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Production of a soluble and functional recombinant apolipoproteinD in the *Pichia pastoris* expression system

Shiva Armanmehr [a, b], Hamid Reza Kalhor [b, *], Alijan Tabarraei [c]

[a] Metabolic Disorder Research Center, Golestan University of Medical Science, Gorgan, Iran
[b] Department of Chemistry, Sharif University of Technology, Tehran, Iran
[c] Department of Microbiology, School of Medicine, Golestan University of Medical Science, Gorgan, Iran

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ABSTRACT

ApolipoproteinD (ApoD) is a human glycoprotein from the lipocalin family. ApoD contains a conserved central motif of an 8-stranded antiparallel β-sheet, which forms a beta-barrel that can be used for transport and storage of diverse hydrophobic ligands. Due to hydrophobic nature of ApoD, it has been difficult to generate a recombinant version of this protein. In the present work, we aimed at the production of ApoD in the robust *Pichia pastoris* expression system. To this end, the ApoD gene sequence was synthesized and subcloned for expression in the yeast host cells. Following integration of the ApoD gene into the yeast genomic region using homologous recombination, the ApoD recombinant protein was induced using methanol, reaching its maximum induction at 96 h. Having purified the ApoD recombinant protein by affinity chromatography, we measured the dissociation constant (K_D) using its natural ligands: progesterone and arachidonic acid. Our results provide a viable solution to the production of recombinant ApoD protein in lieu of previous obstacles in generating soluble and functional ApoD protein.

1. Introduction

ApolipoproteinD (ApoD), a secreted glycoprotein, is a member of lipocalin family. Proteins belonging to this family possess a conserved fold consisting of an 8-strand antiparallel β-sheet forming β-barrel [1,2]. Lipocalins are known as a reservoir and transporter of diverse hydrophobic ligands [3]. Ligand binding experiments of ApoD have revealed that the protein binds to important physiological hydrophobic ligands such as arachidonic acid and progesterone [4–6]. Several studies have suggested that ApoD is a multifunctional and multi-ligand protein; nevertheless, its precise function and all of its endogenous ligands are not known [7]. Despite other lipoproteins that mainly are expressed in the liver, main source of ApoD is in the brain [8]. Upregulation of ApoD expression is observed in several pathological conditions such as Alzheimer's disease [9]. In addition, ApoD is the major component in breast gross cystic disease [10,11].

In order to investigate novel applications of ApoD protein, we decided to efficiently produce a recombinant form of ApoD protein. To this end, the *Pichia pastoris* expression system was chosen to express the recombinant protein. The advantages of using the *Pichia* system include facile genetic engineering, high-level expression in spite of low cost, rapid growth, easy handling and scale up, posttranslational modification, lack of endotoxin and viral contamination of products, proper folding, disulfide bond formation, and intra/extracellular protein production [12–14]. Since the yeast expression system could be manipulated to secrete recombinant proteins into extracellular media, this attribute provides the simplification of downstream procedures including efficient purification of recombinant proteins. Furthermore, due to extracellular expression in the yeast system, the purification steps would be much simpler than in bacterial expression systems [15]. For a high-level expression of the desired gene, a strong AOX promoter is used in *P. pastoris*.

There have been a number of reports in generating the recombinant ApoD protein in the bacterial system. However, due to the difficulty in obtaining the soluble form of ApoD, the authors had to use site-directed mutagenesis in order to generate a soluble form of ApoD [4,6].

In the present study, the soluble ApoD protein was produced under the control of AOX promoter. The secreted recombinant

* Corresponding author.
E-mail address: Kalhor@sharif.edu (H.R. Kalhor).

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protein was subsequently purified using affinity chromatography and its functionality was assessed using natural interacting ligands such as progesterone and arachidonic acid. As a final result and with respect to the previous reports on the recombinant expression of ApoD in the bacterial expression system, we suggest that the *P. pastoris* system is a satisfactory candidate for the ApoD production.

2. Materials and methods

2.1. Reagent and media

ApoD gene sequence was synthesized by Bioneer Corporation (Bioneer, Korea). AOX1 primers were synthesized by Biobasic Company (Biobasic, Canada). Ni-NTA affinity chromatography column was purchased from Biobasic Corporation (Biobasic, Canada). Minipreparation plasmid and gel extraction kits were from Geneall (Geneall, Korea). Other reagents were purchased from Sigma.

2.2. P. pastoris media

The growth of *P. pastoris* was carried out in YPD (1% yeast extract, 2% peptone, 1% dextrose) and BMGY (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer, pH 6.0, 1.34% Yeast Nitrogen Base, 4×10^{-5}% biotin, 1% glycerol) culture media. Protein expression was performed in BMMY media containing methanol as an inducer for recombinant protein production.

2.3. Construction of ApoD − pPICZαA expression vector

In order to obtain a maximum expression in the yeast system, after removal of the native signal peptide (1–20 amino acids), the codon sequence of mature human apolipoprotein D gene (reference sequence: NM_001647.3) was optimized by changing synonymous codons to those preferred by the *P. pastoris* host. The codon usage program (Bioneer Corporation, South Korea) is designed to calculate repeat sequences and invert sequences that cause low-quality codon optimization process. The final amino acid sequences of the synthetic gene were identical to the mature native ApoD. The introduced restriction sites were SalI and EcoRI sites. The optimized ApoD sequence (shown in Supplementary data, S1A) was synthesized in pGEM cloning vector by Bioneer Corporation. Afterwards, the synthesized pGEM-ApoD vector was transformed into chemically competent DH5αA bacterial cells. The ApoD gene was isolated with enzyme digestion and subsequently was ligated into the pPICZαA expression vector with N-terminal α-factor secretion signal and 6-histidine tag in C-terminus with T4 ligase enzyme. The transformed colonies with the recombinant ApoD-pPICZαA vector were selected on Low Salt LB plate with 25 μg/ml zeocin concentration. Ultimately, DNA sequencing and restriction enzyme digestions were used to confirm correct insertion.

2.4. Transformation and selection of positive recombinant P. pastoris yeast cells

The recombinant ApoD-pPICZαA vector was linearized using SacI enzyme. Subsequently, the digested vector was transformed into the *P. pastoris* genome by electroporation method at 1500 V. Recombinant *P. pastoris* was selected on YPDS plate (YPD medium with 1 M sorbitol) with 100 μg/ml zeocin concentration, and the cells were incubated for 3 days, at 30 °C. The successful integration of recombinant plasmid was confirmed using PCR reactions; the template for the PCR reactions was extracted using LiOAc protocol. Eventually, DNA sequencing was performed on the amplified products.

2.5. Expression of recombinant ApoD

For the expression of recombinant ApoD, recombinant *Pichia* cells were cultivated in BMGY mass media for 18 h up to the optical density of equal 2. The biomass was separated with centrifugation and subsequently the cells were inoculated in BMMY expression media for 96 h; 1% methanol was added to the media every 24 h.

2.6. SDS-PAGE and mass spectrometry analysis

In order to detect the protein expression, yeast cells were removed from BMMY media by centrifugation after 96 h induction. Supernatant was analyzed with 12% SDS-PAGE. To further confirm ApoD expression, the corresponding band on SDS-PAGE was cut and analyzed with LC/MS-MS method as the following protocol. The protein band was excised from the gel and chopped into small pieces. In-gel digestion was performed with 500 ng trypsin in 50 mM ammonium bicarbonate overnight at 37 °C. The digested peptides were separated by nano-liquid chromatography using DionexUltiMate 3000 RSLC equipped with an analytical column. Then the peptides were injected into a high capacity trap (HCT) Ultra Ion Trap mass spectrometer (Bruker Daltonics) by electrospray ionization (ESI) at 200 °C drying temperature. Finally, MS/MS spectra were searched against the human UniProt database.

2.7. Purification of recombinant ApoD

A nickel affinity chromatography method was used for isolation of recombinant protein with N-terminal 6-histidine tag. Briefly, the supernatant of expression media was dialyzed in PBS buffer, pH 8. Ni-NTA agarose column was equilibrated with 300 mM NaCl, 50 mM NaH_2PO_4 buffer pH 8. After loading the dialyzed sample onto the column, the elution steps were performed using 20, 50, 100 mM imidazole concentrations. The recombinant protein was eluted using 100 mM imidazole concentration. The quality of purification was assessed with SDS-PAGE on different fractions. The yield of purified ApoD recombinant was measured by Hartree modified Lowery method using BSA as a protein standard [16].

2.8. Functional assay of ApoD

The intrinsic fluorescent of recombinant ApoD was performed using H4 Synergy Hybrid microplate reader instrument. Briefly, fixed concentration of purified ApoD, 1 μM was mixed with 0–100 μM ligands (progesterone and arachidonic acid) concentrations. The working solution of progesterone was prepared in 10% dioxane. The stock solution of arachidonic acid was dissolved in 100% ethanol (HPLC grade), and diluted in 10% ethanol for the desired concentrations. The excitation and emission wavelengths were 280 and 340 nm, respectively. After subtraction of background emission, and scaling data to the initial fluorescence (without ligand) as 100% fluorescence intensity, the dissociation constant (K_D) was calculated using OriginPro Software (OriginLab Corporation, USA) with nonlinear least square regression as previously reported [5]., The following equation was used for protein-ligand complex:

$$F = ([P]_0 - [L]_0 - K_d)\frac{f_0}{2} + ([P]_0 - [L]_0 - K_d)\frac{f_{PL}}{2} + (f_0 - f_{PL})\sqrt{\frac{([P]_0 - [L]_0 - K_d)^2}{4} - [P]_0[L]_0}$$

$[P]_0$ and $[L]_0$ indicate the total concentration of protein and ligand at each titration step, respectively. f_0 was the relative fluorescence

quenching of free protein that was set to 100%/[ApoD] and f_{PL} is the relative fluorescence quenching of protein –ligand complex.

3. Results

3.1. Cloning of synthetic ApoD gene

Since ApoD is a human gene, we decided to express ApoD in a robust eukaryotic expression system. For an enhanced expression of the human gene in the *P. pastoris* system, an optimized sequence of ApoD, lacking its native signal peptide, was synthesized (shown in the Supplementary data, S1A). In order to secrete ApoD into the media, the α-factor signal peptide from the yeast was fused in the N-terminus of ApoD protein as shown in Fig. 1, panel A. The alpha factor signal is designed to be processed prior to it secretes into the media. In order to purify the recombinant ApoD protein using nickel affinity chromatography, 6-histidine residues were designed to be included at the C-terminus. Therefore, the final recombinant product would only contain 6-histidine at the C-terminus (Fig. 1, panel A). Using restriction enzyme, EcoRI and SalI, the synthetic ApoD gene was successfully cloned into the appropriate expression vector; positive transformants containing cloned ApoD were selected and examined using appropriate restriction enzymes as explained in details in Materials and Methods.

3.2. Integration of recombinant ApoD into the P. pastoris genome

After verification of the cloned gene by restriction analysis and DNA sequencing, cloned ApoD was integrated into the *P. pastoris* genome using homologous recombination with electroporation method. The PCR analysis was exploited to confirm homologous recombination, at the appropriate sites, in the *P. pastoris* genome. As shown in Fig. 1 (panel B, lane 3), 2.2 kb and approximately 1000 bp amplified products corresponding to native AOX and ApoD fragments, respectively, confirmed the integration of the ApoD gene into the yeast genome.

3.3. Production of soluble recombinant ApoD in media

To produce ApoD recombinant protein, initially recombinant yeast cells were grown up to OD = 2 and methanol was used to induce the ApoD expression as explained in Materials and Methods. Subsequently, the media were harvested after 96 h and the protein expression was analyzed by SDS-PAGE. As shown in Fig. 2, results indicated that the highest expression of secreted recombinant ApoD (29 kDa) was obtained in 1% methanol after 96 h induction. In order to confirm ApoD expression, the 29-kD band was cut off the gel; after performing trypsin digestion, LC/MS-MS analysis of the trypsinized fragments confirmed the presence of the ApoD protein (Fig. 3).

3.4. The purified ApoD possess ligand binding activity

As explained in Materials and Methods, the media containing

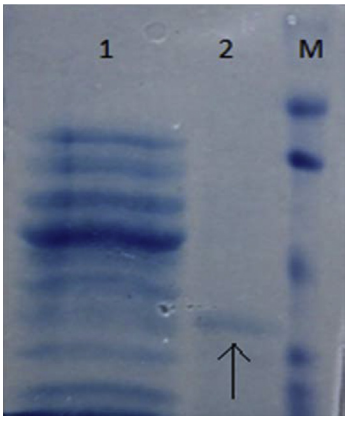

Fig. 2. SDS-PAGE analysis of purified ApoD on 12% polyacrylamide gel stained with coomassie brilliant blue R-250. Lane 1: Supernatant of media culture induced with 1% methanol; lane2: purified ApoD. 29 kDa band is corresponding to recombinant ApoD. The arrow indicates purified recombinant ApoD. From top to button protein marker bands were 78.6, 50.6, 35.9, 27.1, and 19.2 respectively.

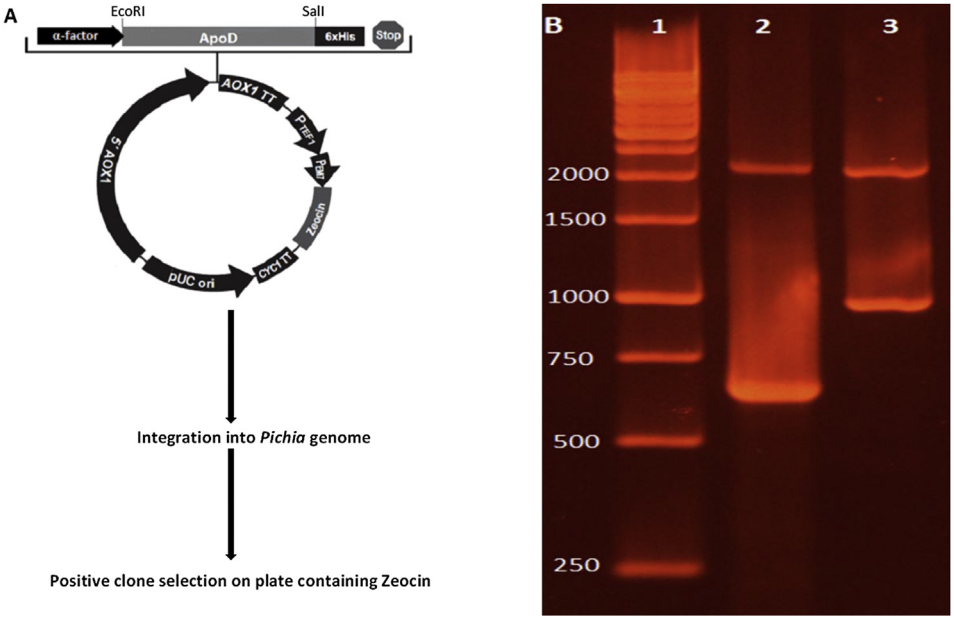

Fig. 1. Outline of cloning and expression of ApoD and PCR amplification to confirm homologous recombination. Panel A: the schematic outline for cloning and expression of ApoD in Pichia pastoris. Panel B: PCR analysis on recombinant Pichia pastoris by AOX1 primers. lane 1: molecular weight marker. Lanes 2 and 3, PCR product of the empty vector and recombinant vector integrated into the Pichia pastoris genome, respectively. 2.2 kb and approximately 1000 bp bands in lane 3 are correspond to native AOX and integrated ApoD in the yeast genome.

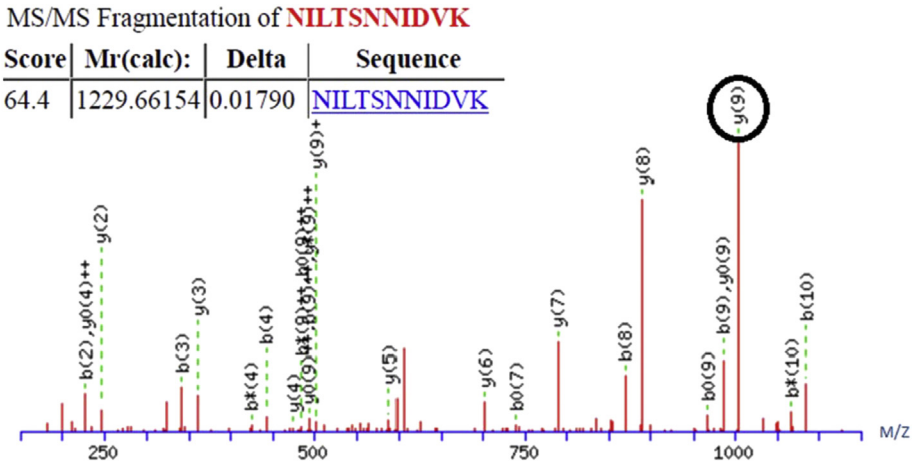

Fig. 3. LC/MS-MS analysis of ApoD expression. The results revealed that ApoD was produced into the media culture successfully. Fragment y [9] with the larger score confirmed the sequence indicated.

ApoD protein was purified using a Ni-NTA affinity chromatography. The high degree of purity of the recombinant ApoD protein was confirmed using silver staining method (S3). In order to assess the function of the recombinant protein, the interaction of the purified recombinant ApoD and its physiological ligands including progesterone and arachidonic acid were investigated. As shown in Fig. 4, different concentrations of ligands were able to quench the intrinsic fluorescence of ApoD. K_D (dissociation constant) values for arachidonic acid and progesterone were 5.8 μM (±1.7) and 3.8 (±1.3) μM, respectively (Fig. 4).

4. Discussion

ApoD is a member of lipocalin family which is abundantly expressed in the number of tissues and plasma. It has been shown that ApoD, due to its structural properties, binds to hydrophobic ligands such as progesterone and arachidonic acid [17]. The precise ApoD function has still remained unknown, but upregulation of ApoD has been reported in the neurodegenerative conditions such as Alzheimer and Parkinson, and also in prostate cancer [9,18–23]. It has been suggested that in Alzheimer's disease, when the inflammatory response is initiated, leading to arachidonic acid derivatives production, the ApoD protein could act as a scavenger to bind the arachidonic acid derivatives leading to ApoD aggregation [24].

Some of the applications of engineered lipocalins known as anticalin have been used in drug delivery and recognition of non-natural ligands [25]. For example, ApoD has been engineered to bind hemoglobin as a ligand [26]. To investigate the recombinant ApoD function in neurodegenerative diseases, we have decided to produce soluble recombinant ApoD. Due to the hydrophobic nature of ApoD, it has been challenging to produce a soluble recombinant version of the ApoD protein [4].

In this work, we have cloned an optimized synthetic ApoD gene in the yeast expression vector. After the integration of ApoD gene into the *Pichia* genome, the secreted recombinant protein was isolated from the gel and its trypsinized fragments were identified using MS/MS analysis. Furthermore, using affinity chromatography,

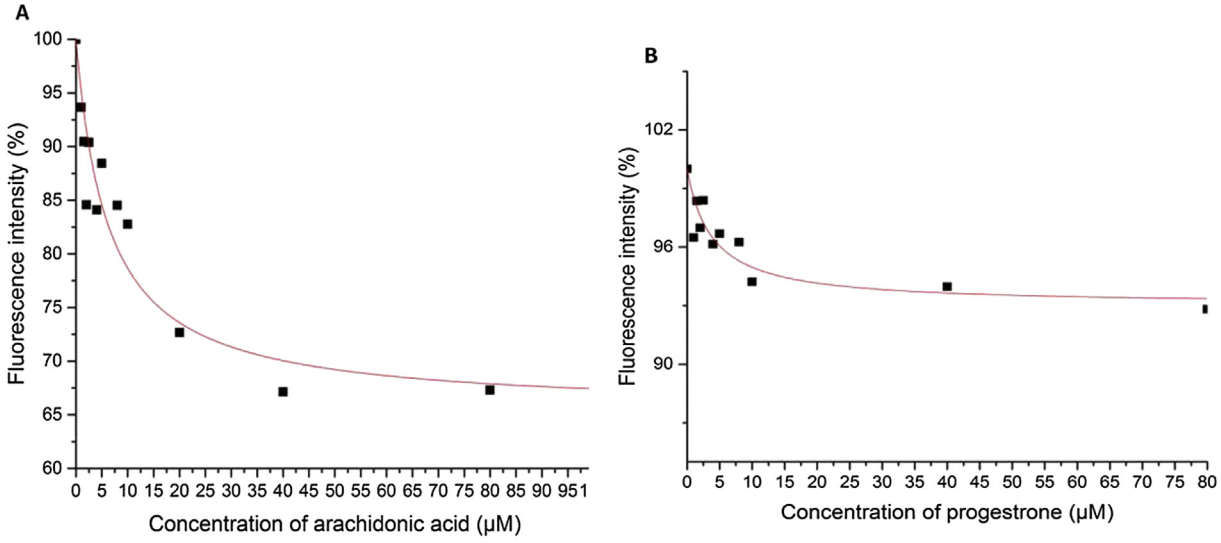

Fig. 4. Determination of the dissociation constant for purified recombinant ApoD. As explained in Materials and Methods, the relative fluorescence intensity of ApoD recombinant protein (vertical axis) is plotted against total ligand concentration (horizontal axis). Panel A: Relative quenching of intrinsic ApoD fluorescence with arachidonic acid (0–100 μM). Panel B: relative quenching of ApoD with progesterone (0–80 μM). The concentration of ApoD was 1 μM in each experiment.

the ApoD recombinant protein was successfully purified, as shown in Fig. 2. Using Lowery assay, the concentration of recombinant ApoD was measured up to 6 mg/L in the final pure sample from 40 mg/L of total proteins that were secreted into the culture media (shown in Supplementary data, S2). This yield, in comparison to the reported bacterial system, is significantly improved.

Fluorescence titration is one of the most common functional assays for lipocalin family members [8]. In order to measure the activity of recombinant ApoD protein, fluorescence titration assay was performed with progesterone and arachidonic acid as its natural ligands. Based on this method, the emission of intrinsic fluorescence of ApoD is quenched after binding of hydrophobic ligands [5].

As shown in Fig. 4, the interaction of arachidonic acid and progesterone with the recombinant ApoD protein led to quenching of intrinsic fluorescence. Indeed, K_D values for arachidonic and progesterone was 5.8 and 3.8 μM, respectively. These values are comparable to the previously reported values of bacterially produced ApoD, 3.2 μM for arachidonic acid and 3.7 μM for progesterone, [5]. It is plausible that posttranslational modifications that could take place in the *P. pastoris* expression system may cause some slight conformational changes of the protein during ligand binding, leading to the differences in K_D value [27]. The mechanism of quenching the intrinsic fluorescence of ApoD by the unsaturated fatty acids is not fully understood. However, it has been suggested that when the hydrophobic ligands interact with the ApoD protein, a Trp residue located on a mobile loop 3 becomes more exposed to solvent, leading to the quenching of the fluorescence [4,5].

Nasreen*et* et al. reported the expression of soluble ApoD in the bacterial system. However, the soluble form was created by introducing a number of mutations in the protein. Owing to the eukaryotic origin of ApoD and with respect to the solubility problem in the bacterial system, we decided to express ApoD, without introducing mutation, in the yeast expression system. *P. pastoris* is a superior eukaryotic expression system compared with the bacterial system due to the simple protein production procedure. In addition the *P. pastoris* expression system is able to secrete a soluble form of recombinant protein; therefore, purification can be a facile one-step process in native condition by affinity chromatography [15].

One of the outcomes in the expression of a recombinant protein in *P. pastoris* is different glycosylation as compared to higher eukaryotic expression system. However, in *P. pastoris*, the recombinant proteins are founded to be glycosylated on the average 8–14 mannose residues per side chain of mainly Asn residues [28]. It is assumed that because of the small number of sugar residues that can be conjugated on the expressed protein in the yeast, glycosylation would not interfere with the function of the recombinant protein [29]. Although we have not shown any evidence of ApoD glycosylation in our system, it is still plausible that ApoD might undergo glycosylation in the yeast expression system.

The expression of functional and soluble recombinant ApoD would allow investigators to better study ApoD complex function including its role in protein amyloid formation in Alzheimer's and other neurodegenerative diseases.

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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.2016.01.016.

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