Cloning, expression, and spectral analysis of mouse betatrophin

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Abstract

Background: Betatrophin, a novel secretory protein from liver and fatty tissues, is believed to be involved in lipid and glucose metabolism. However, its precise physiological role remains unclear. Here, we report the cloning, expression, and purification steps of mouse betatrophin in a prokaryotic system, followed by its structural analysis.

Methods: Specific cloning primers were used to amplify the coding sequence of mouse liver betatrophin. The product was cloned into pET28 and expressed in E.coli BL21 (DE3) cells. The suitability of the refolding procedure was assessed by determining secondary structures of the initial and refolded proteins using circular dichroism spectroscopy.

Results: The polymerase chain reaction resulted in a 549 bp nucleotide sequence, encoding a 183 amino acid polypeptide, with an apparent molecular weight of 21 kDa, which was expressed in an inclusion body. Following an optimization and refolding procedure, the recombinant protein was purified by anion exchange and metal affinity chromatography. CD spectra revealed that the refolded protein has suitable configuration.

Conclusion: We believe that the produced betatrophin is suitable for further biochemical studies on glucose and lipid metabolism.

Keywords: Mouse betatrophin, Recombinant protein, Refolding, CD spectroscopy, Chromatography

Introduction

Betatrophin, also named TD26, LOC55908, RIFL, EG624219, lipasin, and ANGPTL8, is predominately expressed in liver and fat cells; however, its exact function and molecular targets are not still clear. Recent studies show that this recently identified hormone may promote pancreatic beta cell proliferation and improve glucose tolerance in patients with diabetes (1,2). Moreover, it was found that hepatic expression of this molecule has been upregulated in insulin-resistant mouse models (1,3). This overexpression increases the rate of pancreatic beta cell proliferation and the size of islets and their insulin content; therefore, it could be beneficial for glucose homeostasis (1,4). Hence, it seems that betatrophin acts as a he-patic signal for compensatory growth of beta cells in response to insulin resistance; thus, it could be a novel candidate in therapeutic approaches, such as regeneration of beta cells in patients with diabetes (5,6). In addition to glucose metabolism, betatrophin is linked to lipid metabolism (7,8). Betatrophin gene deletion largely reduces serum triglyceride levels in mice, whereas adenovirus-mediated betatrophin overexpression increases circulating triacylglycerol concentrations (9,10). Betatrophin may also affect blood lipid profiles by controlling hepatic very-low-density lipoprotein (VLDL) secretion and lipoprotein lipase activity (7). Notably, betatrophin-deficient mice exhibited no alterations in glucose homeostasis,

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↑What is "already known" in this topic:
Betatrophin is considered a novel candidate molecule for treatment of lipid and glucose metabolism disorders; however, the exact function of this protein is still unknown.

→What this article adds:
This article describes cloning, expression, purification, and spectral analysis of mouse betatrophin. We believe this research provides pharmaceutical companies with useful data about the structure of this potentially therapeutic recombinant protein to produce a functional molecule applicable in both basic and clinical studies.

Table 1. Primer sequences used for cloning of mouse betatrophin and evaluation of the insert size

Vector/Sequence	Primers	Sequence: 5'→ 3'
pET-28a	Forward-Bam H1	ATAGGATCCGTGCGACCCGCTCCAG
	Reverse-Sal1	ATAGTCGACGGCTGGGAGGGCTGC
T7-Promotor/Terminator	Forward	TAATACGACTCACTATAGGG
	Reverse	GCTAGTTATTGCTCAGCGG

when fed normal or high-fat diets (11).

Mouse betatrophin (m-betatrophin) has been identified as the GM6484 gene, positioned on chromosome 9, and its transcript, NM_001080940.1, comprising 3 exons encoding a 198 amino acid protein with a short signal peptide (1). Betatrophin and its orthologs have only been detected in mammals; its homologs are absent in other species (12). Betatrophin has no conserved domain; however, it is considered as a novel atypical angiopoietin-like (ANGPTL) family member (13). The nearest paralog protein is ANGPTL3, which has only 22% amino acid identity with betatrophin (8). The 3 dimensional structure of betatrophin has not yet been determined.

Despite the defined function of betatrophin as a critical regulator of metabolic pathways in preclinical models, little is known about its role in energy metabolism. Recently, betatrophin was reported to be increased in patients with type 1 diabetes (1,5) although no association has yet been found between betatrophin concentration and insulin secretion (14,15). However, the recent discovery that betatrophin is a circulating factor secreted in response to insulin resistance has raised fundamental questions about a potential link between betatrophin and glucose homeostasis as well as lipid metabolism in insulin-resistant individuals (15). In this study, we aimed at producing sufficient amounts of recombinant m-betatrophin in a prokaryotic system for use in further structural and functional studies.

Methods
RNA extraction and cDNA synthesis
Total RNA was extracted from fresh mouse liver using TRIzol reagent (Invitrogen Carlsbad, CA, USA). Genomic DNA contamination in the RNA preparation was eliminated with RNase-free DNase (Thermo Fisher, USA) according to the manufacturer's instructions. The quantity, purity, and integrity of the extracted RNA were determined by measuring absorbance at 230, 260, and 280 nm, and electrophoresing the product of 1% agarose gels. First-strand cDNA was synthesized from the total RNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher, USA) using oligo $(dT)_{18}$ primers according to the manufacturer's protocol.

PCR amplification and cloning of m-betatrophin
The betatrophin coding sequence was retrieved from NCBI, and specific cloning primers containing Bam H1 and Sal1 restriction sites were designed as summarized in Table 1. The PCR was performed in 50 µL volumes containing 2.5 U of ExPrime-Taq DNA polymerase, 5 µL of 10x reaction buffer, 2 mM of dNTPs, 100 pM of each primer, and approximately 100 ng of cDNA. The m-betatrophin gene was amplified by initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 40

seconds, 61°C for 40 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplified DNA was electrophoresed on a 1.3% agarose gel, and the single DNA band was extracted with a DNA extraction kit (Gene All, Korea). Both the PCR product and the pET28a plasmid were then double digested with Bam H1 and Sal1 (Thermo Fisher, USA), and ligated with T4 DNA ligase. E. coli TOP 10 cells were transformed with the ligation product and the selected colonies were analyzed for appropriate insertion of the target sequence by colony PCR using T7 promotor and terminator universal primers (Table 1), followed by DNA sequencing.

Sequence analysis and recombinant protein overexpression
The nucleotide sequence was analyzed using the Blastn (http://www.ncbi.nlm.nih.gov/BLAST). Molecular weight (Mw), theoretical isoelectric point (PI), and the conserved domains were calculated using ExPASy (http://expacy.org). The protein secondary structure was predicted using Sopma (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) software.

E.coli BL21 (DE3) cells were transformed with the recombinant pET28a, containing 6His-tagged m-betatrophin. Luria Bertani medium (Merck, Darmstadt, Germany), containing 50 µg/mL of kanamycin (Sigma, Saint Louis, USA), was inoculated with a single colony harboring the recombinant plasmid and incubated at 18, 25, or 37°C with shaking at 200 rpm overnight until A_{600} reached 0.6. Then, overproduction of the recombinant protein was induced by adding 1 mM isopropyl β-D-1 thio galactopyranoside (IPTG) (Sigma, Saint Louis, USA) for 6 hours. The cells were pelleted by centrifugation, the pellet was then resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, containing 100 mM NaH_2PO_4 and 5 mM DTT), and lysed by 4 freeze-thaw cycles. The lysate was centrifuged at 9000 x g for 10 minutes at 4°C, and the inclusion body was washed 3 times in lysis buffer supplemented with 1 M urea. The final pellet was solubilized in lysis buffer containing 6 M urea by mixing for 30 minutes at 4°C. The protein solution was then clarified by centrifugation at 13000 x g for 30 minutes at 4°C and stored at -80°C.

Protein purification and refolding
The solubilized proteins were refolded in 3 steps by dialyzing against 50 mM Tris-HCl, pH 8.0, and containing decreasing amounts of urea (16). In brief, in the first step, 4 M urea, 2.5 mM DTT, and 1 mM EDTA were added to the dialyzing buffer, while in the second step, the dialysis buffer contained 2 M urea, 1 mM DTT, and 1 mM EDTA. In the third step, the proteins were dialyzed against 20 mM Tris-HCl, pH 8.0, to prepare them for anion exchange

chromatography (17). The refolded product was filtered through a 0.22 μm PVDF membrane, and 500 μL of it was chromatographed on 10 mL of DEAE sepharose 6B resin (Pharmacia, Uppsala, Sweden), which had been pre-equilibrated with 20 mM Tris-HCl, pH 8.0 as the starting buffer. The ingredients were then eluted with the starting buffer containing increasing amounts of NaCl at a constant rate of 0.75 mL/min on an AKTA PRIME PLUS FPLC system (GE Health care Biosciences, Uppsala, Sweden). The effluent was monitored by measuring the absorbance at 280 nm and analyzed on 15% SDS-PAGE.

Fractions containing the greatest soluble m-betatrophin concentrations were chromatographed on Ni-iminodiacetic acid (Ni-IDA) sepharose (Arya Tous Biotech, Mashhad, Iran) that had been pre-equilibrated with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 5% glycerol, and the content was recirculated for 10 minutes in the column to increase the amount of protein bound. The unbound proteins were washed out with 5 column volumes of wash buffer, containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 20 mM imidazole. 6His-tagged m-betatrophin was eluted by washing the column with elution buffer, containing 50 mM Tris-HCl, pH 8.0, 500 mM imidazole, and 150 mM NaCl. The final effluents were collected and purity of the contents was examined by 12% SDS-PAGE.

Secondary structure determination

The secondary structure of m-betatrophin was measured using circular dichroism (CD) at 25°C in a JASCO J-810 spectropolarimeter, equipped with a Peltier-type temperature controller. Because CD spectra analysis requires highly purified sample, the purified protein was dialyzed against 10 mM Tris-HCl, pH 8.0, containing 25 mM NaH2PO4. Far-UV CD spectra were recorded at a protein concentration of 2.2 mg over a wavelength range of 190 to 240 nm with a scan rate of 20 nm/min. Data were correct-ed for the solvent and smoothed using the Savitzky-Golay algorithm. The molar ellipticity was determined as $[θ] = [100 × (MRW) × θobs/(cl)$, where "θobs" was the observed ellipticity in degrees at a given wavelength, "c" was the concentration in mg/mL, and "l" was the light path length in cm (18). For further analysis, the CD spectra data were uploaded onto the K2D2 server to estimate the secondary structural content of m-betatrophin (19). This server compares real and predicted values by means of the Pearson correlation coefficient (r) and the root mean square deviation (RMSD) of proteins with known 3-dimensional structures. The applied algorithm was described in detail elsewhere (20-22).

Results
Gene cloning and sequence analysis

A 549 bp DNA fragment was obtained by PCR using mouse liver cDNA as the template (Fig. 1A). The m-betatrophin predicted mature protein sequence and contained 183 amino acids with a predicted molecular mass of 20.7 kDa and calculated isoelectric point (pI) of 6.18. The protein deduced from the cloned sequence agreed with the previously predicted protein sequence, and the DNA sequence was submitted to NCBI database with the accession number KX357380.

The nucleotide sequence encoding m-betatrophin was amplified with specific primers and ligated into pET28a. The size of the insert amplified by colony PCR using T7 universal primers was approximately 850 bp (Fig. 1B, L2-L4). Of this approximately 850 bp, 549 bp encoded m-betatrophin (Fig. 1A, L2-L4 and Fig. 1B, L5) and 300 bp encoded T7 polymerase. This finding was supported by restriction digestion with Bam H1 and Sal1 and agarose gel electrophoresis (Fig. 1C). DNA sequencing verified the identity of the construct. The aligned sequence was 78% identical with human betatrophin and 91% and 68% similar with Rattus norvegicus betatrophin and porcine
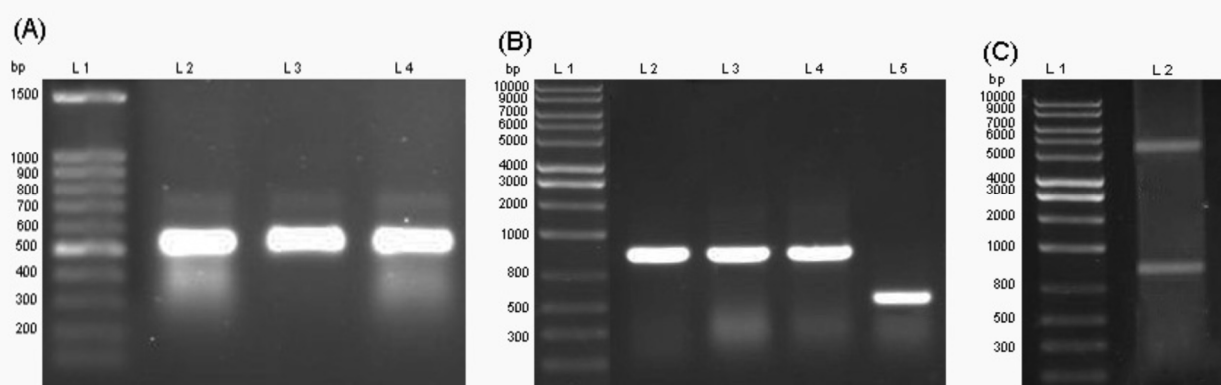

Fig. 1. Cloning of mouse betatrophin in pET28a
  A. Agarose gel of the PCR amplification product of mouse betatrophin coding sequence. L1: DNA molecular weight marker (100-1500 bp); L2-4: Electrophoresis of the amplified betatrophin gene showing amplification of a 549 base-pair DNA fragment.
  B. Agarose gel of colony PCR products amplified with universal T7 promotor/terminator and betatrophin-specific primers. L1: DNA molecular weight marker (300-10000 bp), L2-4 PCR products from three different colonies amplified with T7 universal primers showing amplification of an approximately 850-bp DNA fragment, consistent with the estimated size of the intended target DNA; L5: Electrophoresis of the PCR product with betatrophin-specific primers showing specific amplification of the 549-bp mouse betatrophin gene inserted in the construct.
  C. Electrophoresis of the digested construct. L1: DNA molecular weight marker (300-10000 bp), L2: pET28a-mouse betatrophin construct digested with Sal1 and Bam H1, indicating a 5.4 kbp plasmid backbone and the 549 bp betatrophin gene.

lipasin, respectively.

Expression and purification of m-betatrophin

The induced recombinant protein migrated with an apparent molecular weight of 32 kDa on SDS-PAGE (Fig. 2A). Most of the expressed protein was insoluble; thus, we purified it under denaturing conditions using 2-6 M urea. To purify the protein, we first used anion exchange chromatography to remove major impurities, such as bacterial lipoproteins (Fig. 2D). Desired fractions were then chromatographed on a Ni-IDA column. The recombinant protein was eluted from the column with 500 mM imidazole, and the eluates were analyzed by SDS-PAGE (Fig. 2C). The protein migrated with an apparent molecular mass of approximately 32 kDa, which was consistent with the calculated mass of the construct containing m-betatrophin of 20.7 kDa, the plasmid backbone of 6.5 kDa, and a number

of His-tag repeats of approximately 5.4 kDa. After chromatography, a single 32 kDa band was observed (Fig. 2C).

Refolding of the inclusion body-imbedded proteins is a crucial step to confer appropriate conformation and function to the denatured proteins. However, no standard protocol exists for refolding of such proteins. To refold m-betatrophin, we dialyzed the solubilized inclusion bodies against 50 mM Tris-HCl, pH 8.0, supplemented with stepwise decreasing amounts of urea from 3.0 to 0 M over 48 hours. The final yield of recovered recombinant m-betatrophin was 0.44 mg/mL of bacterial culture.

Secondary structure analysis of the recombinant protein

To estimate the secondary structure, we measured far-UV CD spectra of 190 to 250 nm for denatured (solubilized inclusion body proteins) and renatured (refolded) m-
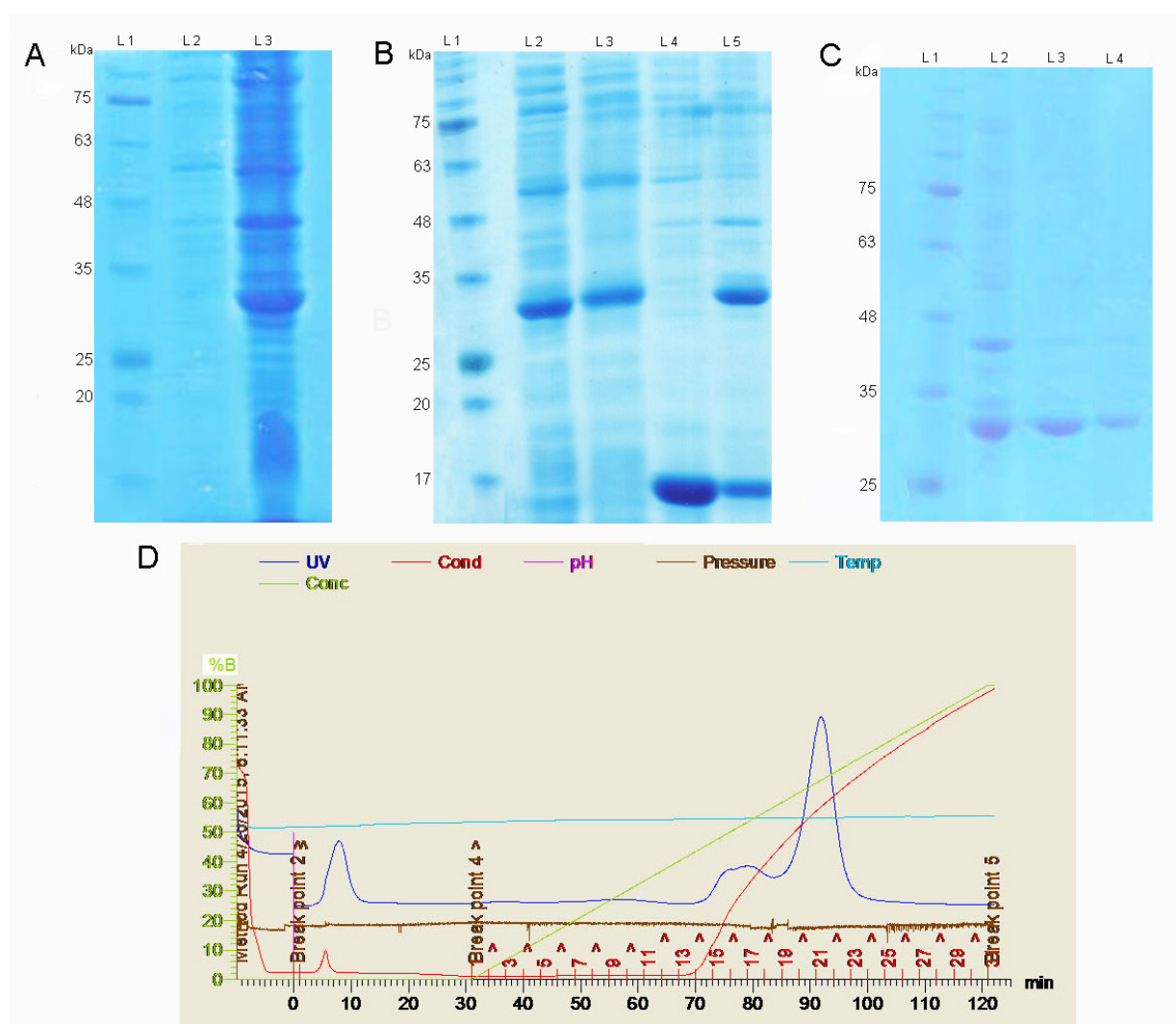

Fig. 2. Purification of recombinant mouse betatrophin
  A. SDS-PAGE showing expression of recombinant betatrophin in E.coli BL21. L1: Low molecular weight protein marker, L2: Supernatant from the bacterial lysate, L3: Precipitated fraction of the bacterial lysate.
  B. SDS-PAGE of fractions eluted from anion exchange chromatography of the bacterial lysate producing recombinant betatrophin, L1: MWM, L2-5: Fractions 9-13 of the anion exchange chromatography.
  C. SDS-PAGE showing the purification of recombinant betatrophin by Ni-IDA column chromatography. L1: MWM, L2: Initial elution product with 20 mM imidazole, L3: Second elution product with 500 mM imidazole, L4: Recombinant protein after dialysis.
  D. Elution profile of anion exchange chromatography.

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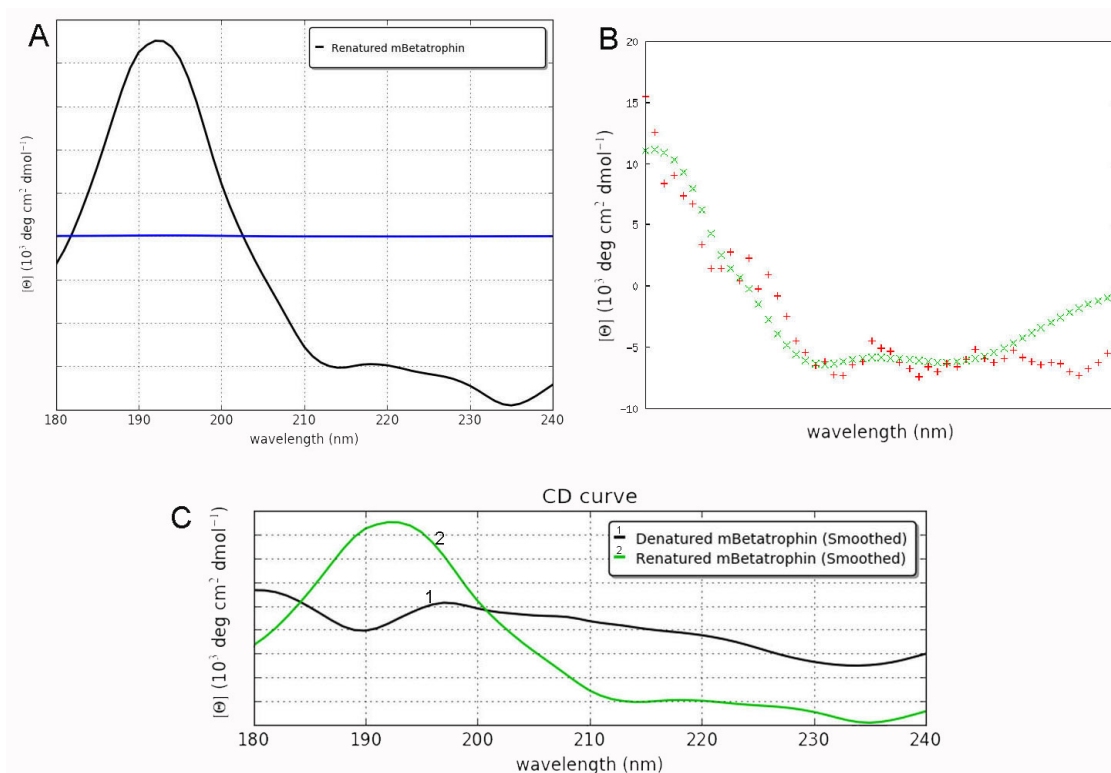
Fig. 3. Far-UV CD spectra of recombinant mouse betatrophin
A. Renatured betatrophin CD spectra curves by SOPMA server
B. Renatured betatrophin CD spectra analysis by K2D3 server and CAPITO server
C. Curves 1 and 2 represent spectra of denatured and renatured betatrophin respectively.

Table 2. Summary of demographic data for mouse betatrophin derived from CD analysis

Mouse betatrophin structures	Alpha helix	Beta sheet	Turn	Random coil
Predicted structure	69.29 %	5.71 %	3.57 %	21.43 %
Denatured structure	17.59 %	2.81 %	--	79.6 %
Renatured structure	63.12 %	5.13 %	2.45 %	29.3 %

betatrophin at 25°C (Fig. 3). K2D3 server and CAPITO software (http://capito.nmr.leibniz-fli.de/index.php) were used to analyze the data. The secondary structural contents of denatured m-betatrophin contained 17.59% alpha helix, 2.81% beta sheet, and 79.6% random coil, and the renatured protein consisted of 63.12% alpha helix, 5.13% beta sheet, 2.45% turn, and 29.3% random coil. Estimation of alpha helix and beta sheet contents by the SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) for renatured m-betatrophin was similar to those predicted from the sequence analysis, exhibiting 69.29% alpha helix, 5.71% beta sheet, 3.57% extended strand, and 21.43% random coil, indicating that the purified protein had refolded properly. The CD analysis is summarized in Table 2.

Discussion

Betatrophin is a member of the angiopoietin-like protein family. Previous studies revealed that suppression or deletion of betatrophin in mice may lead to serum hyperlipidemia, and its molecular variations in humans are associated with blood lipid content (2, 9, 10). Moreover, recent studies revealed that in mice treated with S961, an insulin receptor antagonist, betatrophin controls beta cell proliferation. These findings raised considerable attention on the potential therapeutic role of this mechanism in diabetes treatment (1).

Further studies suggested that betatrophin levels are increased in insulin-resistant diabetes. However, betatrophin levels do not correlate with serum c-peptide level, glucose control, or insulin requirements; hence, the final conclusion was that betatrophin does not play a significant role in beta cell proliferation (11, 23). Overall, the role of betatrophin in type 2 diabetes and obesity remains controversial. One study revealed that betatrophin is elevated in type 2 diabetics (3), whereas in another study no difference in betatrophin levels was found between healthy controls and patients with type 2 diabetes (11). Moreover, recent studies confirmed that betatrophin has no significant role in the control of beta cell function or growth (14).

Betatrophin gene expression has been reported in several organs in mice including brown and white adipose tissues and liver (1). Recently, it was reported that circulating betatrophin is significantly increased in hypothyroidism patients, who show increased thyroid-stimulating hormone (TSH) and decreased free thyroxine (FT4) levels. Furthermore, serum betatrophin concentration is independently associated with thyroid hormones including TSH and thyroglobulin (24).

Further studies suggested that betatrophin regulates postprandial thyroglobulin and fatty acid levels by inhibiting lipoprotein lipase through interaction with ANGPTL3 (12, 25).

Although the mechanism of action of mouse betatrophin is unknown and its receptor has not yet been identified, identification of this newly-identified protein as a hormone, which plays a role in controlling beta-cell proliferation and triglyceride regulation, offers a promising treatment for future glucose and lipid metabolism disorders. Hence, understanding of betatrophin function, structure, and molecular mechanisms rely on the availability of adequate quantities of pure recombinant protein for further studies. In this study, we cloned, expressed, and purified mouse liver betatrophin in a common prokaryotic expression system. The protein was unfortunately expressed as inclusion bodies. Following solubilization, a combination of chromatography methods were used to purify the target protein, which was then refolded and compared with its initial structure by spectroscopy. Although the exact structure of the protein remains unknown, in our study CD spectra analysis revealed similar structural content of the refolded protein with other well-known proteins of this family; moreover, based on initial stability studies (data not shown), this protein could be used in further lipid metabolism and diabetic animal studies. These studies will continue to gain importance with the rising incidence of metabolic disorders in humans (10, 24).

Our study had some limitations. First, the Western blots were performed using an anti-His tag probe (data not presented), as no specific mouse betatrophin-antibody was available. Second, the protein's structure should be further analyzed to gain insight into its function, correlation with other hormones, and possible receptor structure.

Conclusion

In summary, mouse liver betatrophin was cloned and expressed in a prokaryotic system. However, expression of the full-length sequence of this protein in E. coli BL21 (DE3) cells resulted in deposition of the recombinant protein as inclusion body, prompting us to develop a simple procedure for its purification and refolding. The recombinant protein was purified by a combination of anion exchange and metal-affinity chromatography. The refolded protein has its natural configuration and is applicable for further structural and functional studies, especially for lipid and glucose metabolism purposes.

Acknowledgments

Conflict of Interests

The authors declare that they have no competing interests.

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