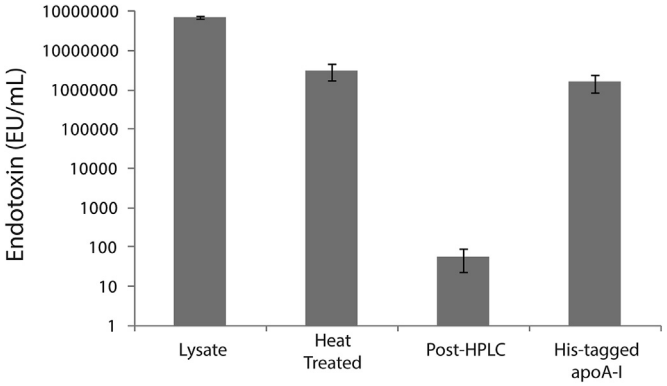


**Fig. 4. Endotoxin levels**. Endotoxin levels were measured by the Limulus Amebocyte Lysate assay at various stages of the purification scheme for His-tag apoA-I and untagged apoA-I. Values reported are the mean ± S.D. (n = 3).

concentration of 5 mg/mL, this corresponds to ~12 EU/mg apoA-I.

### 3.6. Secondary structure content and stability of apoA-I

To determine if heat exposure results in a structurally damaged apoA-I product, far UV CD spectroscopy was performed (Fig. 5). An α-helix content of 48.0 ± 1.7% was calculated for control His-tag apoA-I and 53.3 ± 1.7% for untagged apoA-I. The α-helix contents observed for untagged apoA-I (heat exposed) and control His-tag apoA-I are in good agreement with values (50% α-helix) reported by others [12]. Similarly, untagged apoA-I and His-tag apoA-I displayed a similar ability to resist guanidine HCl-induced unfolding (midpoint of guanidine HCl induced denaturation = 0.97 ± 0.03 M for His-tag apoA-I and 1.03 ± 0.03 M for untagged apoA-I). These data indicate the purification method involving heat exposure yields a protein that has not suffered irreparable loss of secondary structure content or intrinsic stability.

### 3.7. Cholesterol efflux capacity of isolated recombinant apoA-I

A major function of apoA-I is promotion of cholesterol efflux from cells via interaction with members of the ATP binding cassette transporter protein family. To determine if the ability of apoA-I to function as a cholesterol acceptor was compromised as a result of the purification scheme employed, cholesterol efflux activity was measured in J774 macrophages (Fig. 6). Over a period of 24 h no significant differences in cholesterol efflux were observed between

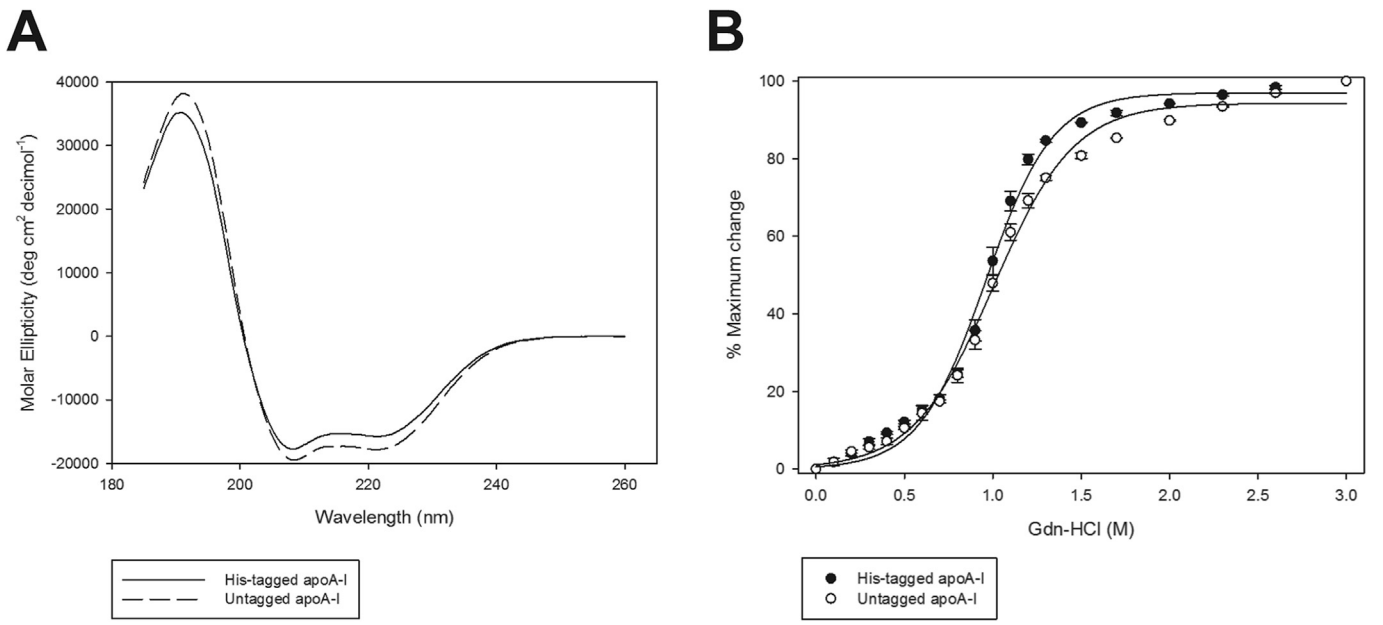


**Fig. 5. Secondary structure content and stability of apoA-I**. A) Far UV CD spectra of His-tag apoA-I and untagged apoA-I. B) Both apoA-I proteins were incubated with increasing concentrations of guanidine HCl and the folded ⇄ unfolded transition midpoint determined from changes in molar ellipticity at 222 nm (B). Results are presented as mean ± S.D. (n = 3).

untagged apoA-I (heat exposed) and control His-tag apoA-I.

### 3.8. ApoA-I dependent formation of rHDL

A major therapeutic application for recombinant apoA-I involves formation of rHDL. To investigate the ability of apoA-I to form these complexes, untagged apoA-I was incubated with an aqueous dispersion of DMPC. Bath sonication of the apolipoprotein/phospholipid mixture at 22 °C induced sample clarification, indicating rHDL formation. Analysis by negative stain electron microscopy revealed a population of discoidal particles with diameters in the range of 12–20 nm (Fig. 7). The particle diameter distribution and discoidal shape of the particles is similar to previously described apoA-I rHDL [4].

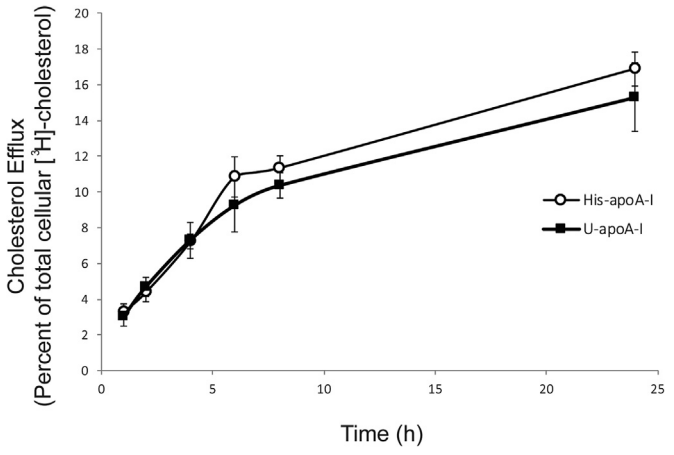


**Fig. 6. ApoA-I mediated cholesterol efflux**. J774 macrophages were loaded with [$^3$H] cholesterol followed by incubation with 10 μg His-tag apoA-I or untagged apoA-I for specified times ranging from 1 to 24 h. Following incubation, the medium and cells were collected and radioactivity measured by liquid scintillation spectrometry. Results are reported as percent efflux: [$^3$H]cholesterol in the media over total [$^3$H]cholesterol (media + cell). Values are the mean ± S.D. (n = 4).

## 4. Discussion

There is a growing need for apoA-I for commercial applications in nanotechnology [21] and as a therapeutic [22]. While it is possible to isolate apoA-I from human blood products, this approach is costly and vulnerable to disease transmission [23–25]. Approaches to generate recombinant apoA-I using yeast, baculovirus or bacteria present their own challenges. Each of these systems has been utilized for apoA-I production. Feng et al. [26] reported a bio-reactor scale *Pichia pastoris*-based expression system that utilized a purification scheme involving acetone precipitation and ion exchange chromatography. Sorci-Thomas et al. [27] and Pyle et al. [28] developed baculovirus systems capable of expressing apoA-I. Drawbacks include the length of time to express the protein and downstream processing complexity. Methods employing bacteria generally append tags or extensions onto the target protein. While this approach streamlines processing and purification, it requires additional steps, and increased cost, to remove the tag. In addition, leftover amino acids and/or non-specific cleavage can present problems [7–9,29]. In the present approach we elected to express mature untagged human apoA-I. Although apoA-I produced in this manner is contaminated with HCP, by taking advantage of the unique ability of the class of exchangeable apolipoproteins to refold following thermal denaturation [12,13,30] it was possible to eliminate the majority of HCP by heat treatment, while maintaining apoA-I in solution. The folding ⇄ unfolding transition of apoA-I has its midpoint between 50 and 60 °C and is readily measured by CD spectroscopy. Far UV CD analysis revealed the α-helix content of untagged apoA-I recovers fully following exposure to temperatures as high as 95 °C. At the same time, after a single 10 min heat exposure step, a large proportion of contaminating HCP precipitate. A subsequent semi-preparative reversed-phase HPLC step yielded highly purified WT apoA-I. When the effect of bacterial culture temperature on apoA-I production was examined, enhanced apoA-I expression was

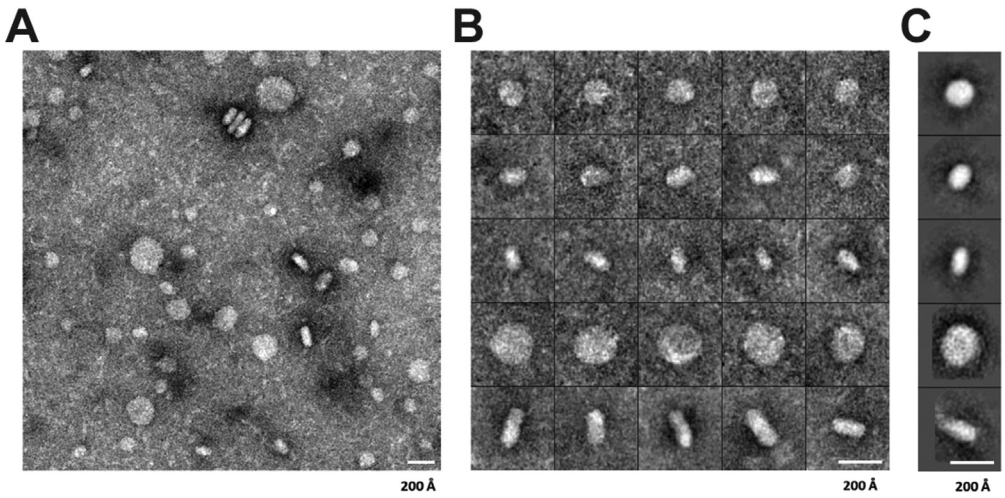


**Fig. 7. Negative stain EM images and reference-free class averages of apoA-I rHDL.** A) Survey view of rHDL prepared by optimized negative staining. B) Twenty-five representative rHDL particles. The first, second and third rows show the top, half-side and side views of the most abundant particle population (~12 nm in diameter). The fourth and fifth rows show top and side views of second most abundant particle population (~20 nm in diameter). C) Five selected reference-free class averages (from a total of ~80 classes) corresponding to representative particles from the left panel.

observed upon lowering the culture temperature from 37 °C to 25 °C. The reason for this is unknown but may be related to a temperature-dependent membrane damaging, detergent-like effect of apoA-I.

Although significant quantities of untagged apoA-I can be prepared using this two-step isolation protocol, we sought to assess functionality of the final protein product. The untagged apoA-I product resisted guanidine HCl-induced denaturation to an extent similar to other apoA-I preparations [31], and cholesterol efflux assays revealed this apoA-I preparation is functional. Likewise, negative stain electron microscopy confirmed that apoA-I forms rHDL upon incubation with phospholipid.

A critical aspect related to use of recombinant apoA-I produced in *E. coli* is the extent to which it is contaminated by bacterial endotoxin. Indeed, LPS contamination is a major problem encountered in the use of bacteria in the manufacture of proteins. While affinity purification of His-tag apoA-I yielded a modest reduction in endotoxin contamination, the protocol outlined herein reduced endotoxin levels by one million-fold. Although the final preparation still contained detectable endotoxin levels, the amounts were close to Good Manufacturing Practice standards. Given that endotoxin limits for protein-based injectables are typically 0.5 EU/mg, an additional endotoxin removal step(s) [32] may be required for use of recombinant apoA-I isolated by the present protocol in humans. Regardless, overall low endotoxin content together with scalability of the two-step process combines to makes this purification scheme an attractive option to produce WT apoA-I for commercial and pharmaceutical applications. Whereas scale up and yield optimization studies have not been pursued, the methods employed are readily amenable to bioreactor fermentation methods without compromising product purity.

## 5. Conclusions

ApoA-I is an integral component of rHDL employed in a growing number of therapeutic and biotechnology applications. Herein, we report a two-step procedure for isolation of recombinant human apoA-I from *E. coli* without extraneous sequence tags. The method employed takes advantage of reversible folding ⇄ unfolding by apoA-I in response to thermal denaturation. The product apoA-I possesses α-helix secondary structure, forms rHDL upon incubation with phospholipid, promotes cholesterol efflux and has low LPS contamination. Thus, the method described is amenable to production scale up and formulation into therapeutics for human use.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2017.03.015.

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