Expression of An Immunogenic Intimin Fragment of EHEC O157:H7 in *Escherichia coli* Periplasm under The Control of A Rhamnose-Based Regulated Promoter

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Abstract

Intimin is the main adhesin of Enterohemorrhagic *E. coli* (EHEC) O157:H7 bacteria which are the most common leading infectious cause of bloody diarrhea and acute kidney failure in children who develop hemolytic uremic syndrome (HUS). Intimin is required for persistent bacterial colonization to eukaryotic host cell and its receptor-binding activity is localized at the C-terminus 282 amino acids (Intimin282). Thus, Intimin282 is an attractive antigen candidate that could be useful in vaccine and diagnostic systems against EHEC infections. Previous studies had reported expression of Intimin in *E. coli* cytoplasm using commonly used prokaryotic expression systems. However, it usually encountered several problems, i.e. low expression level, leaky expression, inclusion body formation, and truncated protein. The pRHA vector, which is tightly regulated by L-rhamnose and D-glucose, represents a viable alternative *E. coli* expression system to overcome such problems. Moreover, *E. coli* periplasm has an advantage of maintaining protein functionality by providing an oxidative environment that is more efficient than cytoplasm. However, to date there is no study about Intimin expression using pRHA expression system and/or in *E. coli* periplasm. Accordingly, we constructed a recombinant pRHA vector harbouring the respective gene to investigate the expression of an immunogenic Intimin fragment of EHEC O157:H7 in *E. coli* periplasm. The gene encoding His6-tagged Intimin282 (Int282) together with *pel*B signal sequence was cloned into the pRHA vector, subsequently expressed in *E. coli* JM109 and purified. Expression and purification of Int282 were verified by SDS-PAGE and Western blot. The result showed that Int282 was successfully expressed in *E. coli* periplasm with a protein size of approximately 32 kDa, which corresponded with the predicted size of the protein based on its amino acid sequence.

Keywords: EHEC O157:H7, immunogenic Intimin fragment, *E. coli* periplasm, rhamnose-based regulated promoter

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Introduction

Enterohemorrhagic *E. coli* (EHEC) O157:H7 is one of the major serotype which is the most virulent among the EHEC pathogenic bacteria (Gyles, 2007). These bacteria have a dangerous risk on human and animal health worldwide as a major cause of fatal human diseases, such as hemorraghic colitis (HC), and hemolytic uremic syndrome (HUS) (Lim *et al*., 2010). Characteristic symptom of HC is bloody diarrhea, whereas HUS could lead to kidney failure, especially in children (Asper *et al*., 2011; WHO, 2011).

The bacterium is distributed worldwide (Gyles, 2007), and has been frequently reported to be associated with the incidence of EHEC infectious disease outbreaks worldwide (Lim *et al*., 2010; WHO, 2011; Fan *et al*., 2012). Although there has not been reliable report about the EHEC infection cases yet, Indonesia is still at risk of EHEC infections. This is associated with several common driving forces of infectious disease incidents (Altekruse *et al*., 1997), such as demographic changes, economic development and land use, microbial adaptation, and disruption of public health infrastructure. Moreover, it is also

associated with local isolates of *E. coli* O157:H7 isolated from dairy products, beef, cattle feces, and human feces (with or without symptoms of diarrhea and kidney failure) which are potent to cause EHEC infections (Suardana *et al*., 2011). The major intermediate host of these bacteria is ruminant, especially cattle, and could sporadically be transmitted directly or indirectly to human, and infected human could also transmit the bacteria to others (Lim *et al*., 2010).

The bacteria enable to cause fatal disease and are highly infectious in human, which occurs in a very low level of infection (Lim *et al*., 2010). Therefore, the appropriate control and handling strategy is required in order to prevent or diminish the colonization and transmission of the bacteria. The strategy includes development of recombinant protein for vaccine and diagnostic systems. One way to reduce the risk of infections in human is to reduce the level of these bacteria at the source point. Vaccine development for cattle, as an important source of these bacteria, is an early effort to prevent cattle from becoming infected by these bacteria, and thus blocking its transmission to human (Fan *et al*., 2012). However, such vaccine has not been commercially available yet, as well as vaccine for human (Fan *et al*., 2012). Moreover, results of the investigations on the prevalence of EHEC O157 in cattle are clearly influenced by the sampling and detection methods adopted (Caprioli *et al*., 2005). It has been reported that the use of specific immunoconcentration procedures for EHEC O157 strongly enhances the sensitivity of the detection methods (Vernozy-Rozand *et al*., 1998). Therefore, strategy in the development of vaccine and detection method requires better understanding of EHEC molecules that could be used as antigen. Furthermore, to study whether an antigen candidate could be used for vaccine and diagnostic systems, it is necessary to develop an appropriate expression system to generate a functional recombinant protein and to enable its production in a large quantity.

Intimin is an interesting and potential antigen candidate for vaccine (for both animal and human) and diagnostic systems against EHEC infections (Dean-Nystrom *et al*., 2002; Son *et al*., 2002; Judge *et al*., 2004; Kühne *et al*., 2004). Intimin is one of the key virulence factors in determining the capability of these bacteria to intensively colonize the host intestinal mucose (Oswald *et al*., 2000). It is an outer membrane protein (major adhesin protein; 934 amino acids), encoded by *E. coli attach-and-efface* (*eae*) gene, and required for intimate attachment of bacterial cell on the surface of the host intestinal epithelial cell membrane. This intimate attachment leads to cell invasion which cause characteristic attachment and effacement (A/E) lesion formation (Kühne *et al*., 2004; Torres *et al*., 2005). With those characteristics, Intimin could raise immunogenic response, and is known to belong to highly immunogenic molecules (Li *et al*., 2000; Karpman *et al*., 2002). Thus, antibodies against Intimin of EHEC O157:H7 would play important role in preventing the attachment of the bacteria to the host cells and protect them from colonization by these bacteria (Kühne *et al*., 2004).

The receptor-binding domain of Intimin is localized at the C-terminus region of 282 amino acids (Intimin282), which mediates the interaction with translocated Intimin receptor (Tir) (Kühne *et al*., 2004). Therefore, Intimin282 is attractive as an antigen candidate that could be useful in vaccine and diagnostic systems against EHEC infections. Previous works had reported expression of full-length or C-terminus fragment of EHEC O157:H7 Intimin as single or fusion proteins in *E. coli* cytoplasm using commonly used prokaryotic expression systems, such as pLac and T7 polymerase (Frankel *et al*., 1995; Son *et al*., 2002; Kühne *et al*., 2004; Gu *et al*., 2009). However, these systems usually encountered several problems, i.e. low expression level and leaky expression (Giacalone *et al*., 2006). Moreover, the recombinant Intimin expressed showed disfunctionality and instability, i.e. inclusion body formation and formation of truncated protein, caused by the limitation of *E. coli* cells which are unable to translocate the protein from cytoplasm into the periplasm.

The pRHA vector has been reported as a viable alternative of *E. coli* expression system for stable cloning and production of functional recombinant proteins (Giacalone *et al*., 2006). Moreover, the periplasmic space has been reported to enable functionality and stability of the protein more efficiently than cytoplasm (Choi & Lee, 2004). However, there is no study about Intimin expression using pRHA expression system and/or in *E. coli* periplasm to date. Accordingly, we developed a

recombinant pRHA vector construct containing *pel*B-Int282 fusion protein designated as pRHA-SDM::*pel*B-Int282. This expression system was constructed to obtain more efficient Int282 expression in order to overcome difficulties found in the commonly used prokaryotic expression systems. The expression vector is tightly regulated by L-rhamnose inducer and D-glucose represser under the control of *rha*B promoter (P_{RHA}) and corresponding regulatory genes (Egan & Schleif, 1993). The signal peptide *pel*B plays a role in modulating direct translocation of the recombinant protein from cytoplasm into periplasm (Choi & Lee, 2004). Thereby, construction of the recombinant vector pRHA-SDM::*pel*B-Int282 would represent an appropriate expression system for more efficient expression of an immunogenic Intimin fragment of EHEC O157:H7.

Materials and Methods

DNA Materials and Bacterial Strains

DNA materials which were used in this research are described in Table 1. *E. coli* DH5α and *E. coli* JM109 were used for cloning and expression, respectively.

Construction of Recombinant Vector

Isolation of the target gene. The target gene encoding His6-tagged Intimin282 containing *Nco*I and *Xho*I restriction sites at 5′ and 3′ ends respectively, was isolated from the plasmid pJExpress404::*syn*EspA128/Intimin282 (Table 1) by polymerase chain reaction (PCR) amplification using Intim1-F and Intim1-R specific primers (Table 2). PCR amplification was carried out using the following steps: predenaturation at 94°C for 1 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 4 min. The PCR product was purified using MinElute PCR Purification Kit according to manufacturer's protocol. Subsequently, it was double digested with the appropriate restriction enzymes. The digestion product was purified using QIAquick Gel Extraction Kit according to manufacturer's protocol, and the purified product was analysed using agarose gel electrophoresis.

Vector engineering. For cloning purpose, the plasmid pJExpress804::77539 as pRHA vector source (Table 1) was modified by site-directed mutagenesis (SDM) through PCR amplification using SDM-F and SDM-R specific primers (Munteanu *et al*., 2012; Table 2). PCR amplification was carried out using the following steps: predenaturation at 95°C for 2 min; 25 cycles of denaturation at 95°C for 1 min, annealing at 55.2°C for 90 s, and extension at 68°C for 10 min; and a final extension at 68°C for 2 min. SDM product (designated as pRHA-SDM) were verified with restriction analysis, and DNA sequencing using SeqM01-F specific primer (Table 2). The verified pRHA-SDM product was then double digested with the appropriate restriction enzymes. The enzymatic digestion was verified using agarose gel electrophoresis, and subsequently the expected vector DNA was isolated from the agarose gel using QIAquick Gel Extraction Kit according to manufacturer's protocol. The purified vector DNA was analysed using agarose gel electrophoresis.

Cloning and transformation. The gene encoding Int282 and *pel*B (generated using SPpelB-F and SPpelB-R specific primers; Table 2) were cloned into the pRHA-SDM vector using the enzyme T4 DNA ligase to develop the construction of pRHA-SDM::*pel*B-Int282 recombinant vector. The resulted recombinant vector was transformed into *E. coli* DH5α using heat-shock method (Chung *et al*., 1989). Positive *E. coli* clones were selected, and recombinant vectors were subsequently isolated. The recombinant vectors were verified with restriction analysis, and DNA sequencing analysis using SeqC01-F and SeqC01-R specific primers (Table 2).

Table 1. DNA materials which were used in the experiments.

DNA materials	Size (bp)	Content	Reference
pJExpress404::*syn*EspA128/ Intimin282	5008	Codon-optimized His6-tagged Intimin282	Kusharyoto *et al*., 2012
pJExpress804::77539	5008	pRHA vector	Andriani *et al*., 2012

Table 2. Nucleotide sequence of primers which were used in the experiments.

Primers[a]	Nucleotide sequence[b]
Intim1-F	5′-GCGAGCCATGGCCTTTGATCAAACCAAGGCCAGCATTAC-3′
Intim1-R	5′-GTCACCTCGAGTTAGTGGTGATGATGGTGATGCGCGGCT-3′
SPpelB-F	5′-TATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGC-3′
SPpelB-R	5′-CATGGCCGGCTGGGCAGCGAGGAGCAGCAGACCAGCAGCAGCGGTCGGCAGCAGGTATTTCA-3′
SDM-F	5′ CGAAAGGGCGTATTCGTATGCGGTGTGAAATACC-3′
SDM-R	5′ GGTATTTCACACCGCATACGAATACGCCCTTTCG-3′
SeqM01-F	5′-GAAGATACCGCGACTTATTTC-3′
SeqC01-F	5′-GTGAACATCATCACGTTCATC-3′
SeqC01-R	5′-GCGAGTCAGTGAGCGAGGAAG-3′

[a]F: forward primer, R: reverse primer

[b]Underlined sequences show specific nucleotide of restriction sites which were used for cloning

Expression of Target Gene

In order to express Int282, pRHA-SDM::*pel*B-Int282 recombinant vector was transformed into *E. coli* JM109 by heat-shock. Overnight culture of the bacteria harbouring the recombinant vector was grown (1/50 dilution) in 100 mL of Luria Bertani (LB) medium containing 0.2% glucose and 100 µg/mL ampicillin at room temperature to an OD_{600} of 0.3-0.4 (6 h) (Chung *et al*., 1989; Giacalone *et al*., 2006). Subsequently, L-rhamnose was added into the culture to a final concentration of 1000 µM, according to the result from optimization of inducer concentration (Giacalone *et al*., 2006; data not shown). Growth was then continued for overnight (16 h; OD_{600} of 1.0). Cells were harvested by centrifugation at 4000×g for 10 min at 4°C, resuspended in lysis buffer (50 mM NaH_2PO_4 (pH 7.5), 300 mM NaCl, and 10 mM of imidazole; 10 mL/100 mL of culture), and subsequently phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 1 mM.

Isolation of the periplasmic fraction (PF) was conducted by lysozyme treatment to a final concentration of 0.5 mg/mL and incubation at room temperature for 1 h (Kusharyoto *et al*., 2002; Amani *et al*., 2010). The supernatant was collected after centrifugation at 20,000×g for 15 min at 4°C, while the cells pellet was resuspended again. Subsequently, cytoplasmic fraction (CF) was isolated by sonication with 5 times pulses for 1 min alternated with 30 s pauses, and the supernatant was collected after centrifugation (Kühne *et al*., 2004). The protein Int282 was then purified from PF by means of Co^{2+} immobilized metal chelate affinity chromatography (IMAC) on TALON™ matrix (McMurry & Macnab, 2004; Giacalone *et al*., 2006). Expression and purification of Int282 were verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using HisDetector™ Ni-HRP Conjugate Western Blot Kit according to manufacturer's protocol.

Results and Discussion

Construction of Recombinant Vector

The construction of the pRHA-SDM::*pel*B-Int282 recombinant vector (Figure 1) has been performed to investigate the expression of an immunogenic Intimin fragment of EHEC O157:H7 in *E. coli* periplasm under the control of a rhamnose-based regulated promoter (P_{RHA}). The immunogenic Intimin fragment used in this research was the C-terminus 282 amino acids (Intimin282) as it is necessary and sufficient for the intimate interaction to its receptor, Tir (Kühne *et al*., 2004). The domain of Intimin282, which directly involved in mediating the binding activity with Tir is localized at 181 amino acids of C-terminus (minimum length), while the rest 94 amino acids of N-terminus contribute to the stability and functionality of the binding domain (Kühne *et al*., 2004). Thus, antibodies towards Intimin282 would be specific enough against Intimin of EHEC O157:H7, and would not give any cross-reactivity with the other types of Intimin (Kühne *et al*., 2004). Eventhough Intimin sequence is highly conserved within the Intimin family (and Invasion family) at approximately two-thirds of its N-terminus, one-third of its C-terminus shows high heterogenicity (Oswald *et al*., 2000). Moreover, the use of a larger fragment of Intimin could lead to less efficient function,

which might be caused by steric hindrance, whereas a smaller one could lead to inactive molecule (Frankel *et al*., 1995). Furthermore, the use of the codon-optimized gene is intended for high level expression as codon optimization could increase transcription and translation processes in a specific host cell, thus optimize gene expression in a given host cell (Fuad *et al*., 2008; Amani *et al*., 2010).

The pRHA vector used in this research represents a viable alternative *E. coli* expression system for stable cloning and expression of functional recombinant proteins (Giacalone *et al*., 2006). This vector is tightly regulated by L-rhamnose inducer and D-glucose represser under the control of *rha*B promoter (P_{RHA}) and corresponding regulatory genes (Egan & Schleif, 1993). The pRHA plasmid belongs to the medium copy number type of plasmid (DNA2.0). It was chosen in order to maintain integrity of the host cell during the expression of recombinant protein. Whereas *pel*B signal peptide fused upstream of Int282 plays an important role in modulating protein translocation from cytoplasm into periplasm of the *E. coli* host. It has been known that periplasm enable to maintain the functionality and stability of the recombinant protein more efficiently than cytoplasm (Choi & Lee, 2004).

The C-terminus of Intimin282 was tagged with His6 to facilitate purification and detection of the expressed recombinant protein (Judge *et al*., 2004; Kühne *et al*., 2004). His6-tag was chosen since it needs a simple process by binding to metal ion (Co^{2+}, Ni^{2+} or Zn^{2+}) with high affinity and it does not require specific three-dimensional structure allowing straightforward purification (McMurry & Macnab, 2004). Another advantage of His6-tag is its small size that is not very immunogenic and usually does not interfere with the function of recombinant protein. The present of the helical linker between Intimin282 and His6-tag is used to facilitate the correct protein folding for maintaining the immunogenicity of Intimin282, since it has a firm structure that can effectively separate the two functional domains and keeps their functionalities independently (Arai *et al*., 2001). It also controls the spatial distance between both domains, thus avoiding unnecessary inter-domain interactions (Arai *et al*., 2001). Moreover, due to hidden structure of C-terminus of Intimin282, the linker could promote the His6-tag to be well exposed for protein purification and detection processes (Figure 2).

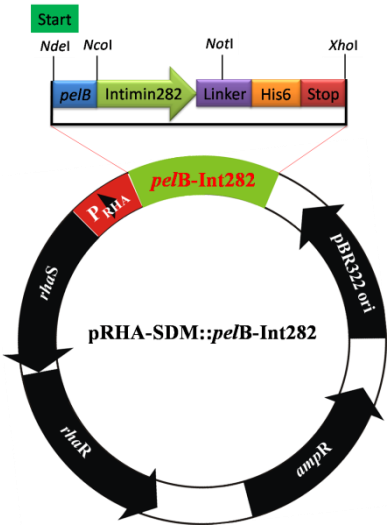

Figure 1. Schematic representation of the recombinant vector pRHA-SDM::*pel*B-Int282.

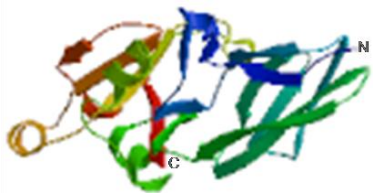

Figure 2. The folding structure of Intimin282 (http://web.expasy.org/cgi-bin/compute_pi/pi_tool; 30.24 kDa). It showed the hidden domain of C-terminus of Intimin282.

Different restriction sites at different positions were employed for efficient cloning and to avoid incorrect orientation of the DNA insert. Each site was selected to have overlap nucleotide sequences with *pel*B and/or Int282. It was necessary to limit the presence of additional nucleotide in the designed construction, which could affect the structure and/or functionality of the protein expressed. Therefore, the recombinant Int282 protein would be relatively similar in its structure to its native protein in the respective fragment. The constructed vector also offers possibility for selection of preferred signal peptides, insert and/or tag in order to further optimize protein expression in future works. Primers were designed to facilitate the cloning purpose (Son *et al*., 2002). For example, *Nde*I restriction site was placed in 5′ end of SPpelB-F, *Nco*I in 5′ end of SPpelB-R and Intim1-F,

and *Xho*I in 5′ end of Intim1-R. The pRHA vector was modified by SDM to facilitate the use of the preferred restriction sites. Specific primers used for SDM were designed to contain the complement of the mutation target nucleotide (Munteanu *et al*., 2012), in such that A was mutated into G.

The result of target gene isolation confirmed that Int282 has been successfully generated showing the expected sizes (approximately 936 and 924 bp for PCR and digestion products, respectively) (Figure 3A). Whereas the pRHA vector had been successfully modified by SDM. The result indicated that the undesirable *Nde*I site within the vector has been successfully removed, and was verified by digestion with the respective enzymes (Figure 3B) and DNA sequencing (data not shown).

Figure 3B showed that a single band was detected with a size of approximately 5008 bp as it was cut with *Nde*I, and two bands with a size of approximately 4177 and 831 bp when cut with *Nde*I dan *Xho*I. Those fragments consistent with that of the expected sizes of recombinant plasmid, vector, and insert of pRHA vector source, respectively. The result indicated that pRHA has been successfully modified to pRHA-SDM having only one *Nde*I site. The result was supported with DNA sequencing analysis which showed that an A has been altered into G as expected within the *Nde*I_{2907} sequence (data not shown).

The cloning of *pel*B-Int282 into the expression vector in the construction of pRHA-SDM::*pel*B-Int282 has been verified with digestions with the respective restriction enzymes (Figure 4) and DNA sequencing (data not shown). Restriction analysis with single digestion showed the presence of a linear DNA fragment of approximately 5161 bp. This is in accordance with the expected size of the recombinant vector. Moreover, it revealed the presence of two DNA fragments of approximately 4177 and 984 bp, respectively, when double digested. Those fragments corresponded to the expected sizes of both vector and insert, respectively. Furthermore, the result was also supported by DNA sequencing analysis that showed that the target gene has exactly the designed *pel*B-Int282 sequence (data not shown).

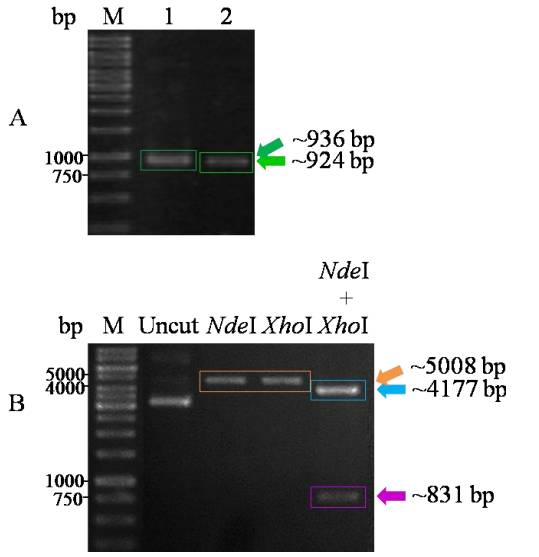

Figure 3. A. Agarose gel electrophoresis analysis result of Int282 DNA fragment. It showed that Int282 DNA fragment has been successfully isolated. B. Restriction analysis result of pRHA-SDM. It showed that pRHA with only one *Nde*I site has been successfully engineered by site-directed mutagenesis (SDM). M. DNA marker, 1 and 2. PCR and digestion products, respectively.

Figure 4. Restriction analysis result of the recombinant vector pRHA-SDM::*pel*B-Int282. It showed that pRHA-SDM::*pel*B-Int282 has been successfully constructed. M. DNA marker.

Expression of Target Gene

We have successfully expressed recombinant Int282 using expression system which was constructed in this research. *E. coli* was chosen as an expression host mostly due to several factors, including fast growing microbe in an inexpensive media, high expression level, and variety of expression vectors readily available. The pRHA vector is an alternative expression plasmid that can be used in any *E. coli* strain or other Gram-negative bacteria and works in a regulated fashion. There were several efforts which were performed in this experiment to facilitate higher expression level, such as addition of

glucose and culture at low temperature. Addition of 0.2% of glucose efficiently blocks leaky expression, whereas low temperature culture facilitates correct protein folding, thus enabling efficient expression and localization of the target protein (Giacalone *et al*., 2006). Purification of protein using Co^{2+} IMAC system offers advantage compared to Ni^{2+} or Zn^{2+}, such as highly specific affinity to His-tagged recombinant protein. Its affinity is very selective and it does not or poorly bind host's native contaminant proteins (McMurry & Macnab, 2004). Thus, the target protein could be easily purified.

Expression of Int282 recombinant protein was verified by SDS-PAGE analysis (Figure 5). We observed the presence of a protein band showing a molecular weight of approximately 32 kDa in PF of induced *E. coli* JM109 harbouring the recombinant plasmid (I). It was not found in PF and CF of wild type *E. coli* JM109 (WT) and *E. coli* JM109 harboring plasmid without insert (P), and PF of non-induced *E. coli* JM109 harboring the recombinant plasmid (NI), although it was still slightly found in CF of I and NI. The protein size is in accordance with the predicted molecular weight based on its amino acid sequence (32.41 kDa; http://web.expasy.org/cgi-in/compute_pi/pi_tool). These results indicated that Int282 has been successfully expressed in *E. coli* periplasm using the designed expression system. Although the result showed suboptimal translocation of the protein from cytoplasm into periplasm with the existence of slight band in CF of I. Moreover, it also showed that there was only slight or no leaky expression with the existence of slight band in CF of NI, which means that pRHA expression system is tightly regulated as previously described (Giacalone *et al*., 2006).

Purification of Int282 followed by SDS-PAGE and Western blot analysis supported these findings (Figure 6), which revealed the presence of a band at approximately 32 kDa in elution fractions (E1-E3), which was mostly found in E2 and E3. Overall, these results indicated that we have successfully used pRHA expression system for expression of Int282 in *E. coli* periplasm. The same vector has also been reported to successfully express nontoxic TphoA, mildly toxic GFP, and toxic MalE-NTR proteins in *E. coli* MG1655 as a functional fully intact full-length protein (Giacalone *et al*., 2006).

Furthermore, these results suggest that the expression system used in this research was able to express Int282 as soluble and untruncated protein, which could not be accomplished by previous works. In the previous works, His6-tagged C-terminal Intimin fragments or full-length Intimin were expressed in *E. coli* BL21(DE3) cytoplasm as inclusion bodies (low solubility), such as Intimin306 and full-length Intimin of EHEC O157:H7, as well as Intimin311 of EHEC O127:H6 using pET28a(+) expression system (Son *et al*., 2002); Intimin188 of EHEC O157:H7 using pET21b (Kühne *et al*., 2004); and Intimin300 of EHEC O157:H7 (as trivalent protein) using pET28a(+) (Gu *et al*., 2009). In contrast, Intimin280 of Enteropathogenic *E. coli* (EPEC) O127 (Frankel *et al*., 1995), and Intimin282 of EHEC O157:H7 (Kühne *et al*., 2004) were able to be expressed as soluble protein following fusion with maltose-binding protein (MBP) in *E. coli* TG1 cytoplasm using pMalc2 expression system. However, the recombinant proteins were produced in truncated forms.

To date, there are several studies that have also expressed Intimin using eukaryotic expression systems, such as expression of Intimin261 of EHEC O157:H7 in *Nicotiana tabacum* cv. Bright Yellow 2 (NT-1) plant cells using pGPTV-Kan binary vector (Judge *et al*., 2004). However, it resulted in glycosylated protein since Intimin261 sequence contains two potential Asparagine (N)-linked glycosylation sites that could be modified by glycosylation in endoplasmic reticulum (Judge *et al*., 2004). The glycosylation disrupted the functionality of the protein, thus affected the immune response which in turn was unable to block adherence of EHEC O157:H7 (Judge *et al*., 2004).

Expression of Intimin282 of EHEC O157:H7 (as trivalent protein) in tobacco plant using pBI121 binary vector, and in canola seeds using pBI1400 binary vector produced functional proteins, though it was still slightly glycosylated since the protein sequence had some potential N-glycosylation sites (Amani *et al*., 2011). Thereby, the expression system constructed in this research could be utilized to overcome such problems which usually encountered with Intimin expression using

commonly used prokaryotic expression systems.

Conclusion

We have successfully expressed a soluble and untruncated immunogenic Intimin fragment of EHEC O157:H7 in *E. coli* periplasm under the control of a rhamnose-based regulated promoter (P_{RHA}). The expressed recombinant Intimin fragment (Int282) has a molecular weight of approximately 32 kDa, which is in accordance with the predicted molecular weight based on its amino acid sequence. These results could raise the possibility of Int282 utilization as antigen candidate in vaccine and diagnostic systems in order to develop preventive measure against EHEC O157:H7 infections. Furthermore, these results could encourage further expression studies using the same expression system of other recombinant proteins, such as which are derived from the virulence factors of EHEC O157:H7, which may also find application as antigen candidates in vaccine and diagnostic systems.

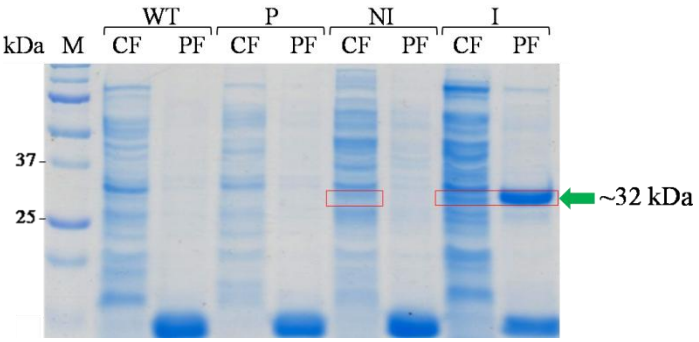

Figure 5. SDS-PAGE analysis result of periplasmic (PF) and cytoplasmic fractions (CF). It showed that Int282 has been successfully expressed in *E. coli* periplasm. M. Protein marker, WT. Wild type *E. coli* JM109, P. *E. coli* JM109 harboring plasmid without insert, NI. Non-induced *E. coli* JM109 harboring the recombinant plasmid, and I. Induced *E. coli* JM109 harbouring the recombinant plasmid.

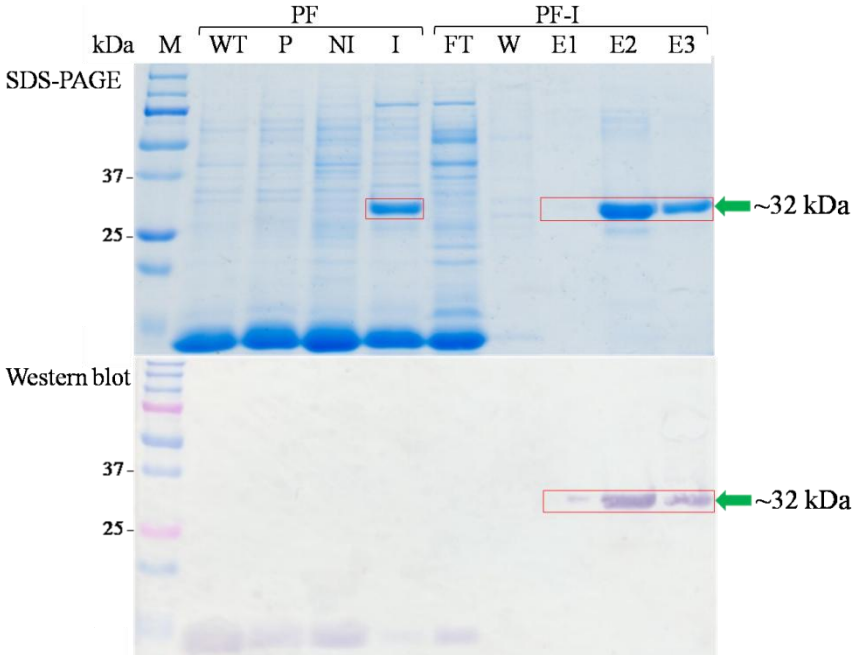

Figure 6. SDS-PAGE and Western blot analysis results of purified Int282. These results showed that Int282 has been successfully expressed in *E. coli* periplasm and purified. M. Protein marker, PF. Periplasmic fraction, WT. Wild type *E. coli* JM109, P. *E. coli* JM109 harboring plasmid without insert, NI. Non-induced *E. coli* JM109 harboring the recombinant plasmid, I. Induced *E. coli* JM109 harbouring the recombinant plasmid, FT. Flow-through fraction, W. Washing fraction, and E. Elusion fraction.

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