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Efficient purification of Apolipoprotein A1 (ApoA1) from plasma by HEA HyperCel™: An alternative approach

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ABSTRACT

HDL-ApoA1 plays a pivotal role in the prevention of atherosclerosis and cardiovascular diseases. ApoA1 purification from blood plasma has always remained tedious, involving multiple steps, large volumes of plasma and substantial loss in the final yield of pure ApoA1. In this study, a two-step method has been developed and optimized for the purification of ApoA1 from plasma. Plasma was first subjected to 60% ammonium sulphate $(NH_4)_2SO_4$ precipitation and subsequently, ApoA1 was recovered using mixed mode chromatographic sorbent, HEA HyperCel™. ApoA1 was found to be enriched in 60% $(NH_4)_2SO_4$ supernatant that was dialyzed and injected onto HEA sorbent with 50 mM phosphate buffer pH 7.4. The bound proteins were eluted by decreasing the pH in step-gradient from pH 7.4 to pH 4.0 and subsequently to pH 3.5 using 50 mM sodium acetate buffer. Gel electrophoresis showed elution of homogeneous apoA1 at pH 3.5, with purity and yield of 63%. An interesting feature of this approach is that the purified ApoA1 was monomeric with a mass of 28,079.30 Da as confirmed by MS analysis. This simple and efficient method of purification of apoA1 serves as an alternative method which can be combined with traditional approaches and has a great potential for biochemical and clinical studies.

1. Introduction

High Desnity Lipoprotein (HDL) – Apolipoprotein A1 (ApoA1) is a lipoprotein consisting of 243 amino acids with a molecular mass of 28.3 kDa. Being the major protein component of HDL around 70%, it is a robust and highly multifunctional protein and contributes several of HDL's function [1,2]. It is important in cholesterol homeostasis where it transports cholesterol from peripheral tissues like vascular macrophages to liver a mechanism termed "Reverse Cholesterol Transport" and this cholesterol is believed to be converted into bile salts and removed by excretion [3]. Apart from regulating cholesterol metabolism, it exhibits various beneficial functions and is well known as an anti-inflammatory, anti-oxidative, and anti-apoptotic molecule. Thus, the HDL-ApoA1 is considered as cardio-protective and plays key role in controlling the progression of atherosclerosis a major pathological condition in cardiovascular diseases (CVD) [4–6].

For structure and function studies, recombinant ApoA1 produced from bacteria, mammalian cell lines, *Pichia pastoris* and baculovirus-insect cell systems have been used. But these systems have their own draw backs with respect to codon optimization, expression of mature protein and yield of the protein [7–12]. However, one of the important aspects which help us to understand a protein's function and its role in a pathological condition are its preparation from complex biological fluids/tissues. ApoA1 is predominantly found in plasma and the regular way of purifying ApoA1 is to first isolate HDL from blood plasma by sequential density gradient ultracentrifugation, followed by delipidation by treatment with organic solvents and then subjecting this preparation for ApoA1 purification by means of chromatography like gel filtration, ion exchange, thiophilic interaction and hydrophobic interaction chromatography [13–19]. But, this whole process is cumbersome, costly and time consuming as it requires large volumes of plasma, hours of density gradient ultracentrifugation before obtaining a reasonable amount of HDL particles and in turn ApoA1, for carrying out various studies.

So in this study, a simple two-step purification method for ApoA1 has been optimized and demonstrated. This involves the use of $(NH_4)_2SO_4$ fractionation followed by a single chromatographic step using mixed mode sorbent HEA (*n*- Hexylamine) HyperCel™.The method described here facilitates simple and efficient purification of ApoA1 with good purity.

Abbreviations: ApoA1, Apolipoprotein A1; CVD, cardiovascular diseases; ESI, electrospray ionization; HEA, *n*-hexylamine; MEP, mercapto ethyl pyridine; PPA, phenylpropyl amine; Q-ToF, quadrupole-time of flight; Vcap, capillary voltage

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2. Materials and methods

2.1. Materials

Chromatographic resin HEA HyperCel™ was purchased from PALL Life sciences(Pall Corporation, Port Washington, NY, USA). Apolipoprotein A1, HRP conjugated anti-rabbit IgG secondary antibody, MS grade acetonitrile and formic acid was purchased from Sigma-Aldrich (St.Louis, MO, USA). Clarity™ Western ECL blotting substrate was purchased from Bio-Rad (Hercules, CA, USA). Amicon Ultra-4, 10 kDa molecular weight cut off device was purchased from Merck Millipore Biosciences (Billerica, MA, USA). Skimmed milk powder was purchased from HiMedia Laboratories Pvt Ltd, Mumbai, India and all other chemicals were purchased from SRL Pvt Ltd, Mumbai, India.

2.2. Sample collection

Institutional human ethical committee approval was obtained for this study and blood samples were collected from healthy donors by institution's healthcare personnel. Subsequently, plasma was prepared from the collected blood samples.

2.3. Purification of ApoA1

Blood plasma – 0.25 mL was diluted 25 folds using 50 mM phosphate buffer pH 7.4 and subjected to 60% $(NH_4)_2SO_4$ precipitation at 4 °C, overnight. To separate the precipated proteins from the supernatant, centrifugation was carried out (Eppendorf 5810R, Germany) at 4630*g* for 20 min at room temperature. The separated pellet was re-suspeded in 50 mM phosphate buffer pH 7.4; both supernatant and resuspended pellets were dialyzed against the same buffer. The dialyzed samples were concentrated using Amicon Ultra-4, 10KDa cut off (Merck MilliporeBioscience) device and protein concentration was determined by Lowry's method [20] and subjected to SDS-PAGE electrophoresis [21] under non-reducing condition.

The supernatant was used for ApoA1 purification using mixed mode chromatographic resin – HEA HyperCel™. The chromatographic experiments were carried out at room temperature with Econo Chromatography system (Bio-Rad, USA) and the absorbance was continuously monitored at 280 nm. A 4 mL packed bed column (I.D-1 × 4 cm) was prepared and operated at 1 mL/min flow rate. About 5 mL supernatant (13.5 mg) was injected onto the column that was pre-equilibrated with equilibration buffer, 50 mM sodium phosphate buffer pH 7.4. The unbound proteins were washed with excess of adsorption buffer until the absorbance reached baseline. Elution of bound proteins was done by discontinuous pH gradient using 50 mM sodium acetate buffer pH 4.0, for the first step of elution, 50 mM sodium acetate buffer pH 3.5, with 50 mM NaCl for the second step of elution and 50 mM sodium acetate buffer pH 3.5 for the final step of elution. Fractions of 2 mL were collected. Respective pH elution fractions were pooled, buffer exchanged to 50 mM sodium phosphate buffer pH 7.4 and concentrated. Protein concentration was determined by Lowry's method.

2.4. SDS-PAGE and Western blot analysis

The pellet and supernatant from $(NH_4)_2SO_4$ precipitation and the chromatographic fractions were analyzed on a 12% SDS–PAGE under non-reducing condition for protein identification and homogeneity, respectively. About 3.5 µg of protein from the pooled and concentrated chromatographic fractions were first separated on 12% SDS-PAGE gel and subsequently transferred onto a nitrocellulose membrane for immuno blot analysis. After transferring the proteins onto the membrane, it was blocked with 5% skimmed milk and washed with phosphate buffered saline/Tween 20 (PBST). In-house developed anti-apoA1 polyclonal Abs (raised in rabbit, 1:8000) was used for the detection of ApoA1. Subsequently, HRP-conjugated anti-rabbit IgG whole molecule, secondary Abs (1:3000) was used for probing and developed using chemiluminescence substrate (Clarity™ Western ECL blotting substrate, Bio-Rad, USA), according to the manufacture's instructions. Similar, SDS-PAGE and immunoblot analysis was performed in which the elution fraction containing purified ApoA1 was compared with commercially purchased ApoA1 (Sigma – Aldrich) along with blood plasma for determining homogeneity and to calculate the yield of purified ApoA1 by densitometric analysis. After probing with secondary Abs the blot was developed using diaminobenzidine tetrahydrochloride (DAB) as substrate. The image of gels and immuno-blots were captured using ChemiDoc™ MP imaging system (Bio-Rad, USA). Densitometry analysis was performed using Image Lab™ software version 4.1 build 16 (Bio-Rad, USA).

2.5. Mass spectrometry characterization of ApoA1

The purified protein (2 µg) was directly infused into LC–MS with 1290 infinity LC and 6540 UHD accurate mass Q-ToF mass spectrometer with Agilent Jet Stream ESI source systems (Agilent Technologies, USA). The mobile phase solvents were water with 0.1% formic acid and acetonitrile (ACN) with 0.1% formic acid and flow rate was 0.2 mL/min. A linear gradient of 2%–100% B was achieved in 4 min. The MS Q-ToF was operated in positive ionization mode. Following were the source parameters used: 300 °C gas temperature, 8L/min gas flow, nebulizer 35 psi, 350 °C sheath gas temperature, Vcap 3500 V, nozzle voltage 1000 V and fragmentor 220 V. The MS acquisition was in the range of 300–3000 *m/z*. The acquisition rate was 1 spectra/s and 1000 ms/spectrum. The data acquisition was done with in-built Agilent Mass Hunter Workstation B.04.00.

Data analysis was done using Agilent MassHunter BioConfirm B.07.00 software. The data were processed using the Maximum Entropy Deconvolution algorithm for deciphering the molecular mass of the protein from the acquired total ion chromatogram. The algorithm used top 90% of peak height for calculating average mass with the mass ranging from 15 kDa to 70 kDa and mass step of 1 Da. The peak signal to noise ratio was 30 and number of iterations allowed was 20.

3. Results

3.1. Purification of ApoA1

About 0.25 mL (24 mg total protein) of plasma subjected to 60% $(NH_4)_2SO_4$ precipitation resulted into 13.5 mg of protein in 5 mL supernatant and 9.2 mg protein in the pellet which was re-suspended in 5 mL of 50 mM phosphate buffer pH 7.4. Fig. 2 represents a typical chromatogram of ApoA1 purified from 60% $(NH_4)_2SO_4$ supernatant on HEA HyperCel™ sorbent. The peak 1 corresponds to the flow-through fraction, peak 2 corresponds to first step of elution with 50 mM sodium acetate pH 4.0 buffer, peak 3 corresponds to second step of elution with 50 mM sodium acetate pH 3.5 buffer containing 50 mM NaCl and peak 4 corresponds to final step o elution with 50 mM sodium acetate pH 3.5 buffer, respectively. Peak 4 revealed the presence of ApoA1 with protein concentration of 0.6 mg, which corresponds to a yield of 63% from 0.25 mL plasma (Table 1).

3.2. SDS-PAGE and Western blot

Figs. 1 and 3a represents electrophoretic analysis of proteins obtained from 60% $(NH_4)_2SO_4$ precipitation and from chromatographic experiments, respectively, on a 12% SDS-gel. Interestingly, it was noted that ApoA1 was enriched in supernatant along with albumin as depicted (Fig. 1, lane 2). Analysis of pooled and concentrated chromatographic peak fractions revealed the presence of ApoA1 at ~28 kDa, in peak 4, eluted by 50 mM sodium acetate pH 3.5 buffer (Fig. 3a, lane 5). Elution with 50 mM sodium acetate buffer pH 4.0 (Fig. 3a, lane 3) contained serum albumin with other proteins. The intermediate wash