

# Over-expression and Purification of RNA Dependent RNA Polymerase from Black queen cell virus in Honeybee

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## Abstract

Honeybee populations infected by numerous viruses could not easily distinguish from the healthy populations because there are no apparent clinical symptoms and signs of infection. Black queen cell virus (BQCV) is one of the most prevalent viruses, resulting in the death of queen larvae and pupae. Like to other picornaviruses, BQCV has the RNA dependent RNA polymerase (RdRp) that catalyzes the replication of viral RNA. The optimal conditions for expression of MBP-BQCV-RdRp protein were confirmed by an induction with 0.1mM IPTG at 25°C for 6 hours and the MBP-BQCV-RdRp protein was purified using amylose resin. Total yield of protein reached approximately 5mg per 50ml of induced cells. Due to its property that is an essential enzyme of virus replication, purified recombinant BQCV-RdRp proteins can be provided to the further lead as an important source to study how to control the BQCV viral disease in honeybee, development of a rapid kit for the diagnosis of BQCV infection in field, or finding out the anti-viral chemical compound for the inhibitor assay to prevent BQCV replication in honeybee.

Key words: Black queen cell virus (BQCV), RNA dependent RNA polymerase (RdRp), Maltose binding protein (MBP), Honeybee

## INTRODUCTION

Honeybee plays a vital role in the development of beekeeping by providing valuable products including beeswax, propolis, pollen and royal jelly. In addition, many agricultural crops require them for pollination to increase the crop yield. However, the occurrence of several honeybee diseases infected by pathogens including viruses and bacteria is considered as a threat to the beekeeping industry. Black queen cell virus (BQCV) is identified as one member of the “picorna-like” group of viruses and belongs to the new genus Cripavirus (Family *Dicistroviridae*) (MayO, 2002). It was originally detected in dead honeybee queen larvae and pupae (Bailey and Wood, 1977). In Australia, BQCV has been reported to be the most comm-

on cause of death of queen larvae (Anderson, 1993).

The complete genome of BQCV is a single-stranded RNA of 8550 nucleotides including two Open reading frames (ORFs) with the specificity of each regions, the 5' proximal ORF encodes a putative replication protein, while the 3' proximal ORF encodes a capsid polyprotein (Leat *et al.*, 2000).

In the replication of the viral genome, the RNA dependent RNA polymerase (RdRp) plays an important role in ensemble with host proteins and viral other proteins (Buck, 1996; Lai, 1998). Previous studies on comparison of structures and the phylogeny of the viral RNA dependent RNA polymerases confirmed the identification of a new conserved motif apparently present in all RdRps (Bruenn, 2003; Koonin, 1991). RdRp was validated as a suitable

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taxonomic marker for the assignment of members of the order Picornavirales, with the potential for use independently of other genetic or phenotypic marker (Baker and Declan, 2008). In Korea, phylogenetic analysis of Black queen cell virus genotypes showed that BQCV may infect both *A. cerana* and *A. mellifera* (Noh *et al.*, 2012).

This study aimed to express and purify RNA dependent RNA polymerases from Black queen cell virus in *E.coli* system. This purified RdRp protein will be used as a potential source of antigen for generating monoclonal antibodies to develop a commercial rapid kit which can detect rapidly and sensitively BQCV in honeybee.

## MATERIALS AND METHODS

### Honeybee sample and total RNA extraction

Honeybee samples infected by BQCV were collected in 2013 in South Korea. Collected honeybee samples were homogenized by MagNA Lyser green Beads (Roche, Switzerland) then were extracted total RNA by using Allspin™ for total DNA & RNA isolation from tissues and cultured cells (GeneAll-Korea), according to the manufacturer's instructions. Finally, RNAs were eluted in 50ul of sterilized RNase-free water and were stored at -70°C until used. Concentration of total RNAs were determined by Biophotometer machine (Eppendorf-Germany).

### Reverse transcription

The total extracted RNAs were reverse transcribed by AccuPower® RT Premix (Bioneer-Korea). RT reaction was performed with 2ug total RNA and 200 pmole of OligodT (Bionics-Korea) for 42°C, 60 mins and 94°C, 5 mins. Subsequently, cDNAs can be used for experiments.

### Molecular cloning of RNA dependent RNA polymerase (RdRp) gene from BQCV

The primer set for BQCV-RdRp was designed base on the published nucleotide sequences (GenBank Accession No. EF517515.1). Details of primers are shown in Table 1. The amplified DNA fragment was cloned into pGEM-3Zf(+) vector (Promega-USA). BQCV-RdRp clones were verified by DNA-sequencing. Afterward, the BQCV-RdRp were subcloned into pMAL c2 expression vector (New England Biolabs). The recombinant pMal-BQCV-RdRp were examined by restriction analysis on 1.5% agarose gel, then were transformed to *E.coli* BL21 (DE3) pLysS for expression.

### Expression of MBP-BQCV-RdRp protein

MBP-BQCV-RdRp in *E.coli* BL21 (DE3) pLysS were cultured in LB medium including Ampicillin (0.05mg/ml) and Chloramphenicol (0.05mg/ml) at 37°C for overnight and then 1ml of bacteria culture was immediately subcultured into 25ml LB medium. To optimize conditions for protein expression, bacterial cultures were grown with different optical densities at 600nm (OD600) and induced with different concentrations of IPTG. In addition, the different temperatures for IPTG induction were examined at 25°C and 37°C. After 1, 3, 6, 12 and 24 hours, aliquots of induced bacterial cells were collected by centrifugation and the cells were suspended in 1X PBS buffer then disrupted by sonication. The expression of MBP-BQCV-RdRp was evaluated by SDS-PAGE.

### Purification of recombinant MBP-BQCV-RdRp protein by amylose resin

MBP-BQCV-RdRp were purified using amylose bind agarose resin (Elpis-Korea) that was designed for rapid purification of recombinant protein fused with MBP. The MBP-BQCV-RdRp binded to amylose, which was

**Table 1.** Sequence of specific BQCV-RdRp primers

Oligo name	Sequence (5'→3')	PCR product (bp)	Reference
BQCV-RdRp-F- <i>Bam</i> HI	AGCGGATCCCTGAGAGACAAGGTACAGGA	903	This study
BQCV-RdRp-R- <i>Sal</i> I	GCGGTCGACGGTCCACATGGGCATTC		

immobilized on the agarose resin before. After unbound proteins were washed away, the MBP-BQCV-RdRp were recovered by elution with maltose containing elution buffer. Concentration of the purified MBP-BQCV-RdRp was determined by Bradford assay with BSA standard (1mg/ml) and was stored at  $-70^{\circ}\text{C}$  until used.

## RESULTS AND DISCUSSION

### Amplification of BQCV-RdRp fragment

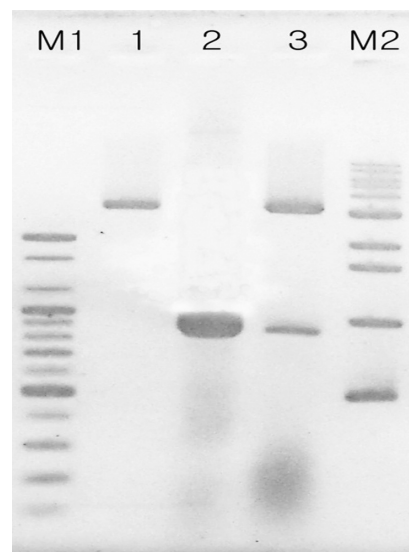
BQCV-RdRp gene was amplified by PCR reaction with specific primer pairs (Table 1). Amplified PCR fragment was cloned into pGEM-3Zf(+) vector and the clone was named as pGEM-BQCV-RdRp. BQCV-RdRp clone was screened by *Bam*HI/*Sal*I restriction analysis (Fig. 1). The sequencing result revealed 93% homology of nucleotide sequences (Fig. 2) and 98% homology of amino acid sequences to deposited sequences (Genbank accession No. EF517515.1) (Fig. 3).

### Expression of MBP-BQCV-RdRp protein

BQCV-RdRp was subcloned into pMAL-C2 vector, then transformed to *E.coli* BL21 (DE3) pLysS host cell. Based on amino acid sequence, the size of MBP-BQCV-RdRp was calculated around 80 kDal. Firstly, the expression of MBP-BQCV-RdRp protein was evaluated both in pellet and supernatant under a normal condition at  $25^{\circ}\text{C}$  induced by 0.6mM IPTG during 6hrs. The result confirmed that MBP-BQCV-RdRp protein expressed well both in pellet and supernatant with expected bands around 80 kDal (Fig. 4). In this experiment, pQE30-gfp-ELP was used as a positive control and expression of pMAL-C2 was performed as a negative control with expected bands about 50 kDa.

### Optimization expression conditions of MBP-BQCV-RdRp protein

Firstly, the optical densities of the cell were examined (Fig. 5). To establish the optimum conditions of subculture, MBP-BQCV-RdRp were grown with different optical



**Fig. 1.** Confirmation of pGEM-BQCV-RdRp clone by restriction analysis. Lane M1 is 100 bp DNA ladder (Bioneer-Korea: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1600, 2000 bp). Lane M2 is 1kb DNA ladder (Bioneer-Korea: 500, 1000, 1600, 2000, 2961, 4000, 5007, 5991, 8000, 10200 bp). Lane 1 is pGEM-3Zf(+) vector which was digested by *Bam*HI/*Sal*I. Lane 2 is PCR product PCR product amplified with BQCV-RdRp-F-BamHI and BQCV-RdRp-R-SalI primer set. recombinant pGEM-BQCV-RdRp DNA which was digested by *Bam*HI/*Sal*I. It is clear to observe BQCV-RdRp that was digested from clone with an expected size around 903 bp and pGEM vector with an expected size around 3179 bp in lane 3. This result is compared with pGEM vector and BQCV-RdRp which were showed in lane 1 and lane 2.

density at 600nm (OD600) values then induced the cells with 0.6mM IPTG at  $25^{\circ}\text{C}$  for 6 hours. In new study, an induction at OD600 = 0.8 was determined as an optimized condition for production of MBP-BQCV-RdRp protein.

Under an optimal OD600 value, IPTG concentration and induced time were evaluated. The results pointed out 0.1mM IPTG was enough to express well MBP-BQCV-RdRp protein, whereas a higher IPTG concentration seemed not to increase dramatically expression levels (Fig. 6). Afterward, incubation time for induction was confirmed that 6 hours was optimal (Fig. 7) and the recombinant BQCV-RdRp could be degraded after long incubations. It is clear to observe that MBP-BQCV-RdRp protein expressed highly at 0.1mM IPTG for 6 hours induction.

Initially, IPTG induction was performed at  $25^{\circ}\text{C}$ . Whether expression of MBP-BQCV-RdRp protein is

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pGEM-BQCVrdrp      TATTTTATAGACACATTGAAAAGATGAGAGAAAACCAAACATAAAAGCTCATAAATCTCGA 120
EF517515.1         TATTTTATAGATACATTGAAAAGATGAGAGAAAACCTAAACATAAAAGCTCATAAATCTCGA 120
*****

pGEM-BQCVrdrp      ATGTTTTCTAATGGCCCAATAGATTACCTTGTGGAGTAAGATGTATTACAATCCAATA 180
EF517515.1         ATGTTCTCTAATGGTCCAATAGATTACCTTGTGGAGCAAGATGTATTTAATCCAATA 180
*****

pGEM-BQCVrdrp      GTAGCAGTGTATCTGAACTGAAAAATGTAGATCATATTTCTGTAGGGTCTAATGTGTAT 240
EF517515.1         GTAGCAGTGTATCTGAACTGAAAAATGTAGATCATATTTCTGTAGGGTCTAATGTGTAT 240
*****

pGEM-BQCVrdrp      TCTACAGATTGGGACGTGATCGCGAGATATTTAAAGTCGAAATCCCATCATATGGTGGCA 300
EF517515.1         TCTACGGACTGGGACGTGATCGCGAGATATTTGAAAGTCAAATCCCATCATATGGTGGCT 300
*****

pGEM-BQCVrdrp      GGGGACTTTGAAGGTTTCGACGCTTCGGAACAGTCTGATATATTGTATGCTGCGGGAGAA 360
EF517515.1         GGAGACTTTGAAGGTTTCGACGCATCAGAACAGTCTGACATATTATACGCTGCAGGAGAG 360
*****

pGEM-BQCVrdrp      GTTCTGCAAGAGTTGAGTAAGAAGATCTTTAACTCAACTGAGGATGAGATTTTACAACAA 420
EF517515.1         GTTTTGC AAGAGTTGAGCAAGAAGATCTTTAACTCAACTGAGGATGAGATTTCTGCAACAA 420
*****

pGEM-BQCVrdrp      AAAGCTATAATACACTCTTTGGTCAATTCTCTTCATATTAATGAAAATGGAATTGCCTT 480
EF517515.1         AAAGCTATAATACACTCTTTGGTCAATTCTCTTCATATCAATGAAAATGGAATTGTCTTA 480
*****

pGEM-BQCVrdrp      CAGTGGTGAAGTCTCTTCCCTAGTGGTCATTATCTCACTGCTATTATAAATCCGCTCTT 540
EF517515.1         CAGTGGTGAAGTCTCTTCCCTAGCGGTCATTATCTTACTGCTATTATAAATCCGCTCTT 540
*****

pGEM-BQCVrdrp      GTAAATTTAGTGATGTGCTCGTATTATGGAGGCTAACC AAAATATTTCATTTACGACT 600
EF517515.1         GTAAATTTAGTGATGTGCTCGTATTATGGAGGCTAACC AAAATATTTCATTTACGACT 600
*****

pGEM-BQCVrdrp      GCTTCCTCATTTTCCCGTGAATGTGGTATAGTCCGATATGGAGACGATCATGTCGCTCT 660
EF517515.1         GCTTCCTCATTTTCCCGTGAATGTGGTATAGTCCGATATGGAGACGATCATGTCGCTCTA 660
*****

pGEM-BQCVrdrp      GTTCCCGAAAAATATTTATCTGTGTTTAAATCAACAGACACTACCCGTTGTGATGAGTAAA 720
EF517515.1         GTTCCCGAAAAATATTTATCTGTGTTTAAATCAACAGACACTACCCGTTGTGATGAGTAAA 720
*****

pGEM-BQCVrdrp      CTCGGGATGTTCTATACCATAGAAACTAAGGATGACACAGAGATCGACTTTCTATCTAGA 780
EF517515.1         TTCGGTATGTTCTATACCATAGAAAACCAAGGATGATACAGAAAATCGATTTTCTTTCTAGA 780
*****

pGEM-BQCVrdrp      AGGTTGGAAGATGTGTCGACTTAAAACGGAATTTTGTGTTATGACGAATCTAGGCAACGT 840
EF517515.1         AAATTGGAGGATGTGTCATACTTGAACGGAATTTTGTGTTATGACGAACCTAGGCAACGT 840
*****

pGEM-BQCVrdrp      TATATTGCTCCTCTCTCTTTGGATGTGATATTGGAATGCCCATGTGGACC 891
EF517515.1         TATATTGCTCCTCTCTCTTTGGATGTGATACTGGAAAATGCCCATGTGGACC 891
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Fig. 2. Nucleotide sequence homology between Genbank EF517515.1 and BQCV Korean strain (pGEM-BQCV-RdRp). The sequence determined was corresponding to RNA dependent RNA polymerase region of EF517515.1 deposited in NCBI databases and symbolized by asterisk (\*) in the consensus line. Alignment to EF517515.1 revealing 93% homology.

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pGEM-BQCVrdrp      LRDKVQECIDAARQKILDHYFIDTLKDERKPKHKAHKSRMFSNGPIDYLVWSKMYNPI 60
EF517515.1         LRDKVQECIEAARQKILDHYFIDTLKDERKPKHKAHKSRMFSNGPIDYLVWSKMYFNPI 60
*****;*****;*****

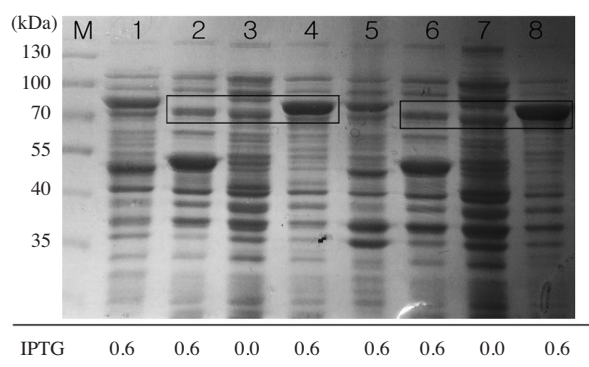
pGEM-BQCVrdrp      VAVLSELKNVDHISVGSNVYSTDWDVIARYLKS KSHHMVAGDFEGFDASEQSDILYAAGE 120
EF517515.1         VAVLSELKNVDHISVGSNVYSTDWDVIARYLKS KSHHMVAGDFEGFDASEQSDILYAAGE 120
*****

pGEM-BQCVrdrp      VLQELSKKIFNSTEDELQKAI IHSLVNSLHINENGIVLQWCKSLPSGHYLTAINSVF 180
EF517515.1         VLQELSKKIFNSTEDELQKAI IHSLVNSLHINENGIVLQWCKSLPSGHYLTAINSVF 180
*****

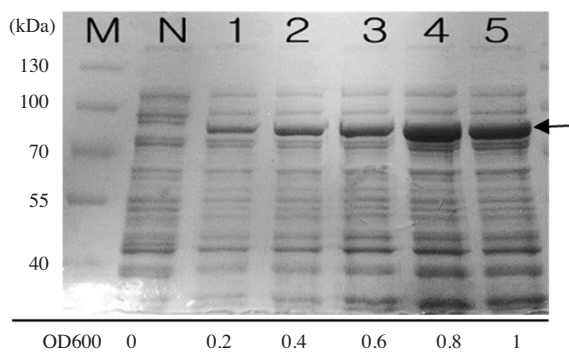
pGEM-BQCVrdrp      VNLVMCLVFMEANQKYSFTTASSFFRECGIVAYGDDHVVSVPKYL SVFNQOTLPVLSK 240
EF517515.1         VNLVMCLVFMEANPKYSFTTASSFFRECGIVAYGDDHVVSVPKYL SVFNQOTLPVLSK 240
*****

pGEM-BQCVrdrp      LGMFYTIETKDDTEIDFLSRRLDVSYLKRN FVYDESRQRYIAPLSLDVILEMPMWT 297
EF517515.1         FGMFYTIETKDDTEIDFLSRKLEDVSYLKRN FVYDEPRQRYIAPLSLDVILEMPMWT 297
*****;*****;*****
    
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Fig. 3. Amino acid sequence homology between EF517515.1 and BQCV Korean strain (pGEM-BQCV-RdRp). The amino acid sequence of pGEM-BQCV-RdRp achieved 98% homology to Genbank EF517515.1 and was marked by asterisk (\*) in the consensus line.

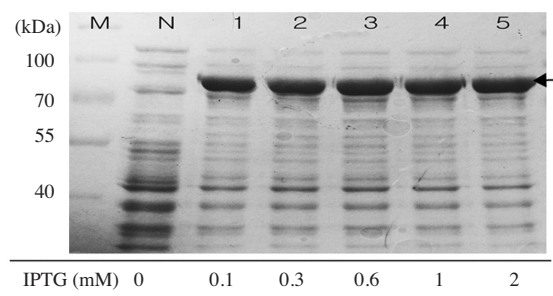


**Fig. 4.** Expression of MBP-BQCV-RdRp protein in *E. coli* BL21 (DE3) pLysS. After sonication, location of MBP-BQCV-RdRp protein was examined both in the supernatant and pellet. Lane M is protein size marker (Thermo scientific-USA; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa). Lane 1 and 5 are induced pQE30-gfp-ELP from supernatant and pellet, respectively. Lane 2 and 6 are pMal-C2 induced IPTG from supernatant and pellet, respectively. Lane 3 and 7 are MBP-BQCV-RdRp protein without IPTG from supernatant and pellet, respectively. Lane 4 and 8 are induced MBP-BQCV-RdRp protein from supernatant and pellet, respectively. The expected bands around 80 kDa of MBP-BQCV-RdRp protein were marked in two boxes and compared with negative controls in lanes 2, 3 and lanes 6, 7.

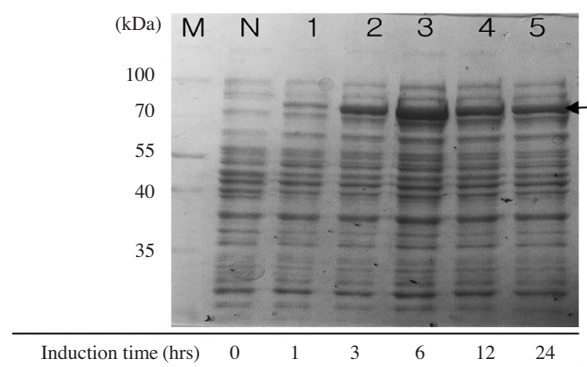


**Fig. 5.** Optimal OD600 value for expression of MBP-BQCV-RdRp protein. MBP-BQCV-RdRp proteins in *E. coli* BL21 (DE3) pLysS were started to induce with 0.6mM IPTG at variable OD600 concentrations. Induced cells were collected after 6 hours. Lane M is protein size marker (Thermo scientific-USA; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa). Lane N is a negative control as no induction. Lane 1 to 5 are induced protein at OD600 = 0.2; 0.4; 0.6; 0.8 and 1, respectively. The black arrow implied the expected protein bands. MBP-BQCV-RdRp protein expressed highest at OD600 = 0.8.

depend on temperature, MBP-BQCV-RdRp protein was induced with two different temperatures. As the result, MBP-BQCV-RdRp protein expressed at 25°C better than



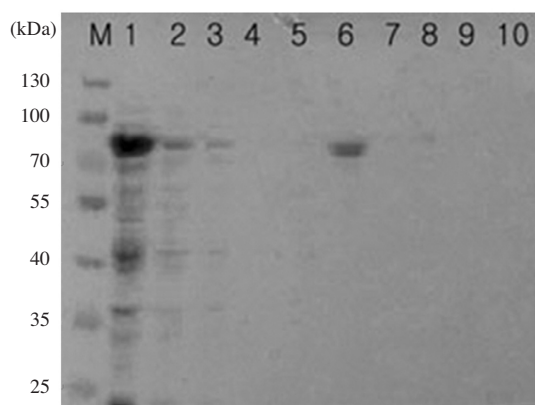
**Fig. 6.** Expression of MBP-BQCV-RdRp protein depends on IPTG concentration. MBP-BQCV-RdRp in *E. coli* BL21 (DE3) pLysS was cultured until OD600 = 0.8 and induced by variable IPTG concentrations. Induced cells were collected after 6 hours. Lane M is protein size marker (Thermo scientific-USA; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa). Lane N is a negative control as no induction. Lane 1 to 5 are induction at 0.1, 0.3, 0.6, 1 and 2mM IPTG, respectively. The black arrow implied the expected protein bands. This result confirmed that 0.1mM IPTG was enough for expression.



**Fig. 7.** Optimal induction time for expression of MBP-BQCV-RdRp protein. The expression of recombinant protein was performed by 10% SDS-PAGE and 1X TGS (Tris/Glycine/SDS) running buffer. Proteins were heated denaturation before loading on the gel. Induction of MBP-BQCV-RdRp protein in *E. coli* BL21 (DE3) pLysS with 0.1mM IPTG was started at OD600 = 0.8 and cells were collected after different induction time. Lane M is protein size marker (Thermo scientific-USA; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa). Lane N is a negative control without induction. Lane 1 to 5 are induced protein at 1, 3, 6, 12 and 24 hours, respectively. The black arrow implied expected protein bands. This result confirmed that 6 hours was an optimal condition for induction time.

37°C (Fig. 8). Compare with other researches, RdRp gene could be expressed well at 30°C (Zhou *et al.*, 2006; Sankar and Porter, 1991). On sum up, the optimal conditions for expression of MBP-BQCV-RdRp protein were pointed out





**Fig. 10.** Purification of MBP-BQCV-RdRp protein using amylose binding resin. Protein after purification was determined by 10% SDS-PAGE and 1X TGS (Tris/Glycine/SDS) running buffer. 16ul of protein was loaded on gel after sonication and heated denaturation. Lane M is protein size marker (Thermo scientific-USA; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDal). Lane 1 is induced total protein. Lane 2 is a part of protein unbound to amylose resin. Lane 3 is washing by column buffer. Lane 4 is washing by column buffer in 1% Tween 20. Lane 5 is washing by column buffer. Lane 6 is eluted MBP-BQCV-RdRp protein. Lane 7 is washing by distilled water. Lane 8 is washing by 0.1% SDS. Lane 9 is washing by distilled water. Lane 10 is washing by column buffer. After purification, one clear band located in lane 6 as a purified MBP-BQCV-RdRp protein form without any other proteins from the cell, this band showed the expected size of MBP-BQCV-RdRp protein about 80 kDal.

initial RNA. Studies of RdRp contribute significantly to understand the replication mechanism of RNA viruses and extend new assays against virus. In this study, the RNA dependent RNA polymerase of Black queen cell virus was successfully amplified. From sequencing results, BQCV-RdRp clone revealed 93% homology of nucleotide sequences and 98% homology of amino acid sequences to deposited sequences (Genbank accession No. EF517515.1). RdRp was well expressed in *E.coli* system and clearly purified by amylose binding resin. The recombinant BQCV-RdRp was highly expressed under optimal conditions at OD<sub>600</sub> = 0.8 with 0.1mM IPTG for 6 hours at 25°C induction. It could be used as an antigen to generate monoclonal antibodies for rapid detection BQCV. Moreover, RdRp will be used in drug assay to find out chemicals that inhibit the activity of this gene.

## ACKNOWLEDGEMENTS

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