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Original research article

Expression of Recombinant *Sugarcane Streak Mosaic Virus* Coat Protein Gene in *Escherichia coli*

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## ABSTRACT

*Sugarcane streak mosaic virus* (SCSMV) is an important virus causing mosaic disease in sugarcane and transmitted through the cutting cane. Commercial antiserum to detect SCSMV and to monitor the disease development is not available. The research was conducted to produce antigen of SCSMV coat protein (SCSMV-CP) through overexpressing it on bacterial expression which will be used for antiserum production. SCSMV-CP was amplified using specific primers for CP gene containing *Bam*HI and *Hind*III restriction enzyme sites and cloned into pTZ57R/T. Subsequently, the SCSMV-CP was subcloned into pET28a and transformed on *Escherichia coli* BL21(DE3) and Rosetta-gami(DE3)pLysS. The concentration of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG), incubation temperature, and bacterial harvesting time after IPTG induction were optimized. SCSMV-CP gene was successfully amplified with size  $\pm$ 855 bp, subcloned into vector expression, and expressed in insoluble fraction either in both bacterial host. Optimal protein expression of SCSMV-CP recombinant was obtained at 25°C with IPTG concentration 0.25–1.00 mM and harvested at 9–12 hours after IPTG induction in *E. coli* BL21(DE3), and at 30°C with IPTG concentration 0.25–1.00 mM and harvested 3–12 hours after IPTG induction in *E. coli* Rosetta(DE3)pLysS. SDS-PAGE analysis showed that protein size of SCSMV-CP recombinant was  $\pm$ 35.4 kDa.

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## 1. Introduction

*Sugarcane streak mosaic virus* (SCSMV) is a member of family *Potyviridae*, genus *Susmovirus* (Xu *et al.* 2010), and has a very close relationship with *triticum mosaic virus* (King *et al.* 2012). SCSMV was first identified by Hall *et al.* (1998) from quarantined sugarcane germplasm showing mosaic symptoms that had been imported from Pakistan to USA. SCSMV was reported infecting sugarcane in India, Pakistan, USA, Thailand, Sri Lanka, Vietnam, Australia, and Indonesia (Hema *et al.* 1999; Chatenet *et al.* 2005; Damayanti and Putra 2011).

SCSMV is relatively a new virus in Indonesia and predominantly infected commercial sugarcane clones in Central Java and East Java, mainly on PS 864 clone. SCSMV spreads rapidly because it is a sett-borne virus and its transmission is facilitated by the cutting knives during preparation of planting materials or during harvesting

(Damayanti and Putra 2011). The incidence of the streak mosaic disease up to 50% decreased the cane tonnage and sugar yield ranged from 16% to 17% and 19% to 21%, respectively (Asnawi 2009). Because SCSMV is sett-borne, the effort to control the virus can be achieved using virus-free setts. Routine detection is necessary to maintain virus-free sugarcane stock, either as setts before planting or as mother plants.

Serology-based detection method is very effective and efficient for plant viruses. The major limiting factor for using these detection methods is the requirement of a good virus purified preparation to be used in immunizing animals for antiserum production. Virus purification from the infected plants favors contamination from host proteins and often gives variable background reaction in serological test (Viswanathan *et al.* 2011). An advanced technique in molecular biology to provide abundance antigen through the expression of a particular gene which inserted into a plasmid expression vector in *Escherichia coli* could overcome the problems. This method avoids cross reaction of antiserum to plant protein (Cotillon *et al.* 2005), suitable to produce antigen for virus with low titer, limited distribution virus in plant tissues, or virus that infects woody plant tissues (Ling *et al.* 2007). Because SCSMV antiserum is

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not available yet commercially, it is necessary to provide abundance of SCSMV antigen by overexpressed CP gene in bacterial expression, *E. coli*.

## 2. Materials and Methods

### 2.1. SCSMV amplification by reverse transcription polymerase chain reaction (PCR)

SCSMV Pasuruan isolates was obtained from Plant Virology Laboratory, Faculty of Agriculture, IPB. Total RNA was isolated from 0.1-g SCSMV infected leaves using cethyl trimethyl ammonium bromide method (Doyle and Doyle 1990). Synthesis of cDNA from 2.5  $\mu$ L total RNA was done as described by Damayanti *et al.* (2010). SCSMV-*Bam*F4 forward primer (5'-AAGGATCCGGAGAGC-AAGGAACA CA-3') and SCSMV-*Hind*R3 reverse primer (5'-TATAAGCTTTCAGTGCTGGGCG CG-3') inserting restriction sites *Bam*HI and *Hind*III (underlined), respectively, were used to amplify the SCSMV-CP gene with expected size of amplicon DNA  $\pm$ 850 bp. PCR was performed as described by Damayanti *et al.* (2010) which amplified in 30 cycles, with an initial denaturation at 94°C for 4 minutes and each cycle with denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute. Final extension was performed at 72°C for 30 minutes to extend the poly-(A) at the end of the amplicon to increase ligation efficiency using TA vector.

The amplification product was separated by electrophoresis on 1% agarose gel in 0.5X TBE (Tris boric acid EDTA) at 50 V for 50 minutes. Gel was soaked in 0.1% ethidium bromide for 15 minutes and in sterile water for 5 minutes. Gel was visualized under UV transilluminator and documented with a digital camera.

### 2.2. Preparation of *E. coli* competent cells

Competent cells used in this study are *E. coli* strain JM 107 (Thermo Scientific, USA), BL21(DE3), and Rosetta-gami(DE3)pLysS (Novagen). The competent cells were prepared using CaCl<sub>2</sub> method as described by Sambrook and Russel (2001).

### 2.3. Cloning SCSMV-CP in pTZ57R/T vector

The amplified SCSMV-CP DNA was ligated into pTZ57R/T vector according to InstAclone PCR Cloning Kit instructions (Thermo Scientific). Ligation reaction contains 3  $\mu$ L DNA SCSMV-CP, 1  $\mu$ L DNA vector pTZ57R/T, 2  $\mu$ L 5X ligation buffer, 1  $\mu$ L T4 DNA ligase (Thermo Scientific), and nuclease free water up to 10  $\mu$ L total volume. The premix ligation was incubated at 4°C for 16 hours to yield pTZ-SCSMV-CP plasmid. The plasmid was then transformed into *E. coli* JM107 cells by heat shock method and spread on Luria Bertani Agar (LBA) containing ampicillin 50  $\mu$ g/mL (LBA-ampicillin; Sambrook and Russel 2001). Plasmid containing expected insert was selected and confirmed by colony PCR using the same primer for the previous amplification. The positive plasmid was extracted using a plasmid Miniprep Kit according to the manufacturer recommended procedure (Thermo Scientific).

SCSMV-CP plasmid was sequenced at First Base, Malaysia. Sequences data were compared with other corresponding sequences deposited in GenBank using Basic Local Alignment Search Tools program (NCBI, 2014). The homologies of nucleotide and amino acid sequences were analyzed using CLC Sequence Viewer 7 and Bioedit V.7.05 software (Hall 1999).

### 2.4. Subcloning of plasmid SCSMV-CP into expression vector

Plasmid pTZ-SCSMV-CP and pET-28a (Novagen) were double digested with *Bam*HI and *Hind*III enzymes (Thermo Scientific, USA) according to protocol provided by Thermo Scientific. Restriction

enzyme digestion was incubated for 24 hours at 37°C. The digested DNA was isolated from 1% agarose gel electrophoresis and was purified using Gel/PCR fragment DNA extraction kit according to the manufacturer's instructions (Genaid, Taiwan).

Ligation, transformation, and confirmation of transformant were conducted as previously described. DNA of SCSMV-CP was ligated into pET28a expression vector to yield pET-SCSMV. The transformation was done by heat shock method into *E. coli* BL21(DE3) and Rosetta-gami(DE3)pLysS and the bacteria was grown in LBA containing kanamycin 25  $\mu$ g/mL.

### 2.5. Expression of recombinant SCSMV-CP gene

#### 2.5.1. Optimization of recombinant SCSMV-CP gene expression

One percent of overnight culture of *E. coli* BL21(DE3) and Rosetta-gami(DE3)pLysS containing pET-SCSMV-CP was grown at 37°C in LB culture medium (25  $\mu$ g/mL kanamycin) and incubated on shaker at 150 rpm to OD<sub>600</sub> = 0.5–0.7. Optimization of culture expression was carried out on three different factors i.e. concentration of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG; 0.25, 0.5, 0.75, 1.0 mM), incubation temperature (25°C, 30°C, 37°C), and bacterial harvesting time (3, 6, 9, 12, 18 hours) after IPTG induction. Cells were harvested by centrifugation of 3-mL culture at 12,000 rpm for 5 minutes.

#### 2.5.2. Purification of SCSMV-CP protein

SCSMV-CP protein was purified in non-denaturing condition using nickel nitrilotriacetic acid column according to manufacturer's instructions (Qiagen) with minor modification on elution step. Elution was done by using two kinds of elution buffer, i.e. buffers E and C with the additional 500 mM imidazole.

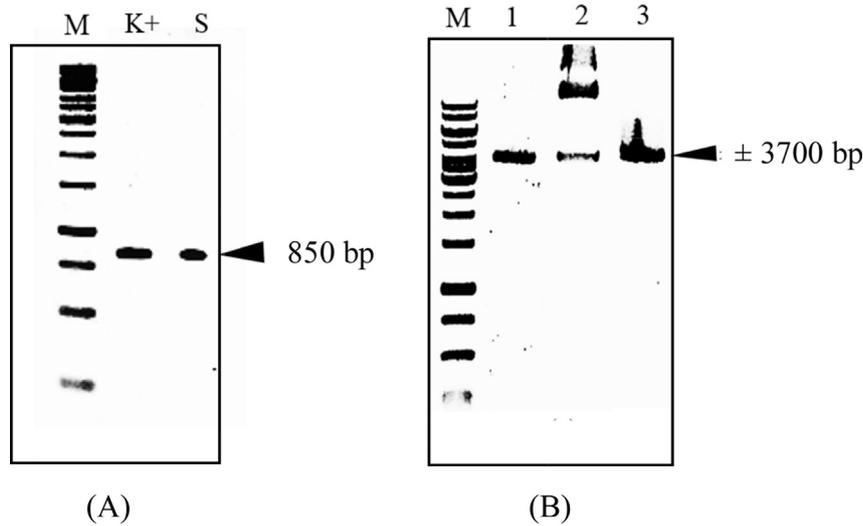
#### 2.5.3. Protein analysis

Protein was analyzed on 12.5% SDS-PAGE (Laemmli 1970) using Vertical Gel Electrophoresis System model V-16-2 (Invitrogen). Bacterial pellet was resuspended in 50  $\mu$ L lysis buffer pH 8.0 and shaken at 150 rpm for 30 minutes. Protein was centrifuged at 12,000 rpm for 5 minutes, and bacterial pellet (insoluble) and supernatant (soluble) were then collected separately. Bacterial pellet was resuspended in 500  $\mu$ L of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.002% bromophenol blue, 5%  $\beta$ -mercaptoethanol, 10% glycerol), and supernatant was mixed with sample buffer. Furthermore, unstained protein marker (Thermo Scientific), soluble and insoluble protein were denatured at 95°C for 10 minutes and cooled down on ice. Electrophoresis was performed using the Bio-Rad power pac 300 for 4 hours at 150 V. Protein was stained using Protein Staining Solution PageBlue™ according to manufacturer's instructions (Thermo Scientific, USA).

## 3. Results

Fragment DNA of  $\pm$ 850 bp in size was successfully amplified using SCSMV-*Bam*F4 and SCSMV-*Hind*R3 primers (Figure 1A). The size of CP gene was similar with previous report (Damayanti and Putra 2011). The plasmid pTZ-SCSMV-CP was successfully obtained having the expected size of  $\pm$ 3700 bp (Figure 1B). Furthermore, colony PCR confirmed the expected CP gene of SCSMV (data not shown).

Three clones of pTZ-SCSMV-CP (clones 1, 2, and 3) were selected and used for sequence analysis. The length of nucleotide sequences for all clones was 855 bp, and they showed 99.4%–99.7% and 99.6%–100% identities among them for nucleotide and amino acid sequence, respectively. Clones 2 and 3 had 100% amino acid homology, whereas clone 1 contains premature stop codon number



**Figure 1.** (A) Amplification of SCSMV-CP gene. M: 1 kb DNA Marker (Thermo Scientific), K (+): A positive control, and S: SCSMV in this study. (B) The plasmid of pTZ-SCSMV-CP clone 1, 2, and 3. SCSMV-CP = sugarcane streak mosaic virus code protein.

130 because of a point mutation (data not shown). SCSMV-CP clone 2 was selected for further subcloning step into expression vector. Nucleotide and amino acid sequences comparison of SCSMV-CP clone 2 showed the highest homology with SCSMV-CP from Indonesia (AB563503) and SCSMV-CP from China (KT257289), respectively (Table). The differences in the amino acid between SCSMV-CP-clone 2 and SCSMV-CP\_China is at amino acid position number 7 (Pro7 Ser) and at number 29 (Iso29Thr; data not shown).

SCSMV-CP gene was successfully subcloned into pET-28a and yielded pET-SCSMV construction as shown in Figure 2A. There is no stop codon in the pET-SCSMV sequence based on analyses using software Proteomic Exspasy Server (Figure 2B).

SCSMV-CP recombinant fragment (pET-SCSMV-CP) was successfully expressed in the insoluble fraction either in *E. coli* BL21(DE3) or Rosetta-gami(DE3)pLysS. It was expressed with molecular weight of approximately 35.4 kDa. Expression of pET-SCSMV-CP gene in *E. coli* BL21(DE3) was optimal for bacteria that was induced by 0.25–1.00 mM IPTG at 25°C for 9–12 hours after IPTG induction (Figure 3A). Expression of pET-SCSMV-CP gene in *E. coli* Rosetta-gami(DE3)pLysS was optimal for bacteria that was induced by 0.25–1.00 mM IPTG at 30°C from 9 to 12 hours after IPTG induction (Figure 3B).

SCSMV-CP recombinant protein from 5 mL bacterial culture was purified by nickel nitrilotriacetic acid column and showed a single protein band with size approximately 35.4 kDa. Purified protein which was eluted by buffer containing imidazole showed thicker

protein band than protein that was eluted by elution buffer with low pH (Figure 4).

#### 4. Discussion

SCSMV is a new emerging disease of sugarcane in Indonesia. Development of serological methods for routine detection of SCSMV either in cutting cane or to monitor the disease development in the field is necessary to manage the virus. Molecular biology techniques are currently being used to express the genes and produce antigen for raising antibodies that are needed in serological detection.

In the present study, pTZ-SCSMV-CP plasmid had a high homology with SCSMV-CP from several countries. In *Potyviriidae*, nucleotide sequence homology >76% and amino acid sequence homology >80% either in the CP or the entire genome are classified as same species of virus (King *et al.* 2012), thus antiserum to be produced from recombinant antigen was expected to detect SCSMV in other countries.

Expression vector pET-28a has some important elements which is necessary in expression gene such as (1) a *lacI* gene that encodes *lac* repressor protein, (2) T7 promoter which is specific to T7 RNA polymerase produced by *E. coli* BL21(DE3) and Rosetta-gami(DE3)pLysS, (3) multiple cloning site which is containing several restriction enzymes, and (4) 6X histidine tags (His-Tag) that are essential for protein purification (Papaneophytou and Contopidis 2014). In addition, insertion of SCSMV-CP in pET-28a is capable of expressing the full length of SCSMV-CP which is characterized by the absence of a stop codon in the expressed gene. *E. coli* BL21(DE3) and Rosetta-gami(DE3)pLysS are able to produce T7 RNA polymerase that is recognized by the T7 promoter in the pET-28a. T7 RNA polymerase has an active role in expression systems so the DNA inserts that are placed under a T7 promoter will be highly expressed by those bacteria (over-expression) (Rosano and Ceccarelli 2014).

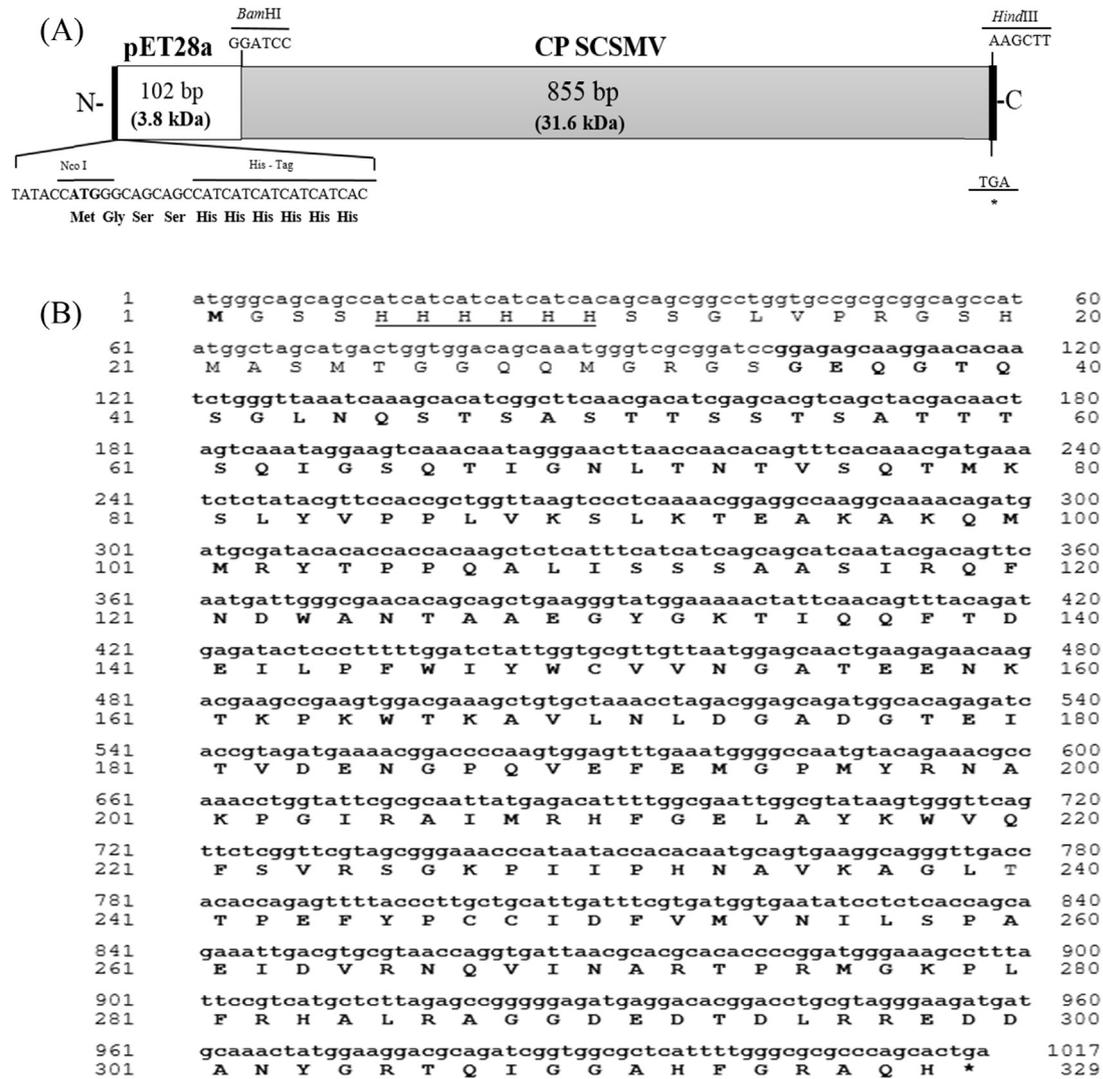
The optimal condition to express the SCSMV-CP recombinant gene in *E. coli* BL21(DE3) and *E. coli* Rosetta-gami(DE3)pLysS was affected by the incubation temperature and bacterial harvesting time. SCSMV-CP recombinant protein can be expressed abundantly although in the low concentration of IPTG on both bacterial expressions.

Table. Homology of nucleotide and amino acid sequences of SCSMV-CP-clone 2 in comparison with other corresponding sequences deposited in GenBank

| Isolate       | Homology   |            | Accession number |
|---------------|------------|------------|------------------|
|               | Nucleotide | Amino acid |                  |
| SCSMV-clone 1 | 99.7       | 99.6       | —                |
| Clone 3       | 99.4       | 100.0      | —                |
| Indonesia     | 97.3       | 98.5       | AB563503         |
| China         | 92.8       | 98.9       | KT257289         |
| India         | 92.5       | 98.2       | AM749393         |
| Pakistan      | 92.5       | 98.2       | U75456           |
| Thailand      | 85.8       | 95.4       | JN163911         |
| SCMV-India*   | 42.8       | 13.3       | DQ842502         |

SCSMV-CP = sugarcane streak mosaic virus code protein.

\* SCMV is used as outgroup.



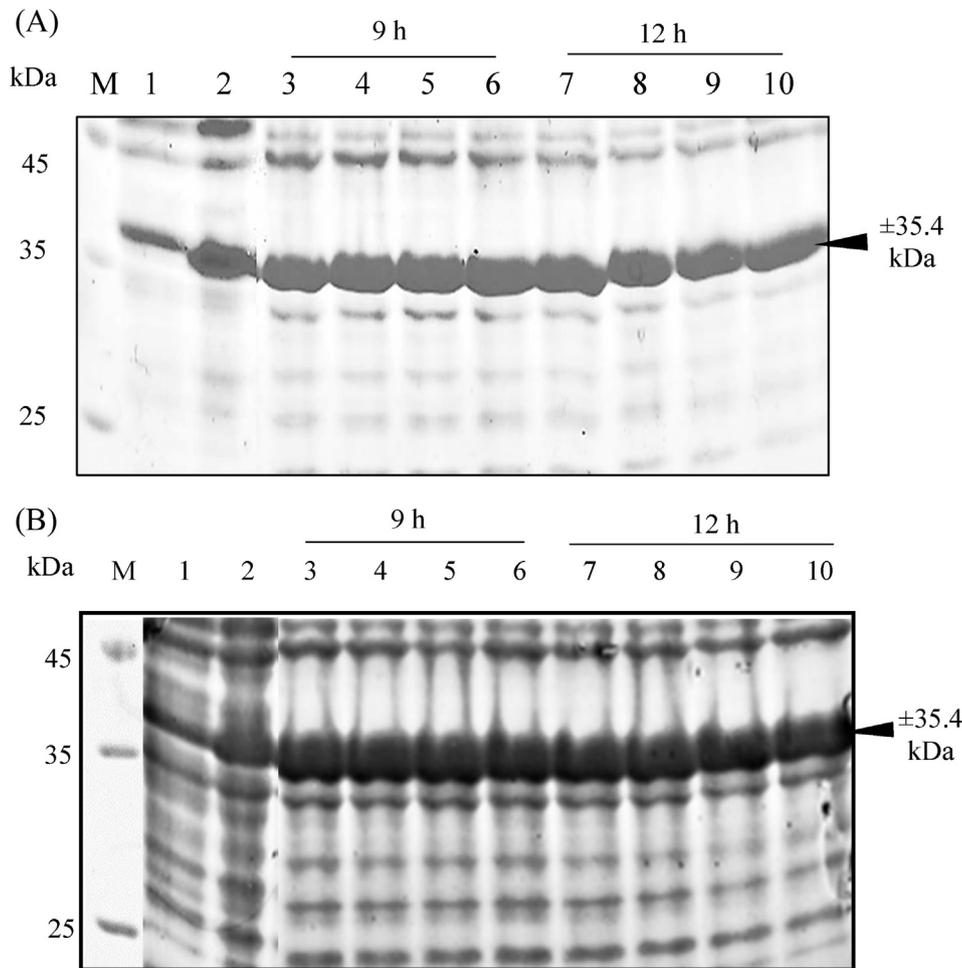
**Figure 2.** SCSMV-CP in pET-28a. (A) Expectation of SCSMV-CP (■) and pET-28a (□) fusion. The translation is started at methionine codon (ATG) of the *Nco*I site in the pET-28a vector and continues until the stop codon (TGA) which is located at the end of the SCSMV-CP sequences (\*). (B) Nucleotides (the first line) and amino acids sequence (the second line) of pET-SCSMV. Histidine tag (H) is the under line. Bold types are nucleotide and amino acid sequences of SCSMV-CP gene. SCSMV-CP = *sugarcane streak mosaic virus* code protein.

Akbari *et al.* (2015) reported that the harvesting time and temperature of incubation have a significant effect than the concentration of IPTG on the expression of anti-Her2 protein scFv which was cloned in pET-22b expression vector and transformed in *E. coli* BL21(DE3)pLysS. IPTG concentrations ranging from 0.5 to 2.0 mM do not affect the expression levels of *cucumber mosaic virus* coat protein (Rostami *et al.* 2014). Santala and Lamminmäki (2004) reported that recombinant protein which is expressed in *E. coli* Origami B optimum occurred at low temperature (24°C). Too high IPTG concentration can reduce the rate of bacterial growth, increase the production of proteinase which can degrade the recombinant protein, and potentially is toxic to the bacterial cell (Akbari *et al.* 2015).

Protein elution is the step to release the recombinant protein target from resin which has 6X His-Tag. Using imidazole in the protein elution step has advantages such as it can be used in original condition (native condition) and denatured conditions (denaturing condition). Imidazole has similar structure with histidine, so the high concentrations of imidazole (greater than

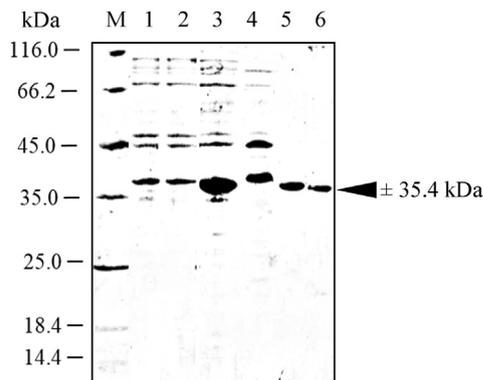
200 mM) can bind to the nickel-charged affinity column and compete with the histidine-tagged recombinant proteins. A decreased pH to 4.5 also can compete the binding of histidin-tagged recombinant proteins but it potentially damages the protein target (Kneusel *et al.* 2000). The preferred method in the elution process is using imidazole because it will not damage the recombinant protein due to pH decreasing.

SCSMV-CP gene can be amplified by specific primers for SCSMV-CP and successfully cloned either into pTZ57R/T cloning vector or into pET28a. SCSMV-CP succeed to expressed in bacterial expression strain either in *E. coli* BL21(DE3) or Rosetta-gami(DE3)pLysS. Expression of pET-SCSMV gene in *E. coli* BL21(DE3) insoluble fraction was optimal for bacteria which is induced by 0.25–1.00 mM IPTG at 25°C for 9–12 hours after IPTG induction. Expression of pET-SCSMV-CP gene in *E. coli* Rosetta-gami(DE3)pLysS insoluble fraction was optimal for bacteria which is induced by 0.25–1.00 mM IPTG at 30°C for 9–12 hours after IPTG induction. SDS-PAGE analysis of purified protein of SCSMV-CP showed a single protein band sized  $\pm 35.4$  kDa. It was the



**Figure 3.** The optimal expression of recombinant protein SCSMV-CP in different bacteria. M: Protein marker unstained (Thermo Scientific), 1: pET28a, 2: Uninduced cell. (A) Expression of SCSMV-CP in *E. coli* BL21(DE3) at 25°C at different harvesting time after IPTG induction with concentration 0.25, 0.50, 0.75, 1.00 mM at 9 (3–6), 12 hours (7–10). (B) Expression of SCSMV-CP in *E. coli* Rosetta-gami(DE3)pLysS at 30°C at different harvesting time after IPTG induction with concentration 0.25, 0.50, 0.75, 1.00 mM at 9 (3–6), 12 hours (7–10). IPTG = isopropyl  $\beta$ -D-thiogalactopyranoside; SCSMV-CP = *sugarcane streak mosaic virus* code protein.

fusion protein of pET28a (3.8 kDa) and SCSMV-CP (31.6 kDa). The purified SCSMV-CP recombinant protein will be used as an antigen to produce SCSMV antiserum. Antiserum production now is under progress.



**Figure 4.** Visualization of purified recombinant protein pET-SCSMV on 12.5% polyacrilamide gel; M: Marker unstained protein, 1: *E. coli* BL21(DE3) carrying pET-28a without SCSMV-CP, 2: Uninduced *E. coli* BL21(DE3) carrying pET-SCSMV, 3: Protein of pET-SCSMV in insoluble fraction (pellet), 4: Soluble fraction (supernatant), 5: Purified protein with elution buffer containing imidazole, 6: Elution buffer pH 4.3. SCSMV-CP = *sugarcane streak mosaic virus* code protein.

### Conflict of interest

Hamdayanty Hamdayanty, Sri Hendrastuti Hidayat, Tri Asmira Damayanti as authors certify that we have NO affiliations with or involvement in any organization or entity with any financial interest (honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, etc; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, and beliefs) in the subject **Expression of Recombinant *Sugarcane streak mosaic virus* Coat Protein Gene in *Escherichia coli*.**

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