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Reports of Biochemistry & Molecular Biology
Vol.6, No.2, Apr 2018

Short article

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Cloning, Expression, Purification and CD Analysis of Recombinant Human Betatrophin

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Abstract

Background: Betatrophin is a member of the angiopoietin-like (ANGPTL) family that has been implicated in both triglyceride and glucose metabolism. The physiological functions and molecular targets of this protein remain largely unknown; hence, a purified available protein would aid study of the exact role of betatrophin in lipid or glucose metabolism.
Methods: In this study, we cloned the full-length cDNA of betatrophin from a human liver cDNA library. Betatrophin was expressed in the pET-21b-*E. coli* Bl21 (DE3) system and purified by immobilized metal-affinity chromatography and ion-exchange chromatography.
Results: Circular dichroism spectroscopy revealed α-helix as the major regular secondary structure in recombinant betatrophin.
Conclusion: The production method is based on commonly available resources; therefore, it can be readily implemented.

Keywords: CD spectroscopy, Human betatrophin, Recombinant protein

Introduction

Betatrophin, also known as lipasin, TD26, ANGPTL8, and RIFL, is a circulating protein that is expressed mostly in liver and white and brown adipose tissue. The human betatrophin gene is located on chromosome 19p13.2, on the strand opposite of the host gene dedicator of cytokinesis 6 (Dock6). The primary structure of betatrophin predicts a 198-amino-acid protein with a signal peptide at the N-terminus and two coiled-coil domains. To date, several betatrophin orthologs have been identified in mammals; however, there is no evidence of betatrophin expression in other species.

Initially betatrophin was detected as a tumor-associated antigen. In 2012, its role in control of serum triglyceride levels or its probable function in lipid metabolism was described (1, 2). Recently, betatrophin was reported to mediate an increase in beta-cell proliferation in response to insulin resistance induced by treatment with the insulin antagonist S961 (3-5). However, the role of betatrophin in beta cell proliferation and lipid metabolism remains controversial (6, 7); particularly, recent findings appear to be in conflict with the Yi-et al. observations (8). Therefore, further studies may be necessary to determine the biochemical properties of this protein. The current study was conducted to produce a recombinant form of this protein in a bacterial host and evaluate its biophysical properties.

Materials and Methods

cDNA cloning of human betatrophin

Primers were designed to the 5′ and 3′ ends of human betatrophin based on the sequence from

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Received: Apr 4, 2017; Accepted: Jun 16, 2017

GenBank accession number NM_018687.6 The forward and reverse primers were: 5' GCCCCCATGGGCGGCCCA 3' and 5' GGCTGGGAGCGCCGCTGT 3', respectively. These primers were used to PCR amplify the coding sequence of betatrophin from a human liver QUICK-Clone™ cDNA library (Clontech Laboratories, USA). *Not*-I and *Xho*-I sites were incorporated into the primers at the 5′ and 3′ ends, respectively, to allow sub-cloning into the pET-21b bacterial expression vector (Clontech Laboratories, USA).

Amplification of the betatrophin coding sequence

Betatrophin DNA was amplified by PCR in a gradient thermal cycler (Eppendorf, Germany). The reaction mixture contained 200 ng of primer, 200 µmol of dNTP mixture, 1X PCR buffer, and 1U of ExPrime Taq DNA polymerase (GenetBio, Korea). Amplification conditions were as follows: 5 min at 96 °C, followed by 35 cycles of 94 °C for 40 sec, 61 °C for 40 sec, 72 °C for 60 sec, and a final extension at 72 °C for 10 minutes.

Molecular cloning of betatrophin

The PCR product and vectors were cut with restriction enzymes *Not*-I and *Xho*-I (Fermentase, Lithuania). The purified restricted PCR product was ligated into pET-21b with T4 DNA ligase (Fermentase, Lithuania). Competent *E. coli* TOP-10 cells were transformed with the ligation product and the transformed cells were grown at 37 °C overnight on Luria-Bertani (LB) plates containing 100 µg/ml of ampicillin (Sigma-Aldrich). Plasmids were isolated from five positive clones using a QIAGEN plasmid extraction and purification kit and the insert size was confirmed by PCR using T7 polymerase-specific primers (T7 Promotor primer; TAATACGACTCACTATAGGG and T7 Terminator primer; GCTAGTTATTGCTCAGCGG). The selected plasmids were sequenced at the Gene-Ray biotechnology company. Nucleotide and presumed protein sequences were examined by the ExPASy tools (http://www.expasy.org/tools/). The nucleotide sequence was searched for homology using BLASTn at NCBI.

Expression of recombinant betatrophin

E. coli BL-21 (DE3) as the expression host was transformed with the pET-21b-betatrophin construct. A positive clone harboring the plasmid was picked and grown overnight in liquid LB with 100 µg/ml of ampicillin at 37°C to control expression of the desired protein. After reaching the 0.6 OD_{600nm}, expression was induced using isopropyl-1-thio-β-D-galactopyranoside (IPTG). Optimization of expression was attempted on 5 ml cultures of LB at of IPTG concentrations of 0.6, 0.8, 1, and 1.2 mM, temperatures of 18, 25, and 37 °C, and incubation times of 8, 12, and 18 hours.

After optimization, the *E. coli* cells were cultivated in larger volumes and the final suspension was centrifuged at 12000 × g at 4 °C for 30 min to remove the cellular debris and resuspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, and disrupted by sonication using 6 x 10 second cycles at 200-300 W. The lysate was centrifuged at 12000 × g for 30 min at 4 °C and aliquots from the supernatant and pellet were used to determine recombinant protein expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining. The inclusion bodies were separated and solubilized using denaturing lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, and 6 M urea (9).

Purification of recombinant betatrophin

The solubilized fraction was dialyzed against binding buffer containing 0.15 M NaCl, 10% glycerol, 0.5% Triton X-100, and 50 mM sodium phosphate, pH 7.8, for 18 h at 4 °C and filtered through a 0.22 µm polyvinylidene fluoride (PVDF) membrane. Three hundred µl of the dialyzed fraction were loaded onto a Ni-IDA resin column (Parstous Co.). The protein was eluted using the recommended buffer containing 0.15 M NaCl, 50 mM sodium phosphate, and 0.5 M imidazole, pH 7.8, at a 0.5 ml/min flow rate. The eluted fractions were collected in 1 ml volumes.

Fraction purity was examined by electrophoresis on 15% SDS-PAGE using 10 µl of each purified fraction per lane. The protein bands were visualized with 0.05% Coomassie brilliant blue G-250. The protein content was measured by the Lowry method using bovine serum albumin (BSA) as the standard (10).

The recombinant protein from the metal affinity-purified fractions was cleared using ion-exchange chromatography. Proteins were resolved by an AKTA prime plus fast protein liquid chromatography (FPLC) system (GE Healthcare, Life Science, USA) using diethylaminoethyl (DEAE) sepharose 6B resin (Pharmacia, Uppsala, Sweden) to remove all impurities, especially bacterial lipopolysaccharides.

The betatrophin-rich fraction was dialyzed against starting buffer containing 20 mM Tris-HCl, pH 8.6, for 18 h at 4 °C and filtered through a 0.22 µm PVDF membrane. Approximately 10 ml of resin was packed into an adjustable column and equilibrated with the same starting buffer. Five hundred µl of the dialyzed extract were loaded onto the column. Proteins were eluted with the starting buffer containing a linear gradient of NaCl at a constant rate of 0.75 ml/min. The eluent was monitored at 280 nm and results were recorded.

Circular dichroism (CD) spectroscopy

The recombinant betatrophin was examined in the far ultraviolet (UV) region of 190-240 nm, which corresponds to peptide bond absorption, using an AVIV model J810 spectropolarimeter (JASCO) to give the content of regular secondary structures. Far UV-CD spectra of a 40 µg/ml solution of purified proteins in PBS buffer, pH 6.5, were obtained with a 1 mm path length quartz cell. The background was corrected against the buffer blank. The data were calculated as molar ellipticity (deg.cm^2/dmol) assuming a mean residue number and average molecular weight of 27.5 kDa for betatrophin using the CD deconvolution software. The molar ellipticity was determined as $[\theta] = [100 \times (MRW) \times \theta_{obs}/(cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength and c is the light path length in cm (11).

Results

The human betatrophin gene was amplified from a cDNA library with 5′ and 3′ cloning primers. A single PCR product of 531 bp was obtained (Fig. 1) and cloned into pET-21b. The recombinant plasmid was confirmed by gene sequencing. The results verified that the betatrophin insert was subcloned into pET-21b and the expression vector was constructed successfully. Also, the nucleotide sequence of the cloned fragment displayed 100% identity with the betatrophin nucleotide sequence in GenBank: KF809856.1.

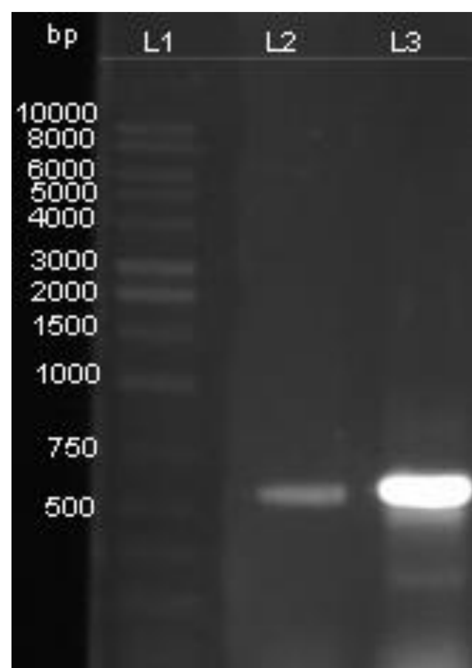


Fig. 1. Agarose gel electrophoresis of betatrophin PCR products obtained from a human cDNA library. Lane 1, DNA 10 kbp ladder, Lanes 2 and 3, 531 bp PCR products.

The *E. coli* BL-21 cells transformed with the construct were grown in LB media at 37 °C until OD reached 0.6; then protein expression was induced with IPTG overnight. The optimal IPTG concentration for protein induction was 1 mM (Fig. 2). The expression at 1 mM IPTG was also corroborated in the time course experiments. Finally, the influence of temperature on betatrophin expression was tested at 18, 25, and 37 °C. The highest-yield expression was reached at 37 °C for 12 hours.

The results indicated that the optimal condition for the expression of the construct in LB media was at 37 °C with 1 mM IPTG for 12 hours.

Assessment of protein expression in the soluble and insoluble cell lysate fractions revealed that a significant amount of protein distributed into the insoluble fraction. As indicated in the SDS-PAGE, a distinct band with considerable intensity was present in the insoluble fraction following treatment with 6 M urea (Fig. 3A).

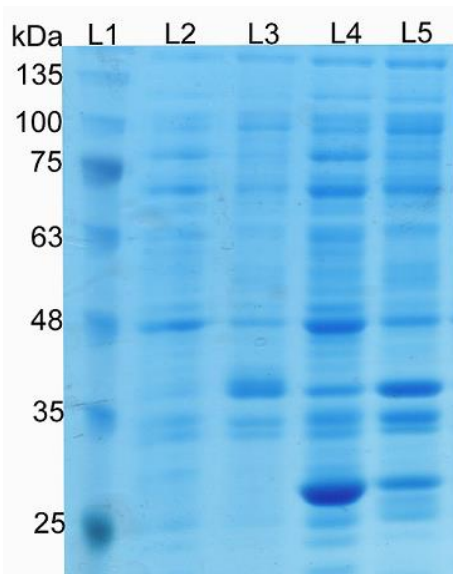


Fig. 2. SDS-PAGE of recombinant betatrophin expressed at different IPTG concentrations. Lane 1; Protein molecular weight marker, Lane 2; 0.6 mM IPTG, Lane 3; 0.8 mM IPTG, Lane 4; 1 mM IPTG, Lane 5; 1.2 mM IPTG. (The expression without IPTG was low, data not shown)

The recombinant human betatrophin was purified by metal-chelate and ion-exchange chromatography. The purification yield was 3 mg/L of culture in Ni-IDA chromatography and 2 mg/L of culture in ion-exchange (Fig. 4A), indicating that a considerable amount of protein was lost during the clarification steps.

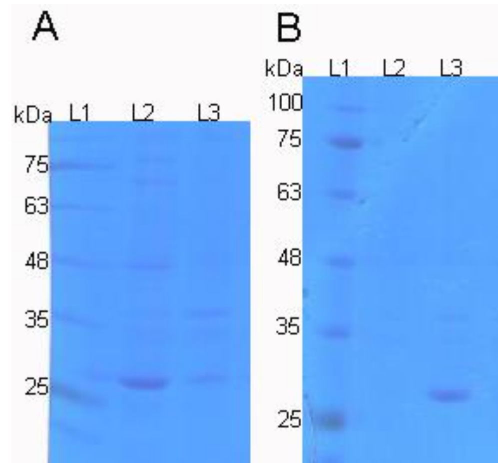
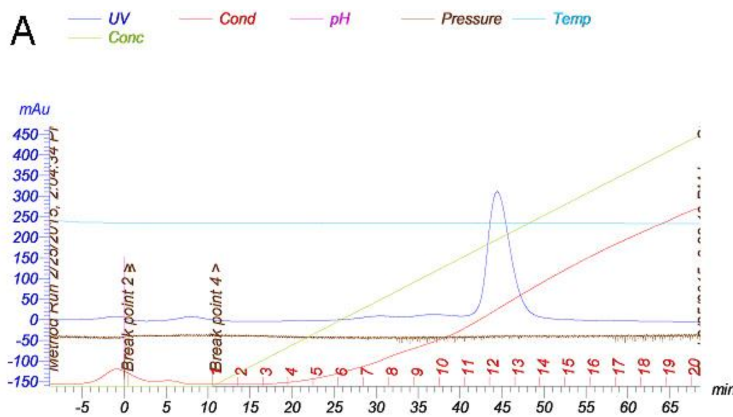


Fig. 3. Purification of recombinant betatrophin A. SDS-PAGE of recombinant betatrophin. In each case, following cell lysis and centrifugation, the cell pellet was isolated and extracted with urea (see methods for details). Lane 1; Molecular weight standard, Lane 2; Cell pellet following the centrifugation of urea-treated-cell extracts, Lane 3; Supernatant following the centrifugation of urea-treated cell extracts.
B. SDS-PAGE of recombinant betatrophin after Ni-IDA chromatography. Lane 1; Molecular weight standard, Lane 2; supernatant, Lane 3; pellet.



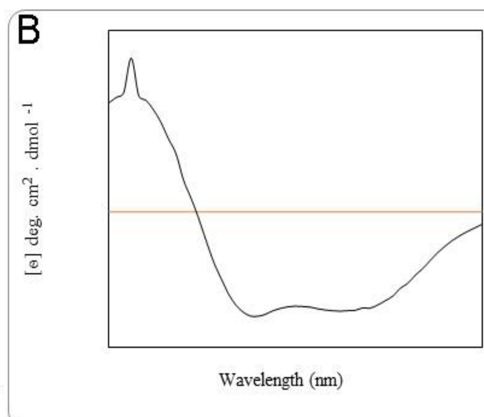


Fig. 4. A. The chromatogram of recombinant betatrophin purification using ion exchange chromatography. **B.** CD spectra of recombinant betatrophin.

The SDS-PAGE of protein fractions obtained from the chromatography showed a single protein band of 27.5 kDa, the expected molecular mass for the recombinant protein based on the estimated molecular mass of human betatrophin of 22 kDa, the 6-His tag of 2.7 kDa, and 2.8 kDa from other amino acids encoded in the plasmid (Fig. 3B).

The results of the CD analysis of the recombinant betatrophin are shown in Fig. 4B and Table 1. The main regular secondary structure found in recombinant betatrophin was α-helix. Notably, no β-sheet structure was revealed for recombinant betatrophin.

Table 1. Percentages of secondary structures in betatrophin

	Secondary structures			
	α-Helix	β-Sheet	β-Turn	Random Coil
Percentage of the structure	40.7	0.0	26.2	33.2

Discussion

Betatrophin is a novel liver-secreted protein with yet unknown function and mechanism. Betatrophin was originally reported to be associated with the control of beta cell proliferation (3). It was reported that betatrophin controls beta cell proliferation in mice treated with the insulin receptor antagonist S961.The direct or indirect mechanism of action for betatrophin has not been described; however, it is clear that betatrophin is not obligatory for beta cell function or compensation of beta cell growth response to insulin antagonist receptors (12, 13). Furthermore, betatrophin overexpression does not increase beta cell proliferation or blood glucose levels (14). Zhang et al. indicated that betatrophin inhibits lipoprotein lipase (LPL) activity and may control serum triglyceride level (15). Their study also showed that betatrophin level was nutritionally regulated.

Generally, many aspects of betatrophin function are unknown and in some cases are controversial. To understand betatrophin's structure and function, pure protein in adequate quantities for detailed biochemical and biophysical studies is needed. Thus, a straightforward procedure for production of recombinant protein with high yield would be valuable. Therefore, we tried to simplify the human betatrophin cloning, expression, and purification process by cloning the gene in the pET system. However, because the structure of most human proteins is relatively complex, their functional recombinant forms may be better expressed in eukaryotic systems (16).

In this study, the optimized condition for expression of recombinant betatrophin was found to be at 37 °C with 1 mM IPTG. These expressions conditions are similar to those found in other reports of recombinant protein expression in bacterial hosts, however, we found no data on betatrophin cloning or expression conditions.

In this study CD spectroscopy was used to determine the secondary structures in recombinant betatrophin. We found that the recombinant betatrophin consists of 40.7% α-helix, 26.2% β-turn, and 33.2% random coil, which agreed with the predicted secondary structure obtained by the Network Protein Sequence Analysis tool (data not shown) (17). We found no published data on betatrophin structure.

In conclusion, in this study, the human betatrophin gene was amplified by conventional PCR from a human cDNA library and cloned into pET-21b. The recombinant protein was expressed in BL21 (DE3) cells, and purified and clarified by metal affinity and anion exchange chromatography, respectively. Circular dichroism revealed that the major secondary structure in recombinant betatrophin is α-helix. The production procedure is easy to implement, which allows the production of large quantities of recombinant protein with no need for special equipment or media. Hence, more studies on betatrophin structure and function could be designed to determine of its effects on beta cell proliferation and triglyceride levels.

Acknowledgement

We are grateful to the deputy of research and technology of Qazvin University of Medical Sciences and Immunology Research Center of Iran University of Medical Science for financial support on this work.

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