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Enhanced production and refolding of human leptin expressed in *Escherichia coli*

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Abstract: The present study describes enhanced production and simplified refolding of a pharmaceutically important protein, leptin, expressed as inclusion bodies in a bacterial expression system. The gene encoding leptin was amplified by RT-PCR methodology, cloned in pTZ57R/T vector by employing dA.dT cloning strategy and then subcloned in T7*lac* promoter-based pET-22b (+) vector to generate pET-LP expression plasmid. *E. coli* strain BL21 (DE3) CodonPlus transformed with pET-LP produced a prominent protein of ~16 kDa upon induction with IPTG/lactose, which represented >30 % of the total *E. coli* cellular proteins. A study of the effect of medium composition on expression level and cell densities revealed that higher cell densities equivalent to ~20 g/L may be achieved in YNG autoinducing medium without compromising the expression level. Whether induced with IPTG or lactose, the target protein expression was in the form of biologically inactive inclusion bodies (IBs), which could be solubilized using a mild detergent i.e., N-lauryl sarcosine (NLS). Refolding was achieved by dilution in the presence of cysteine and cystine followed by stepwise removal of NLS by employing dialysis. The strategy used in the study for refolding is simple, straight-forward and may successfully be employed for proper folding of those recombinant proteins that are expressed as IBs and contain just one or two disulfide bonds.

Keywords: Autoinduction, inclusion bodies, leptin, N-lauryl sarcosine, refolding.
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INTRODUCTION

Obesity has emerged as a serious public health problem of the present age, which has acquired epidemic proportions in developed and developing countries. Its prevalence is increasing globally, with nearly half a billion of the world's population now regarded as overweight or obese[1,2].

Many genes play important role in the control of obesity; *obese* gene (*ob*) is one of them. The product of human *ob* gene is an ~16 kDa protein called leptin, which is comprised of 146 amino acids and contains just a single disulfide bond[3,4]. Though expressed primarily in adipose tissue, leptin is found in many other tissues including placenta, mammary gland, hypothalamus, pituitary, etc.[1,4,5]. The target of leptin is mainly the neurons in the central nervous system through which it controls/suppresses the food intake, increases the energy expenditures and hence normalizes the individual's body weight[6-8].

In the recent years, leptin has also shown promise for abating the symptoms of type-I diabetes mellitus, either alone or in combination with insulin therapy[9]. The present study describes strategy for enhanced production of this pharmaceutically important protein in a bacterial expression system.

MATERIALS AND METHODS

Chemicals and kits

All chemicals used in this study were of the highest purity grade commercially available. Total RNA extraction from placental tissue was performed using QIAgen RNeasy mini kit (QIAgen Inc. California, USA). For plasmid preparation and DNA extraction from gel, QIAprep spin miniprep and QIAquick gel extraction kits, respectively, were used. T4 DNA ligase and restriction endonucleases were acquired from Fermentas Inc. (Manheim, Germany).

Bacterial strains and culture media

T7-*lac* promoter based pET22-b(+) vector, used for *ob* gene expression, was acquired from Novagen Inc. USA. *E. coli* strain DH5α was used for vector propagation while *E. coli* BL21 (RIPL) CodonPlus (Stratagene, USA) for expression studies. Routinely *E. coli* cells were grown on LB medium and agar plates (pH 7.4) supplemented with 100 µg/ml ampicillin. Other media used in this study include TB and YNG[10].

Plasmid construction and expression analysis

Total RNA isolated from the human placental tissue, collected from Social Security Hospital, Multan Road, Lahore, was reverse transcribed using olig-dT primer. The gene encoding leptin was thereafter amplified using a pair of *ob* gene specific primers i.e., LP-F1 and LP-R1 (Table 1). The amplicon was initially cloned in pTZ57R/T vector by employing dA.dT tailing technique and then subcloned in pET-22b(+) vector using restriction digestion and ligase-mediated cloning. The recombinant plasmid pET-LP, thus generated, was used to transform *E. coli* strain DH5α. Positive transformants were selected on LB-plates supplemented with 100 µg/ml ampicillin. Sequence and correct integration of the insert in plasmid was confirmed by sequencing on Beckman Coulter CEQ™ 8000 Genetic Analyzer (School of Biological Sciences, University of the Punjab, Lahore) using T7-promoter and terminator primers (Table 1).

Table 1: Sequence of primers used in PCR amplification and DNA sequencing reactions.

Oligos	*Sequence (5′→3′)*
LP-F1	GCTCGCCATATGGTGCCCATCCAAAAAGTC
LP-R1	GCGAGGATCCTCACTAGCACCAGGGCTGAG
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG

For expression analysis, *E. coli* BL21 CodonPlus (DE3) RIPL cells were transformed with pET-LP according to the standard protocols[11]. 10 ml LB-ampicillin medium was inoculated with well-isolated colony of positive transformant and the cells were grown overnight at 37˚C with constant shaking at 150rpm. 0.3 ml of this overnight culture was used to seed 100 ml LB-amp medium and incubated in orbital incubator shaker until OD_{600} reached ~ 0.5. The expression of *ob* gene was using 0.5 mM IPTG or 10 mM lactose for different time durations (2-16 hours). Three different types of media i.e., LB, TB and YNG[10] were used in order to check the medium, which gives the best expression/yield of the desired protein.

For preparative-scale experiments, 1 liter of bacterial culture grown in YNG medium was auto-induced with 10 mM lactose. After an incubation time of 10-12 hours, cells were harvested by centrifugation at 6,000rpm, 4^{o}C, for 20 minutes. The cell pellet (18.75 g wet cell weight) was resuspended in 1:10 volume of lysis buffer containing 50mM Tris-Cl (pH 8.5), 5 mM EDTA, 100mM NaCl and 1mM freshly prepared phenylmethyl sulfonyl fluoride (PMSF). Cell suspension was subjected to lysis by sonication (15x30 seconds bursts with one minute interval between two successive pulses**)** using Ultraschallprozessor 400S Model Sonicator (Germany). The lysate was centrifuged at 6,000rpm for 20 minutes at 4°C to collect soluble and insoluble protein fractions for SDS-PAGE analysis[12].

Solubilization of inclusion bodies

Prior to solubilization, the IBs containing the recombinant leptin, were washed twice with the washing buffer containing Triton X-100 [50 mM Tris-Cl (pH 8.5), 5 mM EDTA, 0.5 % (v/v) Triton X-100], followed by washing with 50 mM Tris-Cl (pH 8.5). Solubility of IBs was thereafter examined in buffers of different pH and ionic strength both in the presence and absence of denaturing urea (Table 2).

150 µl IBs equivalent to 1 mg protein were taken in 1.5 ml appendorf tubes and centrifuged at 12,000rpm for 1 minute, supernatant was discarded while the pellet was dissolved in 250 µl of each solubilizing solution and incubated at room temperature for 3-4 hours with gentle mixing on rotator shaker.

Table 2: Composition of solutions used for IBs solubilization.

Solubilization solution	*Composition*
A	100 mM Tris, pH 8.5
B	100 mM Tris, 2 M urea, pH 8.5
C	100 mM Tris, 4 M urea, pH 8.5
D	100 mM Tris, 8 M urea, pH 8.5
E	100 mM Tris, pH 10.5
F	100 mM Tris, 2 M urea, pH 10.5
G	100 mM Tris, 4 M urea, pH 10.5
H	100 mM Tris, 8 M urea, pH 10.5
I	100 mM Tris, pH 12.5
J	100 mM Tris, 2 M urea, pH 12.5
K	100 mM Tris, 4 M urea, pH 12.5
L	100 mM Tris, 8 M urea, pH 12.5
M	1 % N-lauroyl sarcosine

Following incubation, the samples were centrifuged at maximum speed (13,000 rpm, 5 min.) and the protein concentration in clarified supernatant was determined by absorbance measurements at 280nm.

Refolding of recombinant leptin

An aliquot of IBs (~50 mg protein) was solubilized in 25 ml of 1 % NLS solution containing [50 mM Tris-Cl (pH 8.5), 0.5mM EDTA, 1mM freshly prepared PMSF]. The solution was kept at room temperature for 30 minutes with gentle shaking and then clarified by centrifugation (20,000rpm, 4^{o}C for 30 minutes).

For refolding, the solubilized protein was gradually added in a 10-volume refolding solution containing 0.5 % NLS, 10 % glycerol, 5 % sucrose, 0.5 mM EDTA, 1mM PMSF, 5mM cysteine and 1mM cystine at 4^{o}C, centrifuged to collect precipitates, if any and then dialyzed against 50mM Tris-Cl to sequentially remove the NLS, sucrose, glycerol, EDTA and PMSF. Dialyzed sample was lyophilized and resuspended in 10 ml of 20 mM Tris-Cl buffer (pH 8.5) and stored at 4^{o}C, until use.

RESULTS AND DISCUSSION

Construction of expression plasmid

Total RNA was isolated from human placental tissue and the gene encoding human *ob* cDNA was RT-PCR amplified. The amplification reaction yielded a single product of 0.45 kb, at an annealing temperature of 65ºC, which was cloned in pTZ57R/T vector followed by transformation in *E. coli* DH5α. Positive transformants were identified by blue white screening and confirmed by *Nde*I/*Bam*HI restriction digestion (Figure 1). Following digestion, the *ob* cDNA was gel purified and then subcloned in similarly

digested pET vector to generate pET-LP expression plasmid (Figure 2). As shown, the construct contains the coding DNA sequence of leptin, in frame with the translational initiation codon, under the regulation of T7*lac* promoter. The recombinant plasmid pET-LP was first transformed into *E. coli* DH5α (cloning host) and then into BL21 (RIPL) CodonPlus (expression host) for expression analysis.

The sequence and correct integration of *ob* cDNA in recombinant plasmid pET-LP was confirmed by colony PCR, restriction digestion and/or sequence analysis (data not shown).

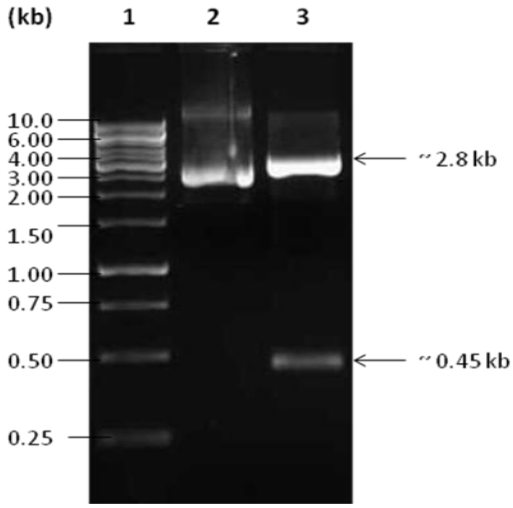

Figure 1: Confirmation of *ob* cDNA cloning in pTZ57R/T vector by restriction digestion. Lane 1: DNA size marker; Lane 2: uncut pTZ-LP; Lane 3: pTZ-LP after double digestion with *Nde*I and *Bam*HI restriction enzymes. ~2.8 kb vector and ~0.45 kb insert are marked with arrows.

Expression of ob gene in E. coli

Expression of human *ob* gene was analyzed in *E. coli* expression host by inducing the transformed cells either with IPTG or lactose. Transformed *E. coli* cells were grown in three different media viz. LB, YNG and TB in baffled Erlenmeyer flasks with good aeration and induced with 0.5 mM IPTG or 10 mM lactose. Cell growth was monitored up to 16 hours in case of lactose and 12 hours in case of IPTG as beyond this time period, substantial increase in growth was not observed in any of the media used (Figure 3).

With IPTG, maximum cell mass (wet cell weight) attained in LB, TB and YNG media was 9.6, 12.7 and 13.6 g/l, respectively. A significant change in cell densities was observed when inexpensive alternative of IPTG inducer i.e., lactose was used to induce *ob* gene expression in *E. coli*. Induction with lactose showed exponential growth of *E. coli* cells to high densities in all three media under study (compares right and left panels of Fig. 3). The cell density increased up to 10-12 hours of cultivation and decreased thereafter. High cell density, equivalent to 19.5 g/l wet cell weight, could be achieved when YNG auto-inducing medium was employed for the expression studies.

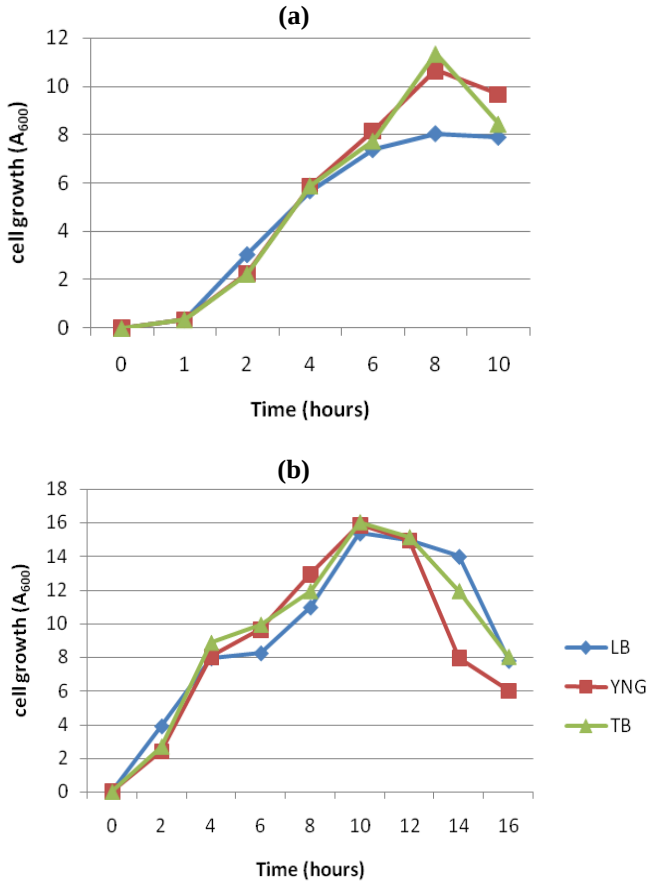

Figure 3: Growth of recombinant *E. coli* harboring pET-LP in different media (LB, YNG and TB) following induction with IPTG (a) and lactose (b). Cell growth was monitored by absorbance measurements at 600 nm.

Lactose based auto-induction strategy originally described by Studier (2005)[13], has previously been employed in our lab. to enhance the biomass and overall expression of bubaline ST in shake flask cultures[10]. In this methodology, inducer (lactose) is added at the beginning of inoculation but the induction is completely repressed due to the presence of glucose in the cultivation medium. Upon glucose depletion, induction and hence the production of recombinant protein starts, automatically. This is advantageous, as unlike IPTG induction, culture growth is not required to be monitored prior to induction.

Following cell growth studies, expression of *ob* gene was monitored by SDS-PAGE analysis of the total *E. coli* cellular proteins (Fig. 4). A prominent band of ~16 kDa protein amounting to ~30 % of the total cell protein could be observed in induced cells. Band of similar intensity, however, was absent in control as well as uninduced cells. Further, the

expression of leptin was examined in both soluble and insoluble fractions.

The recombinant leptin was found associated with the pellet fraction obtained after sonication in an insoluble form while the supernatant representing the soluble fraction didn't show up any protein equivalent to the mass of leptin (compare lanes 'SF' and 'InF' of figure 4).

High-level expression of many proteins, especially of the eukaryotic origin, in *E. coli* generally leads to their aggregation in the form of insoluble aggregates i.e., inclusion bodies (IBs) due to the lack of proper folding machinery[14,15]; expression of leptin as IBs therefore is not an exception.

Solubilization and refolding of leptin

The IBs containing the recombinant leptin were washed twice with 0.5% (v/v) Triton X-100 prior to solubilization. The washed IBs were found to be ~75% pure (Fig. 5) reflecting that working with IBs is somehow advantageous as they facilitate early on purification of the recombinant protein without significant loss (Table 3).

The solubility of IBs was checked in different solubilizing solutions as detailed in Table 2 and the results obtained from A_{280} measurements are summarized in Fig, 6. In Tris-Cl, pH 8.5, the solubility was quite low (~ 25 %) but enhanced significantly both by increasing the pH (10.5 or 12.5) and the molar concentration of urea (2-8 M).

Maximum solubility of IBs was observed in Solution 'D', which had higher concentration (8M) of denaturant i.e., urea as well as alkaline pH, both contributing in complete denaturation and hence solubilization of IBs. However, studies have reported that IBs, although are biologically inactive but they display native-like secondary structure[16].

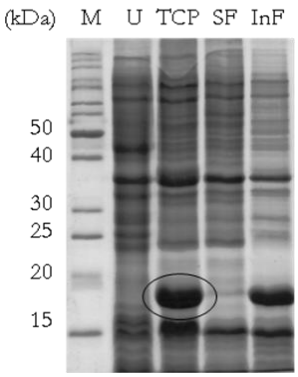

Figure 4: 13 % SDS-PAGE analysis of *E. coli* harbouring pET-LP. Equal amounts of total protein samples mixed with 2 x reducing sample buffer were loaded onto 13 % SDS polyacrylamide gel (8.6 x 6.8 cm; thickness 0.75 mm), run at 150 V for 1.5 hours and stained with Coomassie brilliant blue R250. Lanes M, Protein size marker; U, uninduced; TCP, total cell protein; SF, soluble protein fraction; InF; insoluble protein fraction. Encircled protein represents the desired ~16kDa leptin.

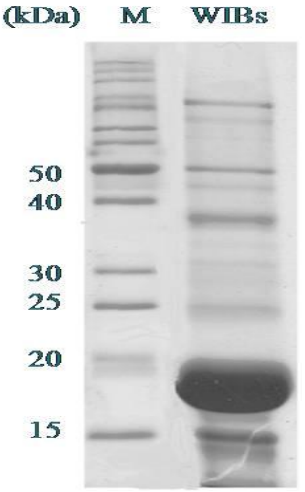

Figure 5: SDS-PAGE analysis of IBs after washing with Triton X-100. M, Protein size marker; WIBs, washed IBs.

A solubilizing solution that may conserve the native-like secondary structure of recombinant protein present in the IBs will likely involve fewer steps in subsequent refolding. Solution 'M' containing 1 % NLS, an efficient but mild detergent, was therefore preferred for IBs solublization, for further studies.

Table 3: Summary of recombinant protein purification and percentage recovery

Steps	TCP (mg)	LP* (mg)	Recovery (%)	Purity (%)
Cell lysate	250	85	100	35
IBs	128	76	89	60
WIBs	82	60	70	75
Solubilization	65	52	61	80
Refolding	60	51	60	85

*The recombinant leptin was isolated from 100 ml of culture containing approx. 2.5 g wet *E. coli* cells.

Dilution of the solubilized protein directly into the refolding buffer is the most commonly used method for small-scale refolding of recombinant proteins. Chelating agent like EDTA used in the renaturation buffer prevents metal-catalyzed air oxidation of cysteines while Tris-Cl containing 10 % glycerol stabilizes the refolding intermediates by preventing the interactions between hydrophobic patches of some partially folded protein molecules[10,17]. In the present study, refolding was accomplished by gradual removal of NLS using dialysis method. No protein aggregation was observed during protein refolding when dialyzed against Tris-Cl of pH 8.5.

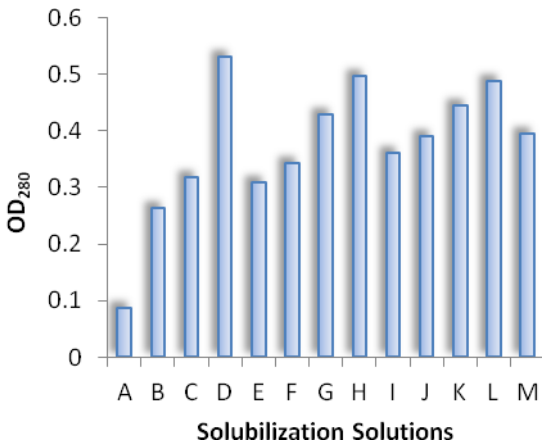

Figure 6: Solubility of IBs in solutions of different pH in the presence and absence of urea. A fixed amount of IBs equivalent to 1 mg/ml was used for solubility analysis.

Slight protein aggregation, however, was observed when the pH was dropped to 7.5 (data not shown). Aggregation of solubilized mixture results in poor recovery of refolded protein and therefore was avoided. Refolding of recombinant leptin was judged by subjecting the oxidized leptin to SDS-PAGE analysis both the in the presence and absence of reducing agent like dithiothreitol (DTT) or β-mercaptoethanol. Faster mobility of leptin in the absence of reducing agent suggests that protein has been folded properly (Figure 7).

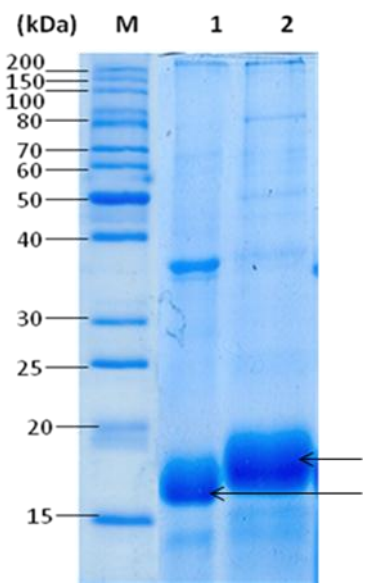

Figure 7: SDS-PAGE analysis of the refolded leptin in the reducing and non-reducing conditions, Lane M, Protein size marker; Lanes 1 and 2, refolded leptin without and after treatment with DTT, respectively.

Using this scheme, the recombinant leptin was not only refolded but also purified up to 85% with a recovery yield of 60% (Table 3). Further studies relating to biological activity assessment of refolded protein are underway.

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