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Original paper

Optimization of expression and purification of recombinant protein in E. coli based on a leptin model

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Abstract

Optimization of expression analyses for enhanced protein production were carried out in *E. coli*, most preferred and excellent host, which enable rapid, high yield, and economical production for heterologous expression of recombinant proteins. Therefore, the study aim was the optimization of parameters involved in bacterial expression conditions, having leptin chosen as a model, a biomarker of appetite and weight control. Optimization of recombinant protein expression in *E. coli BL21 (DE3)* was performed by assessing the growth temperature, the inducer concentration and the post-induction incubation upstream processing parameters, that lead to an increase yield of the protein in its native state. Our data indicates that the highest leptin production in soluble form (442 µg/ml) was achieved at 37°C, 0,1mM IPTG and 5h of post-induction incubation, the purified protein being expressed at the right size of 16 kDa. This approach may also apply to other proteins for efficient expression and purification, and also for downstream *in vivo* or *in vitro* experiments based on leptin, still requiring investigation.

Keywords Bacterial cell host. culture parameters, protein expression optimization, protein quantification, protein purification.

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Introduction

From the production of the first therapeutic proteins in the early 1980s, in the post-genomic era, challenges remain to rapidly express and purify large numbers of proteins for academic and commercial purposes in a high-throughput manner. Trends in the biotechnology industry are forecast to grow related to the development and manufacturing of recombinant proteins. Leptin is a hormone that is crucial to appetite and weight control, showing a 167 amino acids protein sequence of 16kDa, encoded by the ob gene sequence, whose first 21 amino acids function as a signal peptide and are cleaved before the 146 amino acids of the protein is released in the blood circulation (ZHANG et al, 1997; LIEFERS, 2004). Leptin has direct effects on glucose metabolism. The associated fluctuations in the Body Mass Index (BMI) and in the blood sugar and blood fat values ultimately increase the risk of diseases of the cardiovascular system and increase mortality can. At least five to ten percent less weight a year – this is recommended by the guideline 'Prevention and Therapy of Obesity' for people with pathological overweight, depending on the body mass index (FORSTNER and RUSU 2015; TRIF et al, 2016; RUSU et al, 2020). However, falling below this five percent hurdle is very difficult for most obese patients despite numerous diet and lifestyle offers (TRIF et al, 2019). In protein recombinant technology, genetic transformation is usually conducted into *Escherichia coli* host cell often used as a bio-manufacture of protein expression, in regard to maintenance of the genetic construct stability of cloned vector harboring the target DNA sequence to be expressed (MARGAWATI et al, 2017). Moreover, biased codon usage or rare codons in expression host, which might result in translational errors, affecting protein expression should be considered in eukaryotic protein expression, as it can be troublesome, in comparison with the *E. coli* expression system (MURARIU et al, 2011; CHHETRI et al, 2015; MITREA et al, 2017). *E. coli* is most preferred and an excellent host for the heterologous expression of recombinant proteins; moreover its physiology and genetics stands for a fast and cheap economical production of proteins (ABIS et al, 2019), showing advantages like reduced downstream bioprocesses and improved product quality. The protein encoded by the target gene coding sequence would be expressed after IPTG induction, so the assessment of different IPTG concentrations can result in a more effective and efficient in protein production, the optimization process aiming to reduce time and expenses (MARGAWATI et al, 2017; BRAICU et al, 2017; DRULA et al, 2020). Genetically fused purification tag enhances the level of purity and protein recovery (ZHANG et al, 2018). Recently, using DNA recombinant technology, several hormones were successfully produced in *E. coli*, their biological activity being reported, next to expression technologies developed for enhanced production of heterologous proteins in *E. coli*, focusing on protein expression optimization processes to identify the optimal parameters for the culture conditions or based on advanced designed strategies towards (GUPTA and SHUKLA 2016; LU et al, 2015; ABIS et al, 2019; NIEUWKOOP et al, 2019, FREUDL 2018; GAO et al, 2018). The gap between laboratory experiment and industrial product realization is challenging (CHEW et al, 2019). In this study we have assessed the cultivation temperature, inducer concentration and post-induction incubation growth parameters for optimizing the expression of highly pure native leptin.

Materials and Methods

Experimental design

The leptin recombinant protein expression optimization was assessed by preliminary expression of the protein in *E. coli BL21 (DE3)*, set up of the optimal parameters for protein synthesis, the fusion SUMO-leptin protein cleavage, the purification and the quantification of the active protein.

Biologic material and reagents

The biological material resulted from our previous studies (SOCOL et al, 2007, 2008a, 2008b). *E. coli One Shot Mach1-T1* was first transformed using the leptin gene of 441 bp (U84247, NCBI), synthetized through RT-PCR based on the designed primers from sheep adipose tissue, being inserted into pET SUMO vector. The recombinant plasmids were than used to transform *E. coli BL21 (DE3)* for protein expression. All the chemicals and the reagents used were from Sigma-Aldrich, Fluka, Merck, Invitrogen, Thermo Scientific.

Culture media and bacterial cell culture basic condition. Luria Bertani medium (1% triptone, 0,5% yeast extract, 0,5% NaCl) was used in liquid and solid phase (1,5% agar) for cell culture. Bacteria cell culture on liquid phase was carried out at 37°C in skaking incubator at 180 rpm (Infors Unitron, Eppendorf Thermomixer Comfort), and in solid phase in static incubator at 37°C (Memmert INB400).

Preliminary protein expression analyses

Single colony of the recombinant bacterial strain *E. coli BL21 (DE3)* was inoculated in 5 ml of medium LB liquid supplemented with kanamycin (50μg/ml) and glucose 1% and grown overnight in shaking incubator at 37°C 180 rpm overnight. Pre-inoculum (0,1 ml) was inoculated in a 10 ml volume and grown up to DO_{600} of 0,4-0,6, when the culture was spited in two equal parts (5 ml), analyzed according to the bi-factorial scheme 2x7 type (IPTG inducer concentration: 0 mM, 1 mM; incubation period: 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 18 h). Protein bands were analyzed based on electrophoresis gels.

Optimization of recombinant protein production

The culture for protein synthesis optimization. In order to determine the culture parameters for optimizing protein synthesis in *E. coli BL21 (DE3)*, an experimental three-factorial model of 2x4x7 type was assessed (growing temperature: 37°C, 28°C; inducer concentration: 0 mM, 0,01 mM, 0,1 mM, 1 mM IPTG; post-induction incubation period: 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 18 h). LB liquid culture medium supplied with kanamycin (50 μg/ml) and 1% glucoses was used. The *E. coli BL21 (DE3)* overnight pre-culture was used to start up four culture in a 50 ml volume. 56 experimental variants were analyzed based on modified previously described methods (SEMBA et al, 2008; CHHETRI et al, 2015).

The recombinant clones culture for purifying the fusion product. 10 ml of pre-inoculum was grown on LB medium with kanamycin (50 µg/ml) and 1% glucose, in a shake flask liquid culture 180 rpm at 37°C, until reached the DO_{600}=1-2 optical density was further transferred in a 20 ml culture volume; when it reached the DO_{600}=~0,5 optical density IPTG was added (1mM) to induce the recombinant protein expression, according to the optimal parameters determined in the previously.

Protein analysis techniques

Lysis of bacterial cells to obtain the crude protein extract. The bacterial cells were defrost on ice and then 1X SDS-PAGE Sample Buffer (50 mM DTT, 2% SDS, 0,003% Bromophenol Blue) was added to each sample. The bacterial cells lysis was achieved by boiling the samples on a water bath. The 13200 x g centrifugation for 3 minutes and 50 seconds facilitated the separation of the crude protein extract that was kept at -20°C.

The fusion protein purification using IMAC technique

The purification was carried out using HisPur Purification Kit (Thermo Scientific) according to the manufacturer specifications (HisPur cobalt spin columns; equilibration/wash buffer: 50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazol, 0,01% sodium azide; elution buffer: 50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazol, 0,01% azidă de sodiu) throughout IMAC (Immobilized Metal Affinity Chromatography) technique. To equilibrate de resin column, centrifugation was made at 700 x g; the column was washed twice with the equilibration/wash buffer in a 400 µl volume. For protein ligation, the crude protein extract mixed with the equilibration/wash buffer in a 1:1 ratio was incubated 30 minutes at 4°C. Repeated centrifugation steps enabled resin column washes, the samples being monitored at 280 nm OD. The histidine tagged protein was eluted by two washing using the elution buffer.

Enzymatic cleavage reaction of the fusion protein

The dissociation enzymatic reaction mix contain: 0,1 µg/µl fusion protein, 10X SUMO Protease Buffer (500 mM Tris-HCl pH 8,0, 10 mM DTT, 2% igepal (NP-40), 50% glycerol), 1 U/µl Protease SUMO (1U/µl SUMO protease, 5 mM Tris-HCl pH 8,0, 250 mM sodium chloride, 50 µM DTT, 1% igepal (NP-40), 50% glycerol), in a final reaction volume of 250 µl. The reaction mix was incubated at temperatures of 4°C, 16°C, 21°C şi 30°C; 20 µl aliquots were sampled at time intervals of 1 h, 2 h, 3 h, 4 h, 6 h and 18 h to determine optimal conditions for the sub layer hydrolysis.

Proteins quantification method

Protein quantification using the Bradford test was realized with the UV VIS ND 1000 nanodrop Protein Bradford program. Coomassie Plus- The Better Bradford Assay Kit (Pierce) was used according to the manufacturer specifications (Coomassie Plus- reagent: coomassie G-259, methanol, phosphoric acid and other solubilizing agents; albumin vial: 2 mg/ml bovine serum albumin in a 0,9% salt solution and 0,05% sodium azide). Fractions containing pure target protein were pooled and quantified using the Bradford method. 14 bovine serum albumin standards were prepared and used. A mixture of 0.05 ml of each standard/sample and 1.5 ml Coomassie Plus Reagent was made. The quantification was carried out at 595 nm band. The determined standard curves enable to assign a protein concentration.

SDS-PAGE analysis

A 30% acrylamide: 0.8% bisacrylamide solution (Sigma-Aldrich) and Protein Ladder (Fermentas) was used. The separation gel concentration was 15%, and the concentration gel was 4%. Samples were ran using a vertical electrophoresis system (BioRad), being analyzed at 90V for half an hour and at 150 V for approximatively one hour. The coloration solution based on Coomassie R250 (Sigma-Aldrich) was used for the polyacrylamide gels coloration; for discoloration it was used a solution based on acetic acid and ethanol. SDS PAGE was performed according to a previously described method (SCHAGGER and VON JAGOV, 1987).

Results

Preliminary expression analysis. Analyzing the electrophoretic profiles of the fusion protein (Figure 1, Figure 2), an increase in the level of protein was observed when cells were cultivated in the presence of IPTG (1 mM), compared to the non-inducing conditions; the protein production had a slight increase during the first 3 hours, reached a maximum 4-5 hours after the induction and no notable increment was observed after overnight incubation.

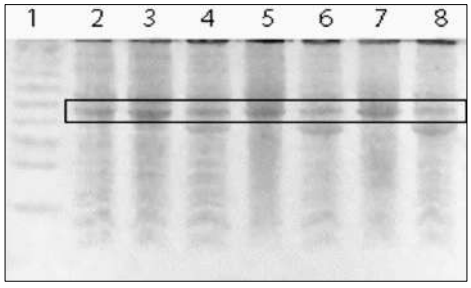

Figure 1. SDS-PAGE of crude proteins in inducing (+IPTG) and non-inducing (-IPTG) conditions at 1, 2 and 3 hours of culture. 1- Protein Ladder. 2-blank. 2-8 - cellular homogenate obtained from cells grown for 1, 2 and 3 h, in the absence (4, 6, 8) or in the presence of 1 mM IPTG (3, 5, 7).

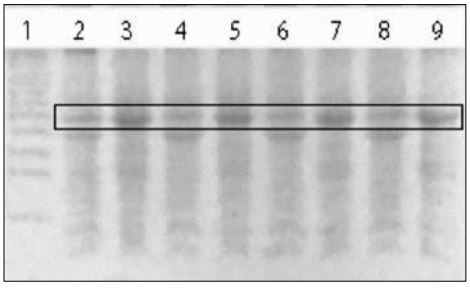

Figure 2. SDS-PAGE of crude proteins in inducing (+IPTG) and non-inducing (-IPTG) conditions at 4, 5, 6 and 18 hours of culture. 1- Protein Ladder. 2-9 - cellular homogenate obtained from cells grown for 4, 5, 6 and 18 h in the absence (2, 4, 6, 8) or in the presence of 1 mM IPTG (3, 5, 7, 9).

Optimization of protein expression. The culture at 37°C and various IPTG inducer concentrations indicated differences in the recombinant protein quantity in periplasm, at different post-induction incubation. Regardless the IPTG concentration a quite uniformed recombinant protein synthesis was observed up to 5 hours of cultivation, then it started to decrease.

Also, a light increase of the recombinant protein quantity during 1-18 hours of culture was noticed for all experimental variants. The culture at 28°C showed similar results to those achieved at 37°C. Low values of IPTG concentration lead to an increase of the protein production level both at 28°C and 37°C. The highest target protein amount is showing one of the experimental variant corresponding to 0,1 mM IPTG concentration at 37°C sampled at 5 hours of culture (Figure 3), similar results being noticed also at 4 hours.

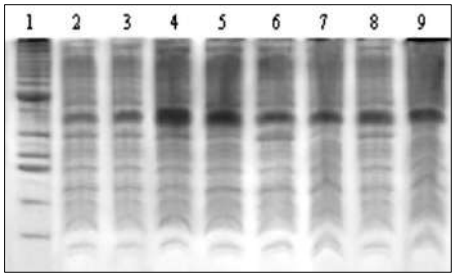

Figure 3. SDS-PAGE analysis of leptin expression. 1- Protein Ladder. 2-9 - cellular homogenate isolated corresponding to experimental variants cultured at 37°C. 2 - 0mM IPTG, 5 h. 3 - 0,01 mM IPTG, 5 h. 4 - 0,1 mM IPTG, 5 h. 5 - 1 mM IPTG, 5 h. 6 - 0mM IPTG, 6. 7 - 0,01 mM IPTG, 6 h. 8 - 0,1 mM IPTG, 6 h. 9 - 1 mM IPTG, 6 h.

Protein purification and quantification. Fusion protein purification using the IMAC technique was made by analyzing the gel in UV white light with the Vilber Lourmat 1000/20M, the profile of the fusion protein leptin-SUMO was outlined based on the molecular weight. The electrophoretic profile reveals the presence of a single band whose apparent size corresponds to the expected size of the fusion protein (29kDa; Figure 4).

The biologic protein in an active form was obtained throughout the enzymatic reaction using SUMO protease, the optimum results being achieved after incubating the reaction mixture for 3 hours at 16°C and 21°C. Due to N- terminal polihistidinic tag, the purification of leptin was carried out by means of chromatography on Co-resine columns, so that the native form of the protein (Figure 5), do not have extra amino acids as a consequence of choosing and implementing such a strategy.

Protein quantification. Protein concentration was assessed using the Bradford test, the protein average concentration was 442 μg/ml.

Discussions

Preliminary expression analysis

E. coli BL21 (DE3) bacterial strain choice was made as it is especially designed for expression of genes that are under the control of T7 promoter (VERMEULEN et al, 2018), but a base level of expression can lead to plasmid instability. Glucose helps to increase the solubility of the recombinant protein and reduce plasmid instability.

The induction level of recombinant protein increases up to the late exponential phase, but expression level reduces in the stationary growth phase, indicating the potential of inducing leptin production at any stage between early exponential and late exponential growth stage. So, an increase in the level of protein was showed in the presence of IPTG (1mM), compared to the non-inducing conditions and also during the first 3 hours, reaching a maximum 4-5 hours after the induction with no notable changes after overnight incubation.

Figure 4. Purified leptin-Sumo fusion protein. 1 - Protein Ladder. 2- leptin-Sumo fusion protein, purified with the IMAC chromatography migrated in polyacrylamide gel (29 kDa).

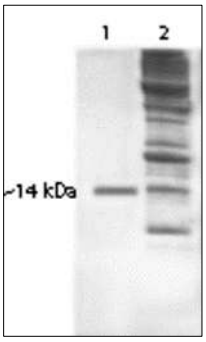

Figure 5. Purified leptin. 1 - Protein Ladder. 2- leptin protein resulted from the cleavage and purification processes with IMAC chromatography migrated in polyacrylamide gel (14 kDa).

Optimization of protein expression

Post-induction incubation period interferes the overall folding, accumulation and productivity of recombinant proteins in *E. coli*. Protein expression is influenced by promoter strength, inducer concentration, post-induction incubation period, protein solubility and other properties of the recombinant protein (BABAEIPOUR et al, 2013). The IPTG inducer interferes in protein expression levels and protein solubility, therefore different concentrations were assessed in order to determine its optimum concentration

for production of high levels of protein. The cultivation at various temperatures was performed to increase the solubility of the recombinant protein leptin, several studies show that protein solubility increases when the culture temperature values are between 28°C-30°C; the solubility increase implies higher amount of recombinant leptin in the periplasm. The culture at 37°C and various IPTG inducer concentrations showed differences in the recombinant protein quantity in periplasm, at different post-induction incubation, with a quite uniformed recombinant protein synthesis up to 5 hours of cultivation and a decrease later.

Even if the induction temperature is influencing the speed of the translation-transcription processes of proteins, for leptin production, the use of the 37°C and 28°C temperatures don't seems to represent a limitative factor in the lab scale experiments using 50 ml culture volume. Since low values of IPTG concentration lead to an increase of the protein production level, is important financially due to the IPTG costs. The optimization strategy for recombinant protein production in general, and leptin in particular, aimed the increase of identical genetic copies out of the initial recombinant bacterial population of biomass and the determination of the optimum culture physiologic conditions. The optimization of protein expression experiments in culture volumes of 50 ml represents an adequate strategy to determine the culture factors that influence the target protein synthesis, before acceding to the next production levels and scaling up processes in higher volumes, including bioreactors.

Protein purification and quantification

Production and purification of proteins using SUMO tag system enables large scale recombinant production (MOHANRAJ et al, 2018). The fusion of the SUMO protein (11 kDa) with leptin (16 kDa) enabled the increase the protein expression level in bacteria clones as well as protein solubility. Also, the N terminal tag of 6 histidin residues, next to leptin and SUMO protein leaded to an increase of the molecular mass of 13 kDa, so that the electrophoretic profile of the fusion protein was the expected one (29kDa).

Cleavage of fusion protein for obtaining the biologic active protein. The biologic protein in an active form was obtained throughout the enzymatic reaction using SUMO protease. The subsequent purification using HisPur Purification Kit (Thermo Scientific) led to the removal of the SUMO fusion protein and of the SUMO proteases from the reaction mixture. The N- terminal polihistidinic tag allowed leptin purification eresulting the native form of the protein with no extra amino acids.

Protein quantification

The cleaved and purified protein shows characteristics that allow its utilization in fine *in vivo* and *in vitro* experiments since it has an identical structure with the leptin synthesized in the animal body avoiding the risk of allergic reactions, immune, virus contamination or other risks specific to the classic leptin isolation are completely removed.

Our results showed that recombinant protein expression can be optimized by means of parameters involved in bacterial expression conditions such as temperature, culture induction and incubation period, yielding enhanced pure protein in native state, required in various R&D e.g. functional, structural, biochemical or biotechnology industries; optimization of recombinant protein synthesis being a concern an showing progress in various studies (BABAEIPOUR et al, 2013; CHHETRI et al, 2015; LU et al, 2015; MARGAWATI et al, 2017; GOLAMI et al, 2018; NIEUWKOOP et al, 2019).

Conclusion

In this study, the data obtained in the preliminary expression analysis and optimization of protein expression experiments indicated that the highest level of native leptin of 16 kDa in purified and soluble form was achieved at 37°C, 0,1 mM IPTG and 5 h of post-induction incubation. The present results can be used further to drive other recombinant protein efficient expression and purification and also to downstream *in vivo* or *in vitro* experiments based on leptin still requiring investigation.

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