Molecular cloning and expression of rat μ-opioid receptor in *Escherichia coli (BL21)*

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ABSTRACT

The μ (mu) opioid receptors, which mediate the effects of morphine, are widely distributed in brain. The purpose of this study was to design a simple expression system for rat μ-receptor in *Escherichia coli (BL21).* In this laboratory study, rat μ-receptor cDNA was isolated from pcDNA3 vector using *Xba1* and *Hind3* restriction enzymes. pET-15b was digested by *Nco1* restriction enzyme. μ-receptor cDNA and pET-15b formed a recombinant DNA that was transformed to *Escherichia coli (BL21).* The insert presence was proved by *Rsa1* restriction enzyme and the induction of its expression was performed using IPTG. Finally, the presence of desired insert was confirmed using *RSA1,* and the colonies that had correct orientation in gene containing plasmid were used for further studies. On the SDS-page gel electrophoresis, a 33 kDa band was observed when IPTG was used at 0.5 and 1 mM concentrations, that is equal to calculated molecular weight of rat μ-receptor. At the end of this project, the expression of rat μ-receptor by IPTG induction was successfully performed.

Key words: μ-receptor; Opioid; Expression; *Escherichia coli* (BL21)

INTRODUCTION

Exogenous opioids and endogenous endorphins and enkephalins play a central role in pain relief and many other physiological activities [1]. The μ, δ, and κ, are the three main types of opioid receptors in the central and peripheral nervous system (CNS, PNS) [2-5]. But each type of these three receptors have the particular distribution [2-5]. Molecular analysis of opioid receptors suggests that these receptors belong to the G-pro coupled receptor super family. The most common cellular response of activation the opioid receptors is depression of neuronal excitability through various mechanisms such as inhibition of cAMP formation, increase in K^+ channel and decrease in Ca^{2+} channel conductivity [6]. Depending on whether they are located on inhibitory or excitatory neurons, the whole effect of opioid receptors activation may be inhibition or excitation [7]. The μ-opioid receptors, which mediate the effects of morphine, are widely distributed in brain [8] and are very interest for several reasons; their propagation and pharmacologic properties place them among the other receptor most identified with the analgesic and addicting properties of opiate drugs [9-11]. μ-receptor is the main target for the production of clinical analgesia, [12] and this came about from the observations that some μ ligands could differentially affect the analgesic response and the unwanted respiratory depression [12].

The expression of μ opioid receptor of different forms of animates was performed by many groups [1, 13] and the aim of this study is to construct the vector containing rat μ opioid receptor, express and optimize its expression in a suitable bacterial host cell for other surveys.

MATERIALS AND METHODS

Materials

E. coli BL21 was purchased from Cinnagen, Iran. The pET15b cloning and expression vector obtained from Novagen Co., USA. Calcium Chloride, Acetic acid, HCl, Ethanol, SDS, Potassium acetate, Sodium acetate, NaCl, and Glycerin were purchased from Merck, Germany. IPTG, *Xba1*, *Hind3*, *Rsa1* and T_4 DNA ligase were purchased from Fementas, Poland. Luria-Bertani (LB) media was prepared according to the guidelines in the laboratory manual offered by Sambrook and Russell. Alkaline phosphatase, RNase, and agarose gel kit were purchased from Roche, Germany,

ampicilin was purchased from Sigma, USA, and aurum plasmid mini kit was provided from BioRad, USA. Finally the rat μ opoid receptor gene was kindly provided by Professor G. Henderson (Bristol, UK).

Transformation of E. coli BL21 with recombinant pET15b plasmids

Five ml of the overnight culture of *Escherichia coli (HB101)* containing insert was centrifuged for 5 minutes. Supernatant was discarded, and the plasmid was extracted using commercial plasmid mini kit [14]. The sample was electerophoresed using 0.7% agarose gel.

Digestion of pcDNA3 containing insert was performed by 1 μl of *Xba1* and *Hind3* restriction enzymes for 1 hr at 37 ℃ and then electerophoresed. The agarose gel containing DNA insert was cut, weighed and added to the buffer solution at 50 ℃ for 10 minutes until the gel was completely dissolved. Separation of the insert was performed using silica column. The amount of insert was determined with agarose gel electrophoresis compared to λ marker. On the other hand, pET-15b was digested by 1 μl of *Nco1* restriction enzyme. Finally 6 μl pET-15b and 1 μl alkaline phosphatase were incubated for 5 minutes at 70 ℃ to dephosphorylate pET-15b and prevent the formation of incorrect circular DNA. Ligation of pET-15b with extracted insert was performed with 1 μl insert, 15 μl pET-15b, 2 μl DNA ligase T_4 and 2 μl buffer at 16 ℃ and for about 2 hrs. Preparation of competent *Escherichia coli (BL21)* and transformation of it was performed using calcium chloride and heat shock method respectively. Colonies from plates containing a recombinant DNA were cultured in 5 ml LB medium and were incubated overnight at 37 ℃ [15]. The next day, plasmids were extracted. One μl of plasmid was digested by *Rsa1* restriction enzyme and was analyzed on agarose gel electrophoresis [16, 17].

Expression of rat μ-receptor

One of the colonies was transferred to 5 ml LB culture and was incubated overnight. After 24 hrs, 1 ml of overnight culture was added to 50 ml LB containing 100 μg/ml ampicillin. When the OD reached to 0.58, the culture was divided in to five 10 ml cultures. Two cultures were induced by two concentrations of IPTG (0.5 and 1 mM). The culture that contained pET-15b without the insert was used for comparison with samples containing insert. Two samples that were used for induction by IPTG, were added to the culture that was induced. IPTG 0.5 mM was also added to the fifth culture. Finally, OD for all samples was measured at 2 and 4 hrs after induction of cultures. Finally all samples were analyzed using SDS-page electerophoresis [18].

Determination of μ-receptor molecular weight

According to the R_f (rate of relative motion) of the standard protein on SDS gel electerophoresis, the molecular weight of proteins were determined in the linear range.

RESULTS

Digestion of pcDNA3 containing insert by restriction enzymes

After extraction of pcDNA3 containing insert from *Escherichia coli (HB101)*, the DNA fragments were analyzed on 0.7% agarose gel electrophoresis to confirm the presence of insert (Figure 1).

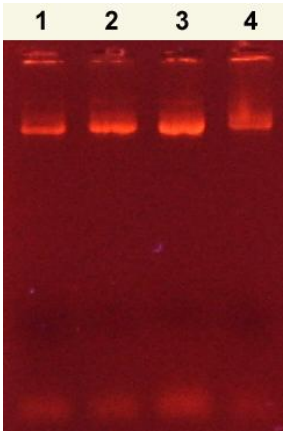

Figure 1. Electrophoresis of pcDNA3 containing insert on 0.7% agarose gel.

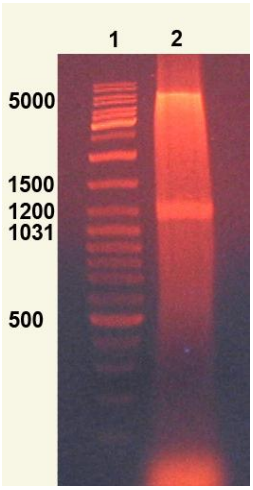

Figure 2. Electrophoresis of digested pcDNA3 containing insert by restriction enzymes (*Xba1* and *Hind3*). Lane 1, DNA marker and Lane 2 the plasmid DNA (5428) containing an insert (1291).

Digestion of pcDNA3 containing insert by restriction enzymes (*Xba1* and *Hind3*), resulted in production of two fragments: the plasmid DNA (5.4 kb) and the insert (1.2 kb). These two fragments were detected using 0.7% agarose gel electrophoresis (Figure 2).

Transformation of Escherichia coli (BL21) with pET-15b containing insert

After extraction of pET-15b from *Escherichia coli (BL21)*, the DNA fragments were separated on agarose gel (0.7%) to confirm the presence of insert (Figure 3).

The linear pET-15b and the insert extracted from the gel after the digestion the pcDNA3, were cut from gel and their weights were determined by λ marker. Values of the pET-15b and the insert were as follows: (Figure 4)

Vector (elution 1): 3.9 ng/µl Vector (elution 1): 3.9 ng/µl
Insert (elution 2): 64.9 ng/µl Insert (elution 2): 40 ng/µl

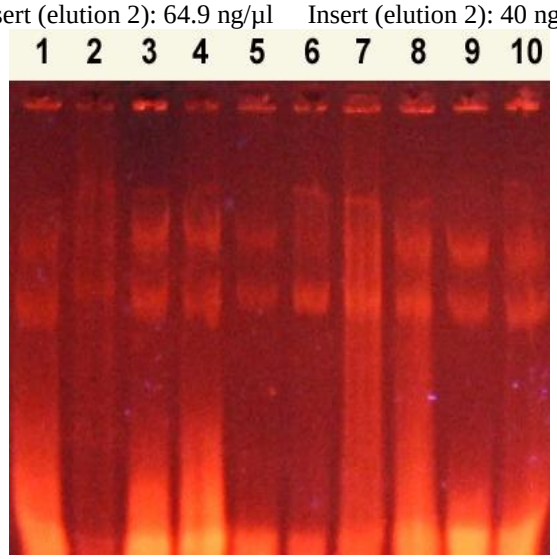

Figure 3. Electrophoresis of pET-15b containing insert on 0.7% agarose gel.
Lanes 1-10: extracted plasmids from transformed *Escherichia coli (HB101)* with pET-15b.

Analysis of pET-15b containing insert by restriction enzyme

pET-15b containing insert and plasmid without insert was cut by the *Rsa1* restriction enzyme. The results showed that two colonies have the correct gene orientation, and for two colonies the gene are linked wrongly to the plasmid. Colonies with the correct gene orientation created 5 bands in agarose gel electrophoresis at about 1500, 1300, 746, 60 and 2263, and Colonies with the wrong gene orientation created 4 bands at about 1500, 580, 680 and 2263 (Figure 5).

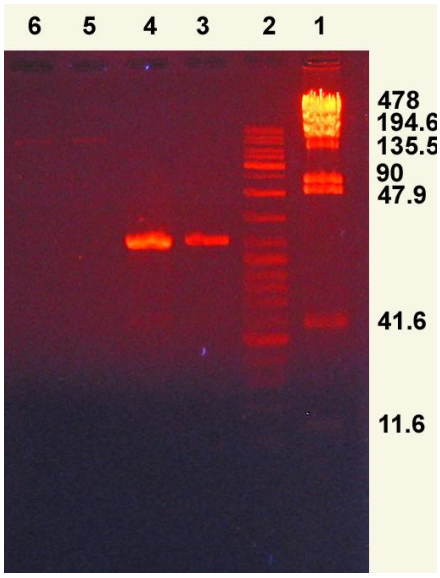

Figure 4. Digestion of DNA by *HindIII* and determination its size using λ Hind3 marker. Column 1: λ Hind3 marker, 2: 100 bp marker, 3: extracted insert from pcDNA3 (elution 2), 4: extracted insert from pcDNA3 (elution 1), 5: digested pET-15b (elution 2), and 6: digested pET-15b (elution 1).

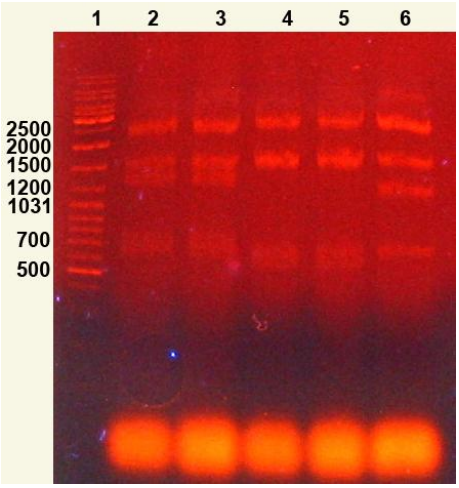

Figure 5. Electrophoresis of pET-15b containing insert by restriction enzyme *RsaI*. Column 1: 100 bp marker, 2 and 3: pET-15b containing insert in correct orientation, 4 and 5: pET-15b containing insert in incorrect orientation, and 6: pET-15b without insert digested by restriction enzyme *RsaI*.

Determination of µ-receptor molecular weight

In SDS-page gel electerophoresis, in addition to the other bands, a band in the induced samples was observed at about 33 KDa. By using of the standard samples with particular molecular weight, the diagram of the logarithm of molecular weight was plotted against of R_f, molecular weight receptor was estimated at about 33 KD (Figure 6).

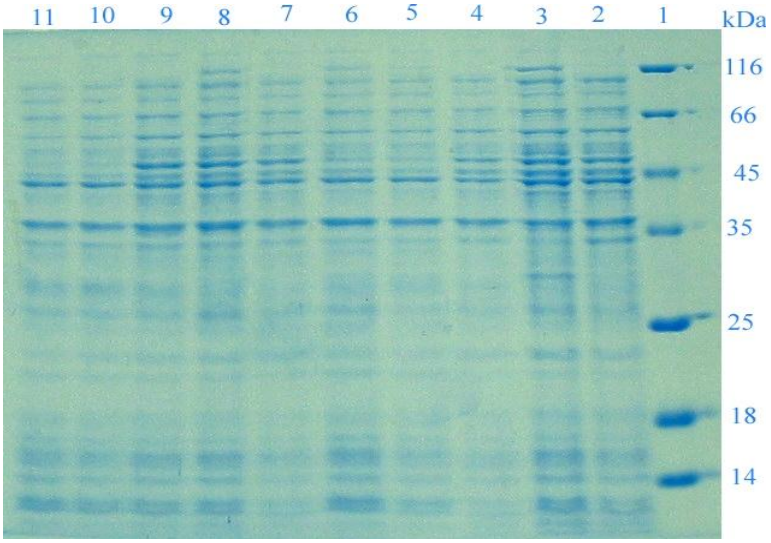

Figure 6. SDS- page electrophoresis of rat μ-receptor protein, Lane 1: molecular weight marker. Lanes 2 and 7: protein sample of pET-15b without insert 4 and 2 hrs after induction using IPTG respectively. Lanes 3 and 5: protein sample of pET-15b with insert 4 hrs after expression without induction. Lanes 6 and 11: protein sample of pET-15b with insert 4 and 2 hrs after induction using IPTG 0.5 mM respectively. Lane 4 and 9: protein sample of pET-15b with insert 4 and 2 hrs after induction using IPTG 1 mM respectively.

DISCUSSION

The purpose of this project was to express the rat μ-receptor gene in *Escherichia coli (BL21)*. *Escherichia coli (BL21)* is able to grow in minimal medium, and is non-pathogen too. It lacks the genes encoding proteases such as ompT and Ion [19, 20], that are involved in degradation of the final recombinant proteins. Expression vector pET-15b system is based on T_7 promoter that has been used in the production of recombinant proteins. This promoter is induced by IPTG [19]. pET-15b vector has several advantages over other vectors including high copy number (20-50 per cell) and has a His-tag sequence so the purification of the recombinant protein is facilitated using affinity chromatography.

The target gene was inserted into pET-15b and its orientation was analyzed by *Rsa1* restriction enzyme. Since the *Rsa1* restriction enzyme has 4 digestion sites in pET-15b, and three on the insert, the digestion of the recombinant vector in the right orientation should result in creation of 7 appropriate fragments sizes (142, 180, 680, 746, 1330, 1567 and 2263). IPTG induction at 0.5 and 1 mM showed no difference on the strength of protein band that was observed at approximately 33 kDa. It seems that the optimal concentration for IPTG in this expression system is between 0.5 and 1 mM. Other studies have also used these concentration of IPTG and the induction was successfully performed [21]. Stanisila et.al expressed the human μ-receptor in *E.coli* in different temperature (20, 30, and 37 ^{o}C) and 0.5 mM, concluded that lower temperature help to increase the amount of expressed receptor. It was due to the correct folding of the recombinant protein [20]. Thus it is suggested the use of lower temperature and concentration of IPTG for optimizing the expression of this receptor.

In some studies by Chen Y, et al and Thompson RC, et al, the expression of rat μ-receptor was performed and the recent cloning of μ-receptor gene [22-24] suggests that it is a 397 amino acid protein and 1197 bp cDNA that is homologous to other seven transmembrane G-pro coupled receptors. The cloned rat μ-receptor has high homology to the δ [23, 25, 26] and κ [27, 28] opioid receptors, particularly in transmembrane domains I-III and V-VII, as well as in the putative intracellular loop.

In this project, we designed a simple system for expression of rat μ-receptor. So it can be increase the level of production of the recombinant μ-receptor by change the induction condition such as the concentration of IPTG and the bacterial growth temperature. Also we can use of other expression systems such as yeasts and other eukaryotic organisms for post translation modification. Research project number: 83287, Isfahan University of medical sciences.

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