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MOLECULAR EXPRESSION OF WINGLESS-TYPE MMTV INTEGRATION SITE FAMILY MEMBER 4 GENE USING *Escherichia coli* BL21

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ABSTRACT

This research was conducted to find out the *Wnt*4 recombinant proteins which expressed by *Escherichia coli* (*E. coli*) BL21 carrying the recombinant DNA *wnt*4 (*E. coli* transformation). Research materials were *E. coli* BL21 transformation and *E. coli* BL21 non-transformation (negative control). The expression of recombinant protein was conducted by culturing *E. coli* for 24 hours in Luria-Bertani (LB) media with *isopropyl β-D-1-thiogalactopyranoside* (IPTG) induction. Recombinant protein was isolated by sonication of pellet bacteria. Protein analysis performed by 15% *sodium dodecyl sulphate polyacrylamide gel electrophoresis* (SDS-PAGE). The results showed that recombinant protein with a molecular weight of 33 kDa has been expressed by *E. coli* BL21 transformation successfully.

Key words: *E. coli* BL21, immunocontraception, wnt4

ABSTRAK

Penelitian ini dilakukan dengan tujuan mendapatkan protein rekombinan Wnt4 yang diekspresikan oleh Escherichia coli (E. coli) BL21 pembawa deoxyribonucleic acid (DNA) rekombinan wnt4 (E. coli transforman). Materi uji ekspresi protein adalah E. coli BL21 transforman dan E. coli BL21 nontransforman (sebagai kontrol negatif). Ekspresi protein rekombinan dilakukan dengan cara menumbuhkan bakteri selama 24 jam pada media Luria-Bertani (LB) cair dengan induksi isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein rekombinan diisolasi dengan cara sonikasi pelet bakteri. Analisis protein dilakukan dengan sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) dengan konsentrasi 15%. Hasil penelitian menunjukkan bahwa protein rekombinan dengan berat molekul 33 kDa berhasil dieskpresikan oleh E. coli BL21 transforman.

Kata kunci: E. coli BL21, imunokontrasepsi, wnt4

INTRODUCTION

Immunocontraception is the process of using immune system in the body to hinder the fertility (Cowan and Tyndale-Bisoce, 1997; Smith *et al*., 1997; Delves *et al*., 2002; Ferro, 2002; Purswell and Korstel, 2006; Peek *et al*., 2008; Powers *et al*., 2011; Samoylova *et al*., 2012; Kachani and Heath, 2014). The concept of immunocontraception can be conducted by the isolation and identification of the gene and or protein, which plays an important role in reproduction process that can be used as antigen (Nie *et al*., 1997; Robinson *et al*., 1997; Bowen, 2006).

The concept of antigen preparation, which is applied to immunocontraception method, is the genetic engineering process (Holland *et al*., 1997; Saito-Diaze *et al*., 2010; Gopal and Kumar, 2013). This process is also known as gene cloning, gene manipulation, deoxyribonucleic acid (DNA) recombination technology, or genetic modification (Nicholl, 2008; Gopal and Kumar, 2013). Technology of DNA recombination has been used to learn or to study the reproductive process, such as analyzing specific characteristic of hormone and gamete (Govind and Gupta, 2000; Clydesdale *et al*., 2004; Saito-Diaz *et al*., 2010; Tubbs *et al*., 2013).

The process of gene cloning according to Nicholl (2008) consists of four steps and the first step is gene isolation (Robinson *et al*., 1997; Muhamed *et al*., 2004: Hsieh *et al*., 2005; Nicholl, 2008; Hayashi *et al*., 2009; Rosano and Ceccarelli, 2014). Gene isolation is performed by the amplification method of nucleic acid compiler or backbone. The product of polymerase chain reaction (PCR) in the form of cDNA is then recombined to plasmid. The next step, plasmid carried recombinant DNA is transformed into bacterial host (Muhamed *et al*., 2004: Hsieh *et al*., 2005; Hayashi *et al*., 2009). The whole process of this recombination and transformation of DNA is the process of enzymatic reaction in vitro (Brown, 2006; Ahison, 2007; Chedrese, 2009). The last step of cloning is the expression of recombinant protein. The expression of recombinant protein can be occurred if sticking gene in recombinant protein suit to the promoter position in the expressed vector (Baez *et al*., 2014; Rosano and Ceccarelli, 2014).

Wnt gene reacts in some stages of the early process of embryo development which has a potency to develop as a source of new antigen in immunocontraception. Wnt protein through the cellular signal system has an important role in basal growth process that determines the cell fate, proliferation, polarity, and cell death during embryo growth (He *et al*., 1997; Paria *et al*., 2002; Miller, 2002; Saito-Diaz, 2013). Sonderegger *et al*. (2010) stated that the cellular signal system which is regulated by wnt protein is playing a part in the embryonic growth, inactivation of the blastocyst, and implantation. McAuley *et al*. (2013) indicated that wnt cellular signal system presents along with the embryo growth so that proves wnt cellular signal system have a

number role in embryo development. The research of Hayashi *et al*. (2009) also indicated that the expression of wnt4 is only found at the implantation site, not on non-implantation site. That expression of genes, whether in mice or human, increases along the process of decidualization (Cory *et al*., 2007; Li *et al*., 2014).

Isolation and detection of wnt gene expression in mice had successfully done by Muhamed *et al.* (2004) and Hayashi *et al*. (2009) using the reverse transcription PCR (RT-PCR) technique. The PCR technique had also been done by Rao *et al*. (2009) in the amplification of wnt gene of monkey and rodentia epididymis. Isolation and cloning of wt4 gene had been reported by Hou *et al*. (2014) with the source of ribonucleic acid (RNA) from *Taenia solium* in pMD18-T vector, then transformed into *E. coli* DH5α. Chen *et al*. (2015) had made a success in isolation and amplification wnt4 gene in *Epinephelus coioides*.

Sitasiwi *et al*. (2015) had succeeded to isolate, amplified, and conduct the cloning of wnt4 gene that was isolated from a seven day pregnant mice. Wnt4 gene had been successfully inserted into pET SUMO as a expression vector and transformed into *E. coli* BL21 as host cell. The research also yielded transformation *E. coli* BL21 carrying the recombinant DNA wnt4. This research is conducted to get wnt4 recombinant proteins that are expressed by transformation *E. coli* BL21. The success expression of this wnt4 recombinant protein that expressed by *E. coli* BL21 makes the open opportunity for the next research focused in purification and understanding the antigenicity of wnt4 recombinant proteins.

MATERIALS AND METHODS

The materials used for the expression of protein were transformation *E. coli* BL21 and non-transformation *E. coli* BL21. The expression of recombinant protein was performed by culturing the bacteria in LB consisted of 10 g tryptone (1st BASE), 5 g yeast (Oxoid), 10 g NaCl (Merck), 15 g agar (Oxoid), glucose (Analar), isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma). Bacterial isolation in big scale was done by preparing 200 ml liquid LB media in three sterile Erlenmeyer tubes. Each Erlenmeyer tube contains 50 mL of media LB, first Erlenmeyer tube was given the induction IPTG with 1000 µL/200 mL media, the second Erlenmeyer tube was only given the liquid LB media. Both tubes were inoculated with bacteria transformation. The third Erlenmeyer tube was used as a negative control that was inoculated with non-transformation *E. coli* BL21. LB media which had been inoculated with the bacteria was incubated for 24 hours at a temperature of 37° C in a rotary shaker with the speed of 900 RPM.

Isolation of recombinant protein was done by isolating the pellet from bacteria colony. The bacteria colony at LB media was poured into sterile tube and centrifuged with the speed of 3000 RPM for 10-15 minutes, the supernatant was discharged. Obtained pellet was cleaned with phosphate buffered saline (PBS) by homogenizing with PBS then centrifuged at a speed of 3000 rpm for 10-15 minutes. The pellet washed three times and the clean pellet was added with PBS with the ratio of 1:1, and then kept in a box containing ice.

The mixture of pellet and PBS was sonicated five times for 30 second. After sonication, the pellet was centrifuged at a speed of 10,000 rpm for 10 minutes at temperature 4° C. Obtained supernatant was taken and kept at temperature -20° C to analyze the protein using the sodium of dodecyl sulphate-polyacrylamide of gel electrophoresis (SDS-PAGE). Concentration separating gel of SDS-PAGE used in this research was 15%, while concentration of stacking gel was 3%. Loading samples used with the amount of 20 µL consisted of 5 µL buffer sample and 15 µL protein sample, and loading protein marker is 5 µL.

RESULTS AND DISCUSSION

The expression of recombinant protein is the indicator process of gene cloning as a whole (Chen and Texada, 2006). The expression of recombinant protein was done by culturing the recombinant *E. coli* and non-recombinant *E. coli* in three Erlenmeyer tubes containing the liquid LB. Each Erlenmeyer contain 50 mL of media LB, first Erlenmeyer inoculated recombinant *E. coli* with the induction IPTG with the amount of 1000 µL/200 mL media, and second Erlenmeyer inoculated recombinant *E. coli* without induction IPTG. The third Erlenmeyer used as negative control inoculated non-recombinant *E. coli*. The isolation of recombinant protein performed by the SDS-PAGE with the concentration of 15% as presented in Figure 1.

The result of SDS-PAGE in Figure 1 indicated that column 1 (M) represented the protein marker, column 2 (K) represented the protein *E. coli* control (non-recombinant), column 3 (R+) represented the protein recombinant with the induction IPTG, while column 4 (R-) represented the protein recombinant without induction IPTG. Protein marker (Sigma) at column 1, from the top to downwards showed the weight of protein molecule alternately from 75, 63, 48, 35, and 25 kDa. The result of SDS-PAGE also show there are some bands of specific protein molecule at column 3 (transformation *E. coli* with the induction IPTG), and column 4 (transformation *E. coli* without induction of IPTG). Protein band at column 3 and 4 show the same band pattern, but protein band at column 4 is thinner compared to a protein band at column 3. Column 2 also shows the different expression band from the protein band expressed at column 3 and 4. Target of Protein band was expressed at column 2 and column 4 (red box) with the molecular weight under 35 kDa.

Specific protein band expressed at column 3 (red box) and column 4 that has a molecular weight of 33 kDa represent the recombinant protein that is expressed by DNA recombinant. Thinner protein band at column 4 (without induction IPTG) which is compared to a protein band at column 3 (with the induction IPTG)

proves that induction IPTG can rate the synthesis of protein in *E. coli*.

Recombinant protein is the fusion of protein (Lee and Keasling, 2006; Lodge *et al*., 2007; Anonymous, 2010; Rosano and Cecarelli, 2014) so that protein size, which is formed representing the affiliation the couple base pair from the result of PCR which was patched at the cloning site of plasmid, added to the base pair from the start to stop codon in the plasmid. Based on that quantification, the weight of the recombinant protein molecule is 33 kDa. The result of this research as presented in Figure 1 shows that recombinant protein with a molecular weight of 33 kDa (red box). This protein size proves that the cloning which was performed in this research using the pET SUMO and *E. coli* BL21 has succeeded to express the expected protein recombinant. This research proves that the induction of IPTG with 24 hours culturing time can rate the expression of recombinant protein without giving the effect to the expected protein expression. Lee and Keasling (2006) expressed that using chemical compound, adding nutrients or inducting temperature at the expression of recombinant protein has a potency to give the negative effect for the growth of cells and the formation of synthesis product. The production of the recombinant protein that used with the addition of inducer (like IPTG) can boost up the production cost and make the contamination of the final product (Lee and Keasling, 2006), changing temperature can kill the bacterium (Caspeta *et al*., 2009: Valdez-Cruz *et al*., 2010) and lacking of nutrient in culture media can inhibit the growth of cell and the synthesis of recombinant protein (Ferenci, 2001; Valdez-Cruz *et al*., 2010). The expression of recombinant protein according to Baez *et al*. (2014) happened because the affiliation of the promoter of gene recombinant protein which is activated by the present a specific factor. The condition culture of transformation *E. coli* was controlled and homogeneous, so that addition IPTG with that concentration is not toxic, but able to trigger the synthesis of recombinant protein. As shown with the specific band in column 3 and 4 in Figure 1.

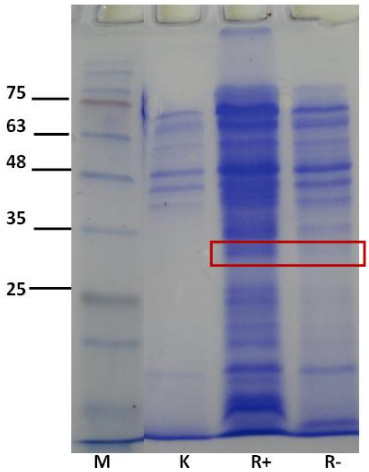

Figure 1. Result of wnt4 recombinant proteins expression in liquid LB media (M= Marker protein (kDa); K= Negative control; R+= Protein recombinant with the induction IPTG; R-= Recombinant protein without induction IPTG; Red box= Recombinant protein)

Cloning vector in this research was pET SUMO. According to Studier and Moffatt (1986) in Rosano and Cecarelli (2014), vector pET has a promoter T7 system. *E. coli* used was BL21 (DE3) strain. BL21 (DE3) strain and its derivate represent the strain which is most commonly used for the expression of proteins. Chromosome BL21 contains the T7Rnap behind lacUV5 promoter, profane λDE3 also inserted into that chromosome. BL21 strain coded T7Rnap, a polymerase that exists in bacterial genomes. Expression T7Rnap is controlled by lac UV5 promoter, so that this system can be induced by lactose or its analogue that is IPTG. The present IPTG in media triggered the expression of T7Rnap then free TNRAP increased the transcription of recombinant gene.

Protein band which is relatively thinner at column 4 proves that the amount of protein is low because the protein expression rate is relative low. The low protein expression according to Lodge *et al*. (2007) is due to two matters. First, cloned gene has a different codon with *E. coli*. This condition due to trauma recognizing cotton is low so that expression of the protein is also low (Chen and Texada, 2006; Chu *et al*., 2014). The production of protein is especially determined by encoding speed at reading frame that is the time needed by ribosome for the translation of MRNA, especially if codon is placed quickly at the end of 5 mRNA (Chu *et al*., 2014). The second matters are the translation, which is not enough, but the running protein turnover is efficient. Final amount of cell protein determined by the rate of synthesis and degradation so that the rate of synthesis is not enough, but the degradation go normally so that the amount of protein that can be detected by SDS-PAGE is also thin.

Another reason of lower protein expression in this research is the possibility of the disruption in tying of protein disulphide bond. The correct forming of disulphide-bond is very important to form the active three dimension conformation. Incorrect disulphide-bond can cause the protein to mis-folding and aggregating in inclusion bodies (Mondal *et al*., 2013; Rosano and Cecarelli, 2014). Inclusion body is an aggregating of inactive protein as a result of mis-folding and degrading of protein, recombinant (Mondal *et al*., 2013). Cysteine oxidation in *E. coli* occurs in the periplasm, where the forming of disulphide-bond change over the reaction catalyzed by a number of enzymes (Messens and Collet, 2006). On the contrary, forming of disulphide-bond in cytoplasm rarely happen, this possibility is caused by residue cysteine that present in the part of catalytic enzyme. Forming of disulphide-bond at cytoplasm will cause the protein inactive, miss folding and aggregating (Derman *et al*., 1993 in Rosano and Cecarelli, 2014), so that the expression does not show in SDS-PAGE.

CONCLUSION

Wnt4 recombinant protein with a molecular weight of 33 kDa has been successfully expressed by *E. coli* BL21 transformation.

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