Efficient Expression of Bioactive Human Leptin in *Escherichia coli* in Soluble Fusion Form

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Abstract Leptin, a 16 kDa nonglycosylated hormone, is produced by mature adipocytes and functions primarily in the hypothalamus to reduce food intake and body weight. To explore a new approach for high-level expression of human Leptin in *Escherichia coli*, the human Leptin gene, synthesized according to the published sequence, was cloned into the vector pET32a to construct a fusion expression plasmid: Trx–Leptin/pET32a. Our data showed that more than 40% of the fusion protein Trx–Leptin was expressed in soluble form. After purified by Ni-IDA affinity chromatography, cleaved by enterokinase and applied Ni-IDA affinity chromatography again, purified Leptin with homogeneity over 96% was achieved. The bio-functional experiments of purified Leptin showed a significant reduction in food intake and body weight of female mice treated with Leptin by comparing with control mice,

and it indicated that the purified Leptin has full biological activity. In addition, our expression system was a very low-cost and efficient prokaryotic expression system. So taken together, our results demonstrated that our expression system of bio-active Leptin provided a new method for producing Leptin in big scale and would be widely applied in commercial Leptin producing industries.

Keywords Human leptin · Trx · *Escherichia coli* · Fusion expression · Enterokinase · Affinity purification

Introduction

Obesity affects up to 35% of the American population and is commonly associated with serious comorbidities, such as type II diabetes, cardiovascular disease, hyperlipidemia, hypertension, and osteoarthritis. So the prevalence of obesity is generally thought to be highest in developed countries [1].

The *ob* gene closely related with obesity was identified in 1994 by positional cloning techniques [2]. Its product, leptin, is a single-chain proteohormone with a molecular mass of 16 kDa that is thought to play a key role in the regulation of body weight. The reported efficacy of leptin is somewhat variable, but effective doses are usually between 0.1 and 10 mg/kg [3]. The dose range mandates the availability in gram of recombinant human leptin (rhLeptin) for in vivo studies. So obtaining leptin in big scale is naturally a problem in the research of leptin in vivo.

It has reported that biologically active leptin can be produced in insect cells [4] or mammalian cell lines [5], but these are inefficient for producing leptin in a commercial scale because of the expensive maintenance and low yield.

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According to the nonglycosylated protein characteristics of leptin, biologically active leptin can be effectively produced in prokaryotic organisms. So many researchers have tried to express the recombinant leptin in *Escherichia coli*. But most of recombinant human leptin expressed in insoluble inclusion bodies (IBs) [6–8]. Purification of recombinant, biologically active leptin from *E. coli* inclusion bodies has also been reported previously. However the procedures were complex and protein refolded from *E. coli* appears to be significantly less potent than that of recombinant leptin secreted from eukaryotic cells [3].

In this work, we synthesized the coding sequence of human leptin by using favored codons for *E. coli*. The vector of pET32a with T7 promoter and Thioredoxin (Trx) as a fusion partner was employed. As we know, fusion partner-Trx has the compatibility with expression of foreign proteins in soluble form [9]. As expected, our data showed that a high expression of soluble fusion rhLeptin was achieved (the soluble fusion rhLeptin was more than 40% of totally fusion protein). After EK digestion the His-tagged Trx and residual undigested fusion proteins were rapidly removed by using another Ni-IDA affinity chromatography, and the purified recombinant human leptin with apparent molecular weight of 16 kDa was obtained; and bio-functional experiments showed that the purified rhLeptin also had the full biological activity; i.e., it reduced food intake and body weight of experimental mice when administered peripherally.

Materials and Methods

Animals

Female Kun-Ming mice were purchased from the Medical Experimental Animal Center, Beijing, China. They were raised in cages in an air-conditioned room (22–24°C) with lights on between 08:00 and 17:00 h. Water and standard mouse chow were available ad libitum. All mice were raised under the same conditions. All animal use in this study was conducted in compliance with approved institutional animal care and use protocols according to NIH guidelines (NIH Publication No. 86-23, 1985).

Bacterial Strains, Vectors and Enzymes

E. coli strain DH5 α was used as the cloning host and strain BL21 (DE3) as the gene expression host. pET32a (Novagen, USA) was used as an expression plasmid. Restriction enzymes and T4 DNA ligase were purchased from Takara Biotech Co. Ltd, and Enterokinase (EK) was from Invitrogen. Plasmid extraction kits and PCR production

purification kits were from Watson Biotech. Other chemical reagents were of analytical grade.

Expression Vector Construction

Human leptin gene sequence synthesized according to the published sequence (GenBank Accession BC060830) was digested with *Kpn*I and *Sac*I, and ligated into the pET32a plasmid at the corresponding restriction sites. The ligation mixture was transformed into *E. coli* DH5 α competent cells for propagation of the recombinant plasmid. The recombinant plasmid pET32a–rhLeptin was confirmed by restriction endonucleases digestion and sequencing.

Expression and Purification of Fusion Protein

The constructed recombinant plasmid pET32a–rhLeptin was transformed into competent *E. coli* BL21 (DE3). One colony of *E. coli* BL21 (DE3) harboring the plasmid pET32a–rhLeptin was grown overnight at 37° in 5 ml LB medium supplemented with ampicillin (100 μ g/ml). The whole culture was inoculated into 500 ml fresh LB medium and the culture was grown to an OD₆₀₀ of 0.4 at 37°. The induction was initiated by adding IPTG to 0.5 mM at 37°C. The induction temperature (25, 30, and 37°C) that affected the level of protein solubility were assayed.

The cells harvested from cultures by centrifugation at 5000 $\times$ g for 20 min were resuspended in binding buffer (20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, and 20 mM imidazole), and lysed on ice by sonication at 400 W for 100 cycles (4 s working, 8 s free). Following centrifugation at 13,000 $\times$ g at 4°C for 30 min, the supernatant of the cell lysate was applied to a Ni-IDA affinity chromatography. After extensive washing with wash buffer (20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, and 60 mM imidazole), the fusion protein was eluted with six column volumes of elution buffer (20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, and 250 mM imidazole). The peak fractions containing the fusion protein were pooled and dialyzed overnight against phosphate-buffered saline (PBS) at 4°C.

Protein Release and Purification

The desalted fusion protein was diluted and incubated with EK at 30°C for 12 h to obtain rhLeptin without Trx tag. The EK reactions (100 μ l) were set up as follows: 50 μ g of Trx–rhLeptin was incubated respectively with 1, 2, 3 U EK in a digestion buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, and 2 mM CaCl₂) at 30°C for 12 h, and the reactions were stopped by the addition of 1 μ l PMSF (100 mM). After digestion, the His-tagged Trx and residual undigested fusion proteins were rapidly removed by using another Ni-IDA affinity chromatography. The flow-through containing

rhLeptin was collected and dialyzed against phosphate-buffered saline (PBS), then lyophilized and stored at -80°C .

Biochemical Characterization and Biological Activity Assay

Amino acid compositional analysis was performed in a manner similar to that described previously [10]. Briefly, Complete hydrolysis of the leptin to liberate the residues with 6 M HCl for 24 h at 110°C in vacuo, followed by reversed-phase HPLC columns analysis and quantification of the liberated amino acids (Use of OPA as a post-column derivatizing agent).

Mice age 5–6 weeks were randomized to two groups ($n = 4/\text{group}$) that had similar mean body weight. Vehicle or human leptin was injected twice daily (08:00 and 17:00) at a dose of 2.5 mg/kg (total daily dose of 5 mg/kg/day) for 5 days [11]. Food intake was determined daily and body weights were recorded at the beginning and the end of the treatment. Percentage changes in food intake were calculated versus the mean baseline intake (calculated using the mean overnight food intake for 4 days during the week prior to injection).

Blood samples were collected by cardiac puncture 16 h after the last intraperitoneal injection. Blood glucose was measured by the Glucotrend monitor and Glucotrend glucose test strips (Roche Diagnostics). Serum insulin was assayed using a human insulin assay kit with human insulin polyclonal antibodies (Navy R.I.A. Technique Center). Data are expressed as mean $\pm$ SD from at least three experiments and statistical analyses were performed by Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

Expression and Purification of Fusion Protein

The construct for rhLeptin expression, containing a $6\times$ His-tag for affinity purification and an EK cleavage site for peptide release, is depicted in Fig. 1. Recombinant plasmid pET32a–rhLeptin sequences were verified by DNA sequencing. After transforming the plasmids into the expression host *E. coli* BL21 (DE3), the fusion protein was over-expressed at 25, 30, 37°C (Fig. 2a). The target fusion protein existed in both the supernatant and pellet of bacteria lysate showed in Fig. 2b. So we chose 25°C as induction temperature for large scale expression. After Ni-IDA purification, the purity of rhLeptin fusion protein increased from 20 to 95% with about 86% target fusion protein recovery (Fig. 3a, b).

Fig. 1 Schematic representation of the expression vector pET32a–rhLeptin. rhLeptin was expressed as a fusion protein with the Trx. To facilitate peptide cleavage from the fusion protein, an engineered EK site, DDDDK (indicated by a vertical arrow), is created between the His-tag and rhLeptin

Cleavage and Purification of Recombinant rhLeptin

In EK digestion, 50 μg of fusion protein Trx–rhLeptin can be cleaved completely with 3 U EK (Fig. 4a). After digestion, Ni-IDA affinity chromatography was applied again. The flow-through was analyzed with SDS-PAGE gels under non-reducing (Fig. 4b, lane 1) or reducing (Fig. 4b, lane 2) conditions. Table 1 summarizes the yields and purity of the fusion protein and released free rhLeptin at each key steps of the purification process. A final yield of 10 mg rhLeptin was achieved from 1 liter shaking flask culture of bacteria.

Biochemical Characterization and Bioactivity of Purified rhLeptin

Table 3 shows the theoretical and found values of the various amino acids in leptin. Asparagine and Glutamine are completely hydrolyzed to Aspartic acid and Glutamic acid, respectively, so the levels of Asparagine and Glutamine found (0 and 0 respect) seem at variance with the literature values (4 and 11, respectively), and the levels of aspartic acid and glutamic acid found (13.6 and 14.6, respectively) seem at variance with the literature values (10 and 4, respectively).

Daily intraperitoneal injection of rhLeptin into normal female mice for 5 days resulted in a significant suppression of daily food intake and an obvious reduction in body weight. Food intake of mice treated with rhLeptin was reduced about 42% during first 24 h (Fig. 5a). Cumulative 5-day food intake of mice treated with rhLeptin was significantly reduced by 20% compared to vehicle control

Fig. 2 Expression optimization of Trx-rhLeptin. **a** Expression of Trx-rhLeptin at 37, 30 and 25°C. Lane M: molecular weight marker; lane 1: non-induced bacteria lysate; lane 2–4 represented bacteria lysate from cell with 0.1 mM IPTG induction at 37, 30 and 25°C. **b** SDS-PAGE analyses of target protein expression under different

temperatures. Molecular weight marker was shown in the left lane; lane 1: non-induced bacteria lysate; lanes 2–4 are soluble proteins when cultured at 37, 30 and 25°C, respectively; lanes 5–7 are the corresponding insoluble proteins. The arrow indicated the location of the recombinant Trx-rhLeptin fusion protein

Fig. 3 Purification of Trx-rhLeptin. **a** Ni-IDA affinity chromatography of Trx-rhLeptin by using LPDataView (BIORAD). **b** The eluted fusion protein showed about 95% or more purity by electrophoretic analysis with 15% SDS-PAGE as analyzed by the BandsCan software (BioMarin Pharmaceutical Inc, UK). Lane M: molecular weight marker; lane 1: supernatant of cell lysate; lane 2: protein fractions eluted from the immobilized metal affinity chromatography

solution (Fig. 5c). After completing the rhLeptin regimen, food intake of treated mice would be increased and then gradually returned to the level of mice treated just with vehicle solution (Fig. 5a, c).

In addition, intraperitoneal injection of rhLeptin into normal female mice (5 mg/kg/day) showed a significant reduction in weight-gain of 76% compared to control animals treated with vehicle control solution (P value <0.05) (Fig. 5d). After completing the rhLeptin regimen, weight-gain increased gradually and was similar to that of control animals finally (Fig. 5b, d).

Table 2 revealed Serum insulin and glucose levels 16 h after the last leptin dose. Serum insulin of mice treated with rhLeptin was significantly reduced by 54% compared to vehicle control solution, while blood glucose levels reduced 10%.

Discussion

So far, the most efficient leptin production has been described by Jeong and Lee [12], who were able to obtain 144.9 mg of leptin from 50-ml culture by the refolding method. However, it has been reported that the leptin re-natured from inclusion bodies was remarkably less potent than that secreted from eukaryotic cells; and furthermore, the biological potency of leptin varied considerably depending on the refolding methods employed [11]. Up to date, however, secreted production of leptin in *E. coli* was rather inefficient. Guisez et al. [13] used the OmpA signal peptide for the secretion of human leptin which was

Fig. 4 SDS-PAGE analyses for the rhLeptin preparation.

a Trx–rhLeptin cleaved by 1, 2, 3 U EK at 30°C for 12 h (12% SDS-PAGE). Molecular weight marker was shown in the left lane; lane 1–4: protein mixture lysed by 0, 1, 2, 3 U EK.

b rhLeptin purified by Ni-IDA affinity chromatography (15% SDS-PAGE). Lane M: molecular weight marker; lane 1–2: SDS-PAGE of rhLeptin in the absence and presence of DTT

Table 1 Isolation of recombinant rhLeptin from pET32a–rhLeptin fusion protein

Purification step	Total protein (mg)	Fusion protein (mg)	Amount of rhLeptin (mg)	Purity (%) ^d
Sonicated supernatant	73.25 ^a	25.2 ^b	12.48 ^c	NA
After affinity chromatography	NA	20.6	10.2 ^c	>95
After EK cleavage	8.4	NA	7.5	NA
Dialysis	NA	NA	5.1	>96

Estimations are based on 500 ml of bacterial culture (about 5 g wet weight)

NA Not applicable

^a Determined using a Bradford assay

^b Percent of rhLeptin fusion protein from total proteins was estimated by SDS gel scanning

^c The amount of rhLeptin was calculated as a fraction of pET32a–rhLeptin fusion protein

^d Purity of protein or peptide was estimated by SDS gels stained by Coomassie Blue

encoded by the modified sequence based on the *E. coli* preferable codon usage. The amount of leptin secreted into the periplasm was 2–4 mg/ml/A600 nm cells, and only 2 mg of human leptin could be purified from a 3-l culture. Rentsch et al. [14] used the PelB signal peptide for the secretion of mouse leptin, but the amount of rhLeptin was also low and only 1 mg of leptin could be purified from a 1-l culture.

Fortunately, these problems are somewhat alleviated when expressed with a fusion partner. As we know, fusing highly stable and compact Trx structure with the foreign protein can facilitate correct protein folding and enhance protein solubility and expression level. In fact, it was reported that the solubility of foreign proteins was increased by using of Trx as a gene fusion partner. The use of a Trx fusion partner has been successfully applied to expression of many foreign proteins in soluble form including antibacterial peptide ABP-CM4 [15], FNII-2 [16], and arginyl-tRNA synthetase [17]. So, here, we put the Trx structure at the N-terminus of the rhLeptin; and then we expressed rhLeptin as Trx-fusion forms in *E. coli*.

The results here demonstrated that more than 40% of the fusion protein Trx–rhLeptin was expressed in soluble form.

Incorporating His-tag and EK recognition sites into the expression vector here could make it easy to obtain purified rhLeptin with native sequences by Nickel affinity chromatography. The fusion protein can be purified by one step with high purity more than 90% as shown in Fig. 3, and 40 mg Trx–rhLeptin was obtained in 1-l culture. Then the fusion protein could be completely digested by EK protease, which is remarkably robust and highly specific, and released soluble free rhLeptin. After second Nickel affinity chromatography, the homogeneity of free rhLeptin protein could be up to 96%, and 10 mg rhLeptin was obtained from a 1-l culture finally.

Biochemical characterization of rhLeptin was measured by Amino acid compositional analysis and SDS-PAGE gels under non-reducing or reducing conditions. Table 3 shows the found values of the various amino acids in rhLeptin were in consistence with theoretical values. Analysis of rhLeptin by nonreducing SDS-PAGE showed a significantly faster mobility in the absence of reducing agent

Fig. 5 Intraperitoneal administration of rhLeptin into normal female mice. **a, b** Graphs showing the average change in food intake or weight gain in percentage over the weight at day 0 of normal female mice injected either with rhLeptin or vehicle control solution. **c,**

d Graphs showing Cumulative food intake or weight gain (in mg/day) for the period of treatment and thereafter. Each value presents mean $\pm$ SD of three independent experiments. * $P < 0.05$ as compared with group in the presence of vehicle control solution only

Table 2 Effects of rhLeptin on serum insulin and blood glucose levels in normal female mice

	Serum insulin (ng/ml)	Blood glucose (mmol/l)
Vehicle	25.22 $\pm$ 1.03	6.03 $\pm$ 0.37
Leptin	11.43 $\pm$ 0.99*	5.45 $\pm$ 0.43

Each value presents mean $\pm$ SD of three independent experiments

* $P < 0.05$ as compared with group in the presence of vehicle control solution only

(Fig. 4b, lane 1). This increase in the mobility is characteristic of the presence of an intramolecular disulfide bond [4]. Both indicated that the purified rhLeptin has the same characterization with the native leptin.

The biological activity of rhLeptin was measured in vitro by detecting its effect on food intake, body weight, serum insulin and blood glucose levels of normal female mice. Intraperitoneal injection of rhLeptin showed a significant reduction in food intake and body weight of normal female mice as shown in Fig. 5.

It has reported that food intake was reduced by 20% during the first 24-h of leptin treatment (1 mg/kg/day). It reached a nadir after the second day that was 30% less than that consumed by the control group [18]. In this paper, food intake of mice treated with rhLeptin was reduced about 42% during first 24 h (27% less than that consumed by the control group). This difference may be due to the different dose of leptin administration (5 mg/kg/day vs. 1 mg/kg/day). A new equilibrium appears to be established when the animal can no longer utilize its lipid reserve. The mice appear to increase feeding rate and spare loss of lean mass by a transient reduction in food intake. Serum insulin of mice treated with rhLeptin was significantly reduced by 54% compared to vehicle control solution (Table 2). This reinforces the early conclusion that leptin can elevate the response of body to insulin, and reduce the secretion of insulin in normal rodents [19]. These results demonstrated that rhLeptin we obtained here was biologically active and bio-functional in vivo. It indicated that our method for expressing rhLeptin in *E. coli* was efficient and suitable for producing rhLeptin in commercial industries.

Table 3 Analytical results of amino acid composition of leptin

Amino acid	Number of amino acid in a leptin molecular	
	Literature value	Found
Serine	17	15.6
Histidine	4	4.4
Glycine	8	7.6
Threonine	11	10.6
Alanine ^a	5	5
Asparagine ^b	0	4
Aspartic acid ^b	10	13.6
Arginine	4	4.2
Tyrosine	2	1.9
Cystine	2	2
Valine	11	10.4
Methionine	3	2.8
Glutamine ^b	0	11
Glutamic acid ^b	4	14.6
Phenylalanine	2	2
Isoleucine	10	9.8
Leucine	23	22.3
Lysine	7	7.2

^a Alanine used as reference

^b Asparagine and glutamine are completely hydrolyzed to aspartic acid and glutamic acid, respectively

In summary, here we developed a low-cost and efficient expression system in *E. coli* and successfully expressed no tagged rhLeptin with high purity and activity. The Trx fusion technology could be widely applied to the production of a variety of cytokine proteins in *E. coli*.

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