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

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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

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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