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Effect of Ethanol and IPTG on the Recombinant Jembrana Trans-Activator of Transcriptation Protein Expression

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Abstract

Jembrana diseases are caused by Jembrana Diseases Virus (JDV). The previous study showed that Jembrana Trans-Activator of Trancriptation (JTAT) recombinant protein is effective as a vaccine for Jembrana diseases. The production of JTAT protein needs to be optimized to obtain a higher amount of vaccine. High expression of JTAT protein will produce a high vaccine product. This study aimed to examine the effect of the addition of ethanol and IPTG in *E. coli* media on the expression of JTAT recombinant protein. This research was experimental research with factorial RAL design with a variation factor of ethanol and IPTG. Qualitatively, the induction of each IPTG, ethanol and interaction between the two could induce the expression of JTAT protein and could be identified with SDS-PAGE at ±11.8 kDa. Statistically, the induction of IPTG, ethanol and interaction between the two were not significantly different. Qualitative and quantitative data show that ethanol can induce JTAT protein expression. This result can be used as a preliminary study to test the effectiveness of ethanol as a substitute for IPTG in inducing the recombinant protein expression.

How to Cite

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INTRODUCTION

Jembrana disease is a disease that only attacks Balinese cattle (*Bos javanicus*) (Kusumawati *et al.,* 2015). Jembrana disease generally attacks adult Balinese cattle with age ranged from 3-4 years (Mardiatmi, 2015). The agent that causes Jembrana disease is called Jembrana Disease Virus (JDV) (Kusumawati *et al.,* 2014). Vaccines from recombinant DNA technology are used as an alternative to overcome the disease (Li *et al*., 2013). The advantages of recombinant-based vaccines include their ability to activate the humoral and cellular immune responses. DNA plasmids are easily produced in large quantities in a short time. Recombinant-based vaccines can also increase the body's immunity against viruses for a long time (Radji, 2009).

Recombinant vaccines are innovations in vaccines development that utilize biotechnology by manipulating a vector. The goal is to express the antigens from pathogenic viruses (Hartawan, 2011). The target gene is inserted into the plasmid (vector) and they both are inserted into the host cell (De, 2016). One JDV functional gene that can be used as a recombinant vaccine ingredient is *tat*. Tat gene encodes the Jembrana Trans-Activator of Trancriptation (JTAT) protein (Cota-Gomez, *et al*., 2002). JTAT protein can induce protective immunity in Bali cattle against Jembrana disease. In addition, the protein has the ability to induce protective immunity in Balinese cattle against Jembrana disease. This makes JTAT protein becomes very potential to be used as a recombinant vaccine material (Margawati, *et al*., 2008).

The expression system using bacteria is the main choice in recombinant protein production. Some factors that influence bacterial expression are the selection of promoters, terminators, vectors, host cell strains, tag fusion and media composition (Joseph *et al*., 2015). One way to optimize the level of expression is by manipulating the composition of the culture medium. In addition to the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the media, ethanol is also known to increase the recombinant protein expression (Chhetri, *et al*., 2015).

This study aimed to examine the effect of the addition of ethanol and IPTG in *E. coli* media on the expression of JTAT recombinant protein. JTAT recombinant protein is the result of genetic engineering to produce recombinant vaccine ingredients. JTAT protein can induce protective immunity in Bali cattle against Jembrana disease. On the other hand, another benefit of this research is to provide information regarding the role of ethanol and IPTG on recombinant protein expression.

METHODS

The study was conducted at the Laboratory of Animal Molecular Genetics, Biotechnology Research Center of LIPI, Bogor in October-December 2017. The source of the recombinant protein construct using the pBT7-C-His construction which was inserted by the *tat* gene as the JTAT protein-coding gene in the *E. coli* NiCo strain belonging to the LIPI Animal Molecular Genetics Laboratory.

Making solid and liquid Luria-Bertani (LB) media

Solid Luria Bertani (LB; Sigma) was made as much as 400 ml. A total of 10 grams LB was weighed and dissolved in sterile distilled water to a volume of 400 ml. Then it was heated and homogenized with a magnetic stirrer (Forma Scientific). LB media was poured into petri dishes. Liquid LB was made as much as 1000 ml. A total of 25 grams LB were weighed and dissolved in sterile distilled water to a volume of 1000 ml. Then the solution was homogenized with a magnetic stirrer. LB media was inserted into 8 erlenmeyer (100 ml each). The remaining solution was poured into a test tube as much as 10 ml. LB media was sterilized by autoclave (Everlight TA630) at 121°C for 15 minutes.

E. coli culture carrying JTAT construct

LB media was made in liquid and solid form. LB media were sterilized by autoclaving at 121°C for 15 minutes. Amount of 20 µl glycerol stock of *E. coli* NiCo was inoculated in solid LB medium with 400 µl of ampicillin (Sigma) (100 µg/ml), and then incubated at 37°C. Amount of 20 µl of *E. coli* glycerol stock was added to 5 ml liquid LB medium with 5 µl ampicillin (100 µg/ml) in the shaker incubator (New Brunswick Scientific) at 37°C with the speed of 150 rpm for an overnight. Amount of 1 ml overnight yield was added to 100 ml liquid LB with 100 µl of ampicillin (100 µg/ml) in the shaker incubator at 37°C with 150 rpm for 2 hours. The inoculum was added with ethanol (Sigma) solution of 0%, 1%, 2% and 3%, then incubated at shaker incubator at 37°C with 150 rpm for 1 hour. Amount of 100 µl IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) was added in LB medium (specifically for treatment with the addition of IPTG), re-incubated for 1 hour at shaker incubator with the speed of 150 rpm and 37°C of temperature. Cells were

harvested by centrifugation at 4,000 rpm and temperature of 4°C for 15 minutes. Cells were then analyzed using the freeze-thaw and sonication methods.

Purification of JTAT protein

The supernatant resulted from sonication was added with 60 µl Ni-NTA resin (Thermo Scientific), then they were processed using the rotator for an overnight at low temperature (bath capture). Results of the previous process were centrifuged at 10°C with 1000 rpm, for 5 minutes. Amount of 1 ml supernatant was taken as an inner volume (IV). Pellets were added with 10 ml of washing buffer (imidazole 20 mM; Sigma) and centrifuged at 10°C with 1000 rpm for 5 minutes. Amount of 1 ml supernatant was taken as washing 1 (W1). Pellets were added with 7 ml washing buffer (imidazole 20 mM) and then centrifuged at 10°C with 1000 rpm for 5 minutes. Amount of 1 ml supernatant was taken washing 2 (W2). Pellets were added with 100 µl elution buffer (imidazole 400 mM) and processed using rotator at low temperature for overnight. The results were then centrifuged at 10°C with 1000 rpm for 5 minutes. Amount of 100 µl supernatant was taken as elution 1 (E1). Pellets were added with 100 µl elution buffer (imidazole 400 mM) and centrifuged 5 minutes at 10°C with 1000 rpm. Then, 100 µl supernatant was taken as elution 2 (E2).

Visualization of JTAT protein with SDS-PAGE

The SDS-PAGE gel was made with a concentration of 12%. The composition of the gel ingredients is presented in Table 1.

Firstly, the resolving gel was made and then followed by making the stacking gel. Then, 20 µl of the sample was denatured with the addition of 10 µl of loading dye (Fermentas) and incubated at 95°C for 15 minutes. Then, the running process was performed. The gel was inserted into the SDS-PAGE set. SDS 1x buffer then was added to the tank. Samples of 15 µl and 4 µl marker protein (Bio-Rad) were included in the well. Running process was carried out for ±2 hours at 100V. Electrophoresis gels were stained with coomassie blue (Fermentas) for an overnight. The gel was washed with a destaining solution for 15 minutes twice. Colored gels were documented.

Quantification of JTAT protein

The purified protein was quantified with a GeneQuant spectrophotometer. Amount of 3 µl protein sample was put in the cuvette. The protein concentration was then measured (mg/ml) at a wavelength of 280 nm (A280).

RESULTS AND DISCUSSION

This study began with the culture process of *E. coli* as a carrier of the construct of JTAT NiCo in solid Luria Bertani (LB) media. The new media contained more nutrients, thus supporting a better culture development (Wulandari & Sulistyani, 2016). Culture rejuvenation can activate bacterial isolates and optimize cell growth (Handayani, Sulistiani, & Setianingrum, 2016). The results of culture in solid LB media are presented in Figure 1.

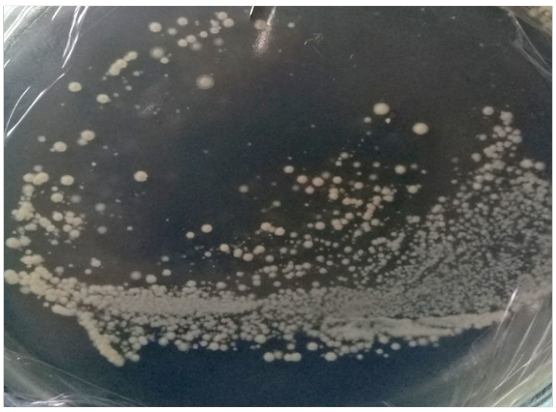

Figure 1. Results of refreshing the JTAT clone

The results of culture in Figure 1 show the morphology of bacteria with features of a round shape, smooth surface and white color or tend to

Table 1. The component of resolving and stacking gel

Resolving Gel	Amount	*Stacking Gel*	Amount
Sucrose (Sigma)	1.8 grams	dH_2O (Sigma)	3.05 ml
dH_2O (Sigma)	4 ml	Acrylamide 30% (Applichem)	0.65 ml
Acrylamide 30% (Applichem)	6 ml	Tris pH 6.8 (Bio-basic)	1.25 ml
1.5 M Tris pH 8.8 (Bio-basic)	3.75 ml	SDS 10%	50 µl
APS (Biomedicals)	100 µl	APS (Biomedicals)	50 µl
TEMED (Amresco)	10 µl	TEMED (Amresco)	10 µl

be colorless. According to the study of Islam, *et al*. (2014) that morphology of *E. coli* when cultured on solid media are in the form of circular, smooth and colorless. *E. coli* culture was performed on LB media, because it had a complete nutritional content for bacterial growth. LB media provided nitrogen and carbon as energy sources. Yeast extract supplied vitamins and NaCl to maintain the osmotic pressure of bacterial cells (Sezonov *et al.*, 2007). In addition, the LB media also was added with ampicillin which served to prevent the growth of bacteria other than *E. coli* (Walewangko *et al.*, 2015). Ampicillin also functions as a selectable marker of success during the screening process (Law *et al.*, 2002). *E. coli* cells carrying JTAT constructs were resistant to ampicillin. The resistance to ampicillin was caused by the plasmid pBT7-C-His which was inserted into the cell. These modified plasmids had ampicillin-resistant genes.

The process of protein visualization was done by SDS-PAGE. Purification of JTAT recombinant protein in pBT7-C-His plasmid was performed using Ni-NTA resin (nickel-nitrilotriacetic acid). Ni-NTA is a resin for plasmids containing 6xHistidine fusion tags. The interaction between 6x histidine tags and Ni-NTA is based on the selectivity and high affinity of Ni-NTA (Iba, 2012). The protein was denatured with heat, β-mercaptoethanol, and SDS. Proteins will be separated by load and size. Protein in the gel will form a separate band according to their molecular weight.

The presence of protein in the gel can be seen with the help of staining (Majek *et al.*, 2013). In this study, the SDS-PAGE was stained using Coomassie Brilliant Blue. The binding mechanism of Coomassie Brilliant Blue with protein occurs through a combination of hydrophobic interactions and heteropolar bonds with amino acids (Chu & Bob, 2000). The advantages of using Coomassie Brilliant Blue as a dye are a lower cost, easier to use and faster coloring times (Dong *et al.*, 2011). Figure 2 can be seen to be expressed under 15 kDa protein markers. JTAT protein was known to be ± 11.8 kDa (Margawati *et al*. 2017).

In addition to the visualisation of SDS-PAGE, the protein concentration of JTAT protein was also measured by GeneQuant spectrophotometry. Measurement of protein concentration was carried out at a wavelength of 280 nm (A280). The value of the protein concentration obtained was expressed in units of mg/ml. The JTAT protein concentration value was then analyzed using the Two way ANOVA test. The results of the Two way ANOVA test of JTAT protein concentration is presented in Table 2.

Table 2 shows that there is no significant difference between the IPTG treatment group, ethanol group and the interaction between the two. Data in the table indicates that IPTG and ethanol have no significant effect on JTAT protein concentration. The use of a narrow range of ethanol concentrations between samples is indicated to be the cause of this nonsignificant different result. Further research is needed with the same theme, but using a wider range of ethanol concentrations.

The expression vector used in this research was pBT7-C-His. This vector was modified by carrying several genes including ori, ampicillin

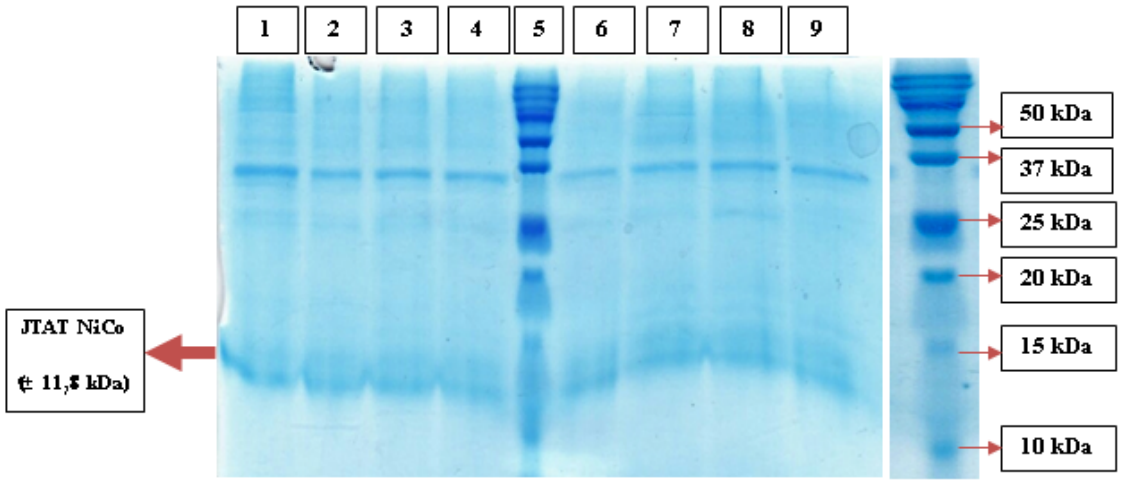

Figure 2. SDS-PAGE electrophoresis results
Note: 1= sample N1T1 (IPTG induction +EtOh 0%); 2= sample N1T2 (IPTG induction +EtOh 1%) 3= sample N1T3 (IPTG induction +EtOh 2%); 4= sample N1T4 (IPTG induction +EtOh 3%); 5= protein marker; 6= sample N0T1 (EtOh 0%); 7= sample N0T2 (EtOh 1%); 8= sample N0T3 (EtOh 2%); 9= sample N0T4 (EtOh 3%)

Table 2. Two-way ANOVA test result of JTAT recombinant protein concentration

Source of diversity	Number of squares	df	Central square	F count	F table 1%	F table 5%
IPTG	0.002	1	0.002	0.006*	8.53	4.49
Ethanol	0.273	3	0.091	0.363*	5.29	3.24
Interaction IPTG x Ethanol	0.218	3	0.073	0.289*	5.29	3.24
Error	4.013	16	0.251			
Total	4.506	23				

Note: (*) = not significantly different

resistant gene, T7 promoter, multiple cloning sites (MCs), 6x His Tag and T7 terminator (Bioneer, 2011). In the sample that with the addition of IPTG into the medium, gene expression will occur through the binding of IPTG as an inducer with a repressor. The bond causes the repressor not to stick to the operator so that the RNA polymerase can run the processes of transcription and translation (Marbach & Bettenbrock, 2012). By adding IPTG, the expression of T7 RNA polymerase fused in the chromosome of E. coli is increased. The result T7 RNA polymerase recognizes the T7 promoter at the upstream position of the target gene (Savalas *et al*., 2017).

JTAT protein was also expressed in samples that were added with ethanol without the addition of IPTG. Ethanol affects the activity of adenylyl cyclase (AC), thereby increasing the production of Cyclic Adenosine Monophosphate (cAMP) to regulate gene expression (Hill *et al*., 2016). Ethanol is known to influence the increased ATP production in cells (Cao *et al*., 2017) and increased AC activity (Qualls-Creekmore *et al*., 2017). When cAMP concentration increases, cAMP will bind to Crp and become active (McDonough & Rodriguez, 2013). The cAMP-Crp complex will bind to the lac promoter and begin the transcription process (Shimizu, 2013). This process causes the ethanol to induce the expression of JTAT recombinant proteins without the presence of IPTG.

The induction mechanism of interaction between ethanol and IPTG is the same as the induction mechanism of ethanol. This is because the time of ethanol induction is done before IPTG induction. So that the induction process is first carried out by ethanol with the mechanism of the formation of a cAMP-Crp complex. Whereas, in samples that were not induced by Ethanol and IPTG both qualitatively and quantitatively, JTAT protein was also obtained. This is because the T7 system can produce low amounts of recombinant protein expression during bacterial growth before the induction (Briand *et al*., 2016).

Limitations of this study include the use of induction time, number of samples and E. coli cells as well as further testing methods with western blot. The most straightforward method to detect the shift of the interacting proteins is by SDS-PAGE and Western blot analysis (Rosadi *et al*., 2016). Subsequent research needs to be done with the use of western blot test to determine the specific JTAT recombinant protein using JTAT anti-protein antibodies. Variations in ethanol induction also need to be known in inducing recombinant protein expression. In addition, the number of samples needs to be increased and the number of E. coli cells needs to be calculated. This research is just initial research, it is necessary to do further research related to ethanol as a substitute for IPTG in inducing recombinant protein expression.

CONCLUSION

Statistically, the induction of IPTG, ethanol and their interactions were not significantly different. However, the addition of IPTG, ethanol and their interactions can induce the expression of JTAT recombinant proteins.

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