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Production of Polyclonal Antibodies Specific to the Recombinant Coat Protein of *Blackeye cowpea mosaic virus* and Its Use in Disease Detection

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ABSTRACT

The coat protein gene of *Blackeye cowpea mosaic virus* (BICMV-CP) was amplified by reverse transcription polymerase chain reaction and cloned into the expression vector pQE-80L. This plasmid was transformed into *Escherichia coli* DH5α competent cells. The BICMV-CP gene was expressed as a fusion protein containing a fragment of 6xHis-tag. Bacterial cells were disrupted by repeated freeze-thawing three times and the BICMV-CP fusion proteins were purified under denaturing conditions by affinity chromatography with Ni-NTA Agarose.

A sample of 500 μg of purified protein was mixed with Freund's complete adjuvant at a ratio of 1:1 (volume to volume). Initially, the emulsion was subcutaneously injected into a New Zealand White rabbit, followed at weekly intervals by three additional immunizations with 500 μg of the purified protein mixed with Freund's incomplete adjuvant. Bleeding was done every week during weeks 5-12 and titers of the antisera ranging from 800-51,200 were obtained. Up to a dilution of 1:320 of the BICMV-infected yardlong bean sap could be detected by indirect enzyme-linked immunosorbent assay. The produced antiserum reacted specifically with the BICMV-infected plant without any cross reaction with other virus species tested. However, a weakly positive reaction with *Bean common mosaic virus* could be observed.

Keywords: *Blackeye cowpea mosaic virus*, recombinant coat protein, cowpea, reverse transcription polymerase chain reaction, enzyme-linked immunosorbent assay

INTRODUCTION

Blackeye cowpea mosaic virus (BICMV) belongs to the genus *Potyvirus* within the family *Potyviridae*. This virus is an economically important and major limiting factor for cowpea (*Vigna unguiculata* (L.) Walp.) production in Asian countries such as India, Japan, Malaysia, Thailand and Taiwan (Mali *et al*., 1989). The virus is distributed by seed-borne transmission with a frequency rate of up to 30% and yield losses as high as 40% under field conditions being recorded (Arakere *et al*., 2009; Udayashankar *et al*., 2012). In addition, mixed infection with BICMV and *Cucumber mosaic virus* (CMV) results in severe stunting and causes yield loss in cowpea of up to

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86.4%; however, While BICMV can be controlled through integrated approaches, the most effective method has focused on host plant resistance (Ouattara and Chambliss, 1991).

The serological methods using polyclonal (PAb) and monoclonal (MAb) antibodies are efficient analytic tools for the detection of BICMV for both screening and virus control purposes (Hao *et al*., 2003). In Thailand, MAb specific to BICMV has been produced by the hybridoma technique and applied successfully to detect the virus (unpublished data). However, maintaining these hybridoma cell lines *in vitro* is unstable under long-term storage. New MAb generation is laborious, time consuming and may be genetically unstable (Pasqualini and Arap, 2004; Balogh *et al*., 2010). In contrast, production of PAb is relatively simple and cheap to produce in the short term compared with MAb (Nelson *et al*., 2000). Generally, the production of high-quality virus-specific antisera to plant viruses requires purified viruses and large amounts of viruses for the immunization animal (Nickel *et al*., 2004). However, the purification in the preparation of *Potyvirus* species is difficult because of their tendency towards irreversible aggregation during extraction and concentration with consequent virus loss during low speed centrifugation (Bashir and Hampton, 1995). To circumvent these problems, molecular biology techniques are currently being used to produce virus-specific antisera using fusion protein from cloned virus genes expressed in *Escherichia coli*. Bacterial expression of protein is simple, fast and inexpensive for the production of high amounts of purified proteins with minimal post-translational modifications (Hartley, 2006). In addition, a fusion tag to the proteins expressed in *E. coli* further improves their expression, solubility and stability and facilitates purification. The purified expressed plant viral protein as a fusion protein has been successfully used as an antigen for raising virus-specific antisera for immunodiagnostics (Raikhy *et al*. 2007; Gulati-Sakhuja *et al*., 2009).

In an attempt to screen yardlong bean (*V. unguiculata* ssp. *sesquipedalis*) and cowpea accessions resistant to BICMV by enzyme-linked immunosorbent assay (ELISA), the purpose of the current research was to produce polyclonal antibodies by using recombinant coat protein expression technology to detect BICMV in infected plants.

MATERIALS AND METHODS

Virus source and symptoms

Leaf samples of cowpea showing a typical systemic mosaic, leaf deformation, vein yellowing and stunting were collected from different fields in Hat Yai, Songkhla province, Thailand. Detection of BICMV in the collected leaf samples was carried out using a monoclonal antibody against BICMV (anti-BICMV MAb) by indirect ELISA (Clark and Adams, 1977), modified by coating crude sap at 1:5 (weight to volume) dilution in 1× phosphate buffer saline (PBS; 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl and 136.8 mM NaCl) at pH 7.4. The monoclonal antibody supernatant was kindly provided by the Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand.

Virus isolation and propagation

The virus isolation was applied according to Lima *et al*. (1979). BICMV was biologically purified through a single local lesion technique on *Chenopodium amaranticolor* (Coste & A. Reyn.) (Zettler *et al*., 1967) by mechanical inoculation. The virus was propagated and maintained in yardlong bean (Huguenot *et al*., 1993) until further use.

Primer design

The published coat protein (CP) gene sequences of the BICMV from GenBank were used for designing the primers CACP1 5′

GGATCCTCAGGAACTGGAC 3′ and CACP2 5′ ***AAGCTT***TTACTGCGGGGAAC 3′ The forward primer CACP1 5′ primer and the reverse primer CACP2 5′ primer were designed to contain a *Bam*HI site and a *Hin*dIII site, respectively.

Nucleic acid extraction and reverse transcription polymerase chain reaction

Total RNA was extracted from infected yardlong bean using a Total RNA Mini Kit (Plant) (Geneaid; Taipei, Taiwan), according to the manufacturer's instructions. Subsequently, DNA was synthesized by a reverse transcription polymerase chain reaction method (RT-PCR) using a Qiagen®OneStep RT-PCR Kit (Qiagen; Hilden, Germany) using the above-mentioned primers. The Qiagen®OneStep RT-PCR mixture in 50 μL contained a total of 1 μL of the RNA solution, Q-Solution (1×), 10mM dNTP, 100 pmol each of primers CACP1 and CACP2 to amplify a 864 bp fragment of the BICMV-CP. Reverse transcription was carried out for 30 min at 50°C with a hot start for 15 min at 95 °C to activate the HotStarTaq™ DNA Polymerase in the reaction mix. PCR followed immediately, with 35 cycles of 94 °C for 45 s, 52 °C for 60 s and 72 °C for 60 s. The final extension step consisted of 10 min at 72 °C. The amplified product was analyzed by 2% agarose gel electrophoresis and purified using a Gel/PCR DNA Fragment Extraction Kit (Geneaid; Taipei, Taiwan).

Cloning, sequencing and analysis of the *Blackeye cowpea mosaic virus* coat protein gene

The amplified fragment containing the BICMV-CP was ligated in the pGEM-T Easy vector (Promega; Madison, USA) using T4 DNA ligase (Promega; Madison, USA) for 1 hr at room temperature. The recombinant plasmids were transformed by heat shock into *E. coli* competent cells DH5α. After addition of 2×YT medium (yeast extract 10 $g.L^{-1}$, tryptone 16 $g.L^{-1}$ and sodium chloride 10 $g.L^{-1}$) to complete 1 mL, the reaction was incubated in a shaker at 37 °C at 200 rpm for 1 hr and plated on selective 2×YT agar, containing ampicillin (100$\mu g.mL^{-1}$), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG). Colonies were grown overnight at 37 °C. The positive clones, selected on plates containing X-Gal, were detected by a PCR method using T7 and SP6 universal primers. One positive clone was sequenced. The resulting nucleotide and amino acid were compared and analyzed to the BICMV-CP gene sequences in GenBank using the Clustal Omega multiple sequence alignment program (Sievers *et al*., 2011).

Expression and purification of the fusion proteins

Both the pGEM-T Easy vector containing the BICMV-CP gene and pQE-80L expression vector (Qiagen; Hilden, Germany) were digested with *Bam*HI (New England Biolabs; Beverly, USA) and *Hin*dIII (New England Biolabs; Beverly, USA). The BICMV-CP fragment and pQE-80L vector were run on 1% agarose gel electrophoresis. A sample of approximately 150 ng of the insert was ligated into pQE-80L in-frame with the fragment of 6×His-tagged protein, according to the manufacturer's instructions using T4 DNA ligase. The transformation of recombinant DNA into *E. coli* DH5α and selection of the positive clones were performed as described above. One BICMV-CP positive clone was selected for expression study.

A total of 1,000 mL 2×YT medium, with 100 $\mu g.mL^{-1}$ ampicillin, was inoculated with 20 mL of fresh overnight culture of *E. coli* DH5α bearing recombinant plasmid pQE-80L/BICMV-CP. The bacterial cell suspension was incubated in a shaker at 37 °C at 200 rpm for 2 hr. Expression of fusion proteins carrying 6×His-tagged protein were induced by 1mM IPTG and the cultures were incubated on a shaker at 37 °C at 200 rpm for 6 hr. Expressed fusion proteins were extracted from

bacterial cells by repeated freeze-thawing three times and purified using Ni-NTA Agarose (Qiagen; CA, USA), according to the manufacturer's instructions. The concentration of the BICMV-CP fusion proteins was measured by Bradford assay (Bradford, 1976) and spectrophotometry using bovine serum albumin (BSA) as a standard.

Analysis of *Blackeye cowpea mosaic virus* recombinant coat protein

Expression of recombinant proteins was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A sample of 10 µL of each protein was denatured by boiling for 10 min with an equal volume of 2× loading buffer (0.1% bromophenol blue, 4% SDS, 5% β- mercaptoeethanol, 10% glycerol and 0.5 M Tris-HCl, pH 6.8). Gel was stained with Coomassie brilliant blue R-250. A prestained protein molecular weight marker (Fermentas; Hanover, MD, USA) was used for protein molecular weight estimation.

Production of polyclonal antibody

A White New Zealand rabbit aged 2 mth initially was immunized subcutaneously with 500 µg of purified BICMV-CP fusion proteins and 500 µl of Freund's complete adjuvant emulsion at a ratio of 1:1 (volume/volume). At weekly intervals, the immunization was followed by three additional subcutaneous injections with 500 µg of purified fusion proteins mixed with Freund's incomplete adjuvant. The rabbit was bled eight times every week, starting 5 wk after the first injection. The antiserum obtained from each week was designated #S1 – #S8 and stored at -20°C until use.

Estimation of polyclonal antibody titer, efficiency and specificity

The suitability of prepared polyclonal antibody for use in indirect ELISA was tested. Samples of 100 µL were placed in each well in all steps and two wells per sample were used.

Titers of the obtained antisera, the polyclonal antiserum #S1 – #S8 developed against BICMV-CP fusion proteins were evaluated for titering by indirect ELISA, applied from Clark and Adams (1977). The ELISA plate wells were coated with 10 $\mu g.mL^{-1}$ of purified BICMV fusion proteins which was diluted in 50 mM sodium carbonate buffer, pH 9.6 and incubated at 37 °C for 1.5 hr. The plate was washed three times with PBST (0.05% Tween 20 in 1× PBS). Subsequently, 100 µL of a two-fold dilution (1:50 to 1:409,600) of each antiserum in blocking solution (PBS + 5% skim milk) were added and incubated at 37 °C for 1.5 hr. Then, each plate was washed and incubated again at 37 °C for 1 hr with 100 µL of a 1:10,000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich; Saint Louis, MO, USA). After washing, 100 µL of the enzyme substrate *p*-nitrophenylphosphate (1.0 $mg.mL^{-1}$) were added and incubated at 37 °C for 30 min. The absorbance was recorded using an ELISA plate reader at A_{405nm}. Rabbit normal serum and sodium carbonate buffer were used in parallel as the negative control and blank, respectively. If the absorbance value of the antiserum was twice the negative, the result was recorded as positive.

To evaluate the efficiency of the antiserum in virus detection, the crude polyclonal antiserum which gave the highest antibody titer was chosen to investigate its ability to detect BICMV-infected yardlong bean by the indirect ELISA process as described above. Purified BICMV-CP (10 $\mu g.mL^{-1}$) was used as a positive control. The ELISA plate wells were coated with leaf yardlong bean extracts infected with single lesion inoculation in *C. amaranticolor* (BICMV-PSU1) diluted to 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 in sodium carbonate buffer and incubated at 4 °C overnight. Crude polyclonal antiserum was diluted to 1:200 and 1:500 in blocking solution. In the experiment, healthy yardlong beans at the same dilutions were used as a negative control. The results were analyzed using the S/H ratio (sample OD405 / healthy control OD405) according to

Gulati-Sakhuja *et al*. (2009).

Specificity testing can be based on the sap dilution which gives the best S/H ratio and this test was used in the current experiment. The positive viruses which have been reported to infect cowpea— *Cucumber mosaic virus* (CMV) subgroup I and II (genus *Cucumovirus*), *Cowpea mosaic virus* (CPMV; genus *Comovirus*) and *Bean common mosaic virus (*BCMV; genus *Potyvirus)* (Arakere *et al*., 2009)—were purchased from Agdia Inc. (Elkhart; IN, USA) and used for coating the ELISA plate. All viruses were tested in freshly prepared extracts or resuspended freeze-dried extracts from infected plants.

RESULTS AND DISCUSSION

Virus isolation and propagation

Virus isolates from naturally infected cowpeas samples showing severe mosaic, leaf deformation, vein yellowing and stunting (Figure 1a) were collected from three different fields in Hat Yai, Songkhla, Thailand. After successive single lesion inoculation in *C. amaranticolor* (Figure 1b), the resulting BICMV isolate (BICMV-PSU1) was propagated and maintained in yardlong bean for future use. The observed symptoms on yardlong bean inoculated with BICMV-PSU1 were the formation of a systemic mosaic on the trifoliate leaves after about 30 d at 25 ^{o}C, followed by leaf deformation (Figure 1c).

Analysis of *Blackeye cowpea mosaic virus* coat protein gene

The BICMV-CP encoding gene, 864 bp, was amplified successfully as shown in Figure 2. The DNA fragment was ligated into pGEM-T Easy vector and one positive clone was sequenced. The result confirmed that the sequence contained the full length of the BICMV-CP gene and the restriction site in the correct open reading frame. Sequence alignment showed that the coat protein gene of BICMV-PSU1 shared 98% nucleotide identity and 99% amino acid identity with the full length BICMV-CP in GenBank access numbers AY575773, AF395678, AJ312438, AJ312437, AH004380 and S66253, respectively (Figure 3). This sequence was submitted to GenBank (accession number FR775796).

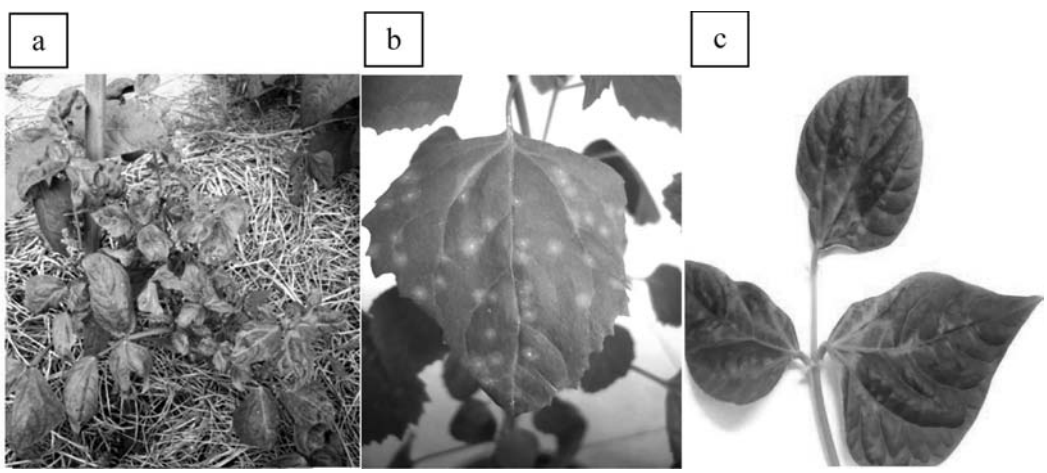

Figure 1 Symptoms of *Blackeye cowpea mosaic virus* (BICMV) on naturally infected cowpea plants: (a) severe mosaic, leaf deformation, vein yellowing and stunting; (b) *Chenopodium amaranticolor* showing chlorotic local lesions on an inoculated leaf after inoculation with BICMV for 6-7 d at 25 ^{o}C; and (c) yardlong bean showing systemic mosaic and leaf deformation on trifoliate leaves after inoculation with BICMV single lesion inoculation in *C. amaranticolor* for 30 d.

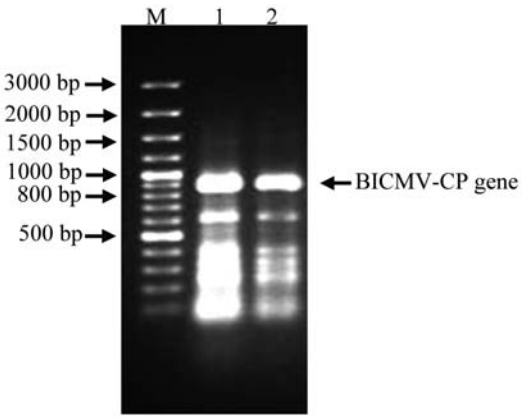

Figure 2 Agarose gel electrophoresis at 2% showing the *Blackeye cowpea mosaic virus* coat protein gene (BICMV-CP) which was amplified at 864 bp from naturally infected cowpea (lane 1) and yardlong bean inoculated with BICMV single lesion inoculation in *C. amaranticolor* using CACP1 forward primer and CACP2 reverse primer, respectively (lane 2). M is the 100 bp plus DNA ladder marker (Fermentas; Hanover, MD, USA).

Expression and purification of BICMV-CP

Production of the BICMV-CP fusion proteins containing a fragment of N-terminal 6×His-tagged protein, recombinant DNA (pQE-80L/BICMV-CP) was chosen to transform in *E. coli* DH5α. After induction for 6 hr, the bacterial cells were harvested and extracted using urea as a denaturant. As shown in Figure 4, the molecular weight of the BICMV-CP fusion proteins, at approximately 33.0 kDa, was clearly observed by 10% SDS-PAGE. The fusion proteins had a relative molecular weight of the BICMV-CP amino acid sequence plus 6×His-tagged which was calculated using the Compute pI/Mw program (Expert Protein Analysis System; Expasy; Swiss Institute of Bioinformatics; Lausanne, Switzerland). The BICMV-CP fusion proteins were further purified by Ni-NTA Agarose and the result showed 100% purity. The yield of BICMV-CP fusion proteins purified from 1,000 mL of *E. coli* cells was about 6.0 mg after estimation by Bradford assay and spectrophotometry.

To check the possibility of BICMV-CP fusion proteins for polyclonal antiserum production against BICMV, anti-BICMV MAb was used to detect purified fusion proteins by indirect ELISA and the results showed that anti-BICMV MAb could react with BICMV-CP fusion proteins as well as with the crude extract of yardlong bean infected with BICMV-PSU1 giving absorbance (A_{405nm}) values of 1.75 and 2.10, respectively. These results suggested that the BICMV-CP fusion proteins and native coat protein had a common epitope (Helias *et al*., 2003). The current study obtained BICMV-CP fusion proteins which can be used for producing PAb against BICMV.

Production and estimation of polyclonal antibody

The polyclonal antisera (#S1–#S8) against the BICMV-CP fusion proteins were evaluated using 10 $mg.mL^{-1}$ of purified proteins by indirect ELISA. The titers of antisera ranged from 800 to 51,200 and the highest titer (1:51,200) was from antisera #S6 and #S7, respectively, while antisera #S2 produced the lowest titer (1:800) as shown in Figure 5.

The efficiency of the prepared crude polyclonal antiserum #S7 was tested with yardlong bean leaf extracts infected with BICMV-PSU1 using indirect ELISA. This antiserum showed cross reactivity with healthy yardlong bean in the same assay. Similar results of non-specificity have been observed with antiserum prepared from the fusion proteins of other viruses (Kumari *et al*., 2001; Gulati-Sakhuja *et al*. 2009). To solve the problem and increase the specificity of the antiserum, polyclonal antiserum #S7 was cross absorbed using healthy yardlong bean extract to remove the cross reactive antibodies before use (Pinner and Markham, 1990). Subsequently, the sensitivity testing of antiserum #S7 was repeated and the new

result clearly showed that the produced antiserum gave higher absorbance values compared to the respective healthy controls. The S/H ratios of crude polyclonal antiserum diluted to 1:200 were 2.1, 2.77, 2.67, 4.00, 4.32 and 6.00 for leaf extracts diluted to 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320, respectively, while the antiserum diluted to 1:500 produced values of 2.0, 3.1, 2.4, 2.98, 3.63 and 4.0, respectively (Table 1). However, while all of the diluted antisera and BICMV-infected plant sap were effective in distinguishing healthy plants using indirect ELISA, the best S/H ratio (diluted antiserum 1:200 and BICMV leaf extract 1:320) were chosen to use in the detection of a BICMV-infected plant.

Polyclonal antiserum (#S7) specificity was evaluated by testing with CMV subgroup I and II; genus *Cucumovirus*, CPMV; genus *Comovirus* and BCMV; and genus *Potyvirus*, respectively. The results showed that the polyclonal antiserum gave higher absorbance values for both the BICMV-CP recombinant protein (1.56) and yardlong bean infected with BICMV (2.05) than for the negative control (0.239) and viruses tested (CMV

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AJ312437        SGTGQPQPPIVDAGVDAGKDKRERNNRGKEPENREGSGNNSRGAGDSTMRDKDVNAGSKG 60
AJ312438        SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPEGREGSGNNNRGAGDSTMRDKDVNAGSKG 60
AF395678        SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPEGREGSVNNNRGAGDSTMRDKDVNAGSKG 60
AY575773        SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPEGREGSVNNNRGAGDSTMRDKDVNAGSKG 60
FR775796        SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPESREGSVNNNRGAGDSTMRDKDVNAGSKG 60
AH004380        SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPESREGSVNNNRGAGDSTMRDKDVNAGSRG 60
S66253          SGTGQPQPPIVDAGVDAGKDKRERSNRGKDPESREGSVNNNRGAGDSTMRDKDVNAGSRG 60
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AJ312437        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPEQTDLFNTRATKMQFEMWYNAVKGEY 120
AJ312438        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPEQTDLFNTRATKMQFEMWYNAVKGEY 120
AF395678        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPEQTDLFNTRATKMQFEMWYNAVKGEY 120
AY575773        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPEQTDLFNTRATKMQFEMWYNAVKGEY 120
FR775796        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPEQTDLFNTRATKMQFEMWYNAVKGEY 120
AH004380        KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPVQTDLFNTRATRDQFEMWYNAVKGEY 120
S66253          KVVPRLQKITKRMNLPMVKGNVILNLDHLLDYKPVQTDLFNTRATRDQFEMWYNAVKGEY 120
                ********************************** **********: *************

AJ312437        EIDDAQMSIVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMVENAKPTLRQIMH 180
AJ312438        EIDDAQMSIVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMVENAKPTLRQIMH 180
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AY575773        EIDDAQMSIVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMVENAKPTLRQIMH 180
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AH004380        EIDDDQMSIVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMVENAKPTLRQIMH 180
S66253          EIDDDQMSIVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMVENAKPTLRQIMH 180
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AJ312437        HFSDAAEAYIEMRNSEKPYMPRYGLLRNLRDKNLARYAFDFYEVTSKTSDRAREAVAQMK 240
AJ312438        HFSDAAEAYIEMRNSEKPYMPRYGLLRNLRDKNLARYAFDFYEVTSKTSDRAREAVAQMK 240
AF395678        HFSDAAEAYIEMRNSEKPYMPRYGLLRNLRDKNLARYAFDFYEVTSKTSDRAREAVAQMK 240
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AH004380        HFSDAAEAYIEMRNSERPYMPRYGLLRNLRDKNLARYAFDFYEVTSKTSDRAREAVAQMK 240
S66253          HFSDAAEAYIEMRNSERPYMPRYGLLRNLRDKNLARYAFDFYEVTSKTSDRAREAVAQMK 240
                ***************:******************************************

AJ312437        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
AJ312438        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
AF395678        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
AY575773        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
FR775796        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
AH004380        AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
S66253          AAALSNVSSKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMGSPQ 287
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Figure 3 Alignment of the deduced amino acid sequence of *Blackeye cowpea mosaic virus* coat protein gene (BICMV-CP) isolated from this study (FR775796) with the full length sequences available in GenBank by accession numbers AY575773, AF395678, AJ312438, AJ312437, AH004380 and S66253, respectively.

subgroup I and II and *CPMV)* as shown in Table 2. However, the polyclonal antiserum produced a weak cross-reaction with BCMV, which suggested a serological relationship to BICMV (Zettler and Evans, 1972). Compared to other results on anti-BICMV polyclonal antiserum production, the specificity of antibody obtained in the present study was similar to the study by Taiwo and Gonsalves (1982).

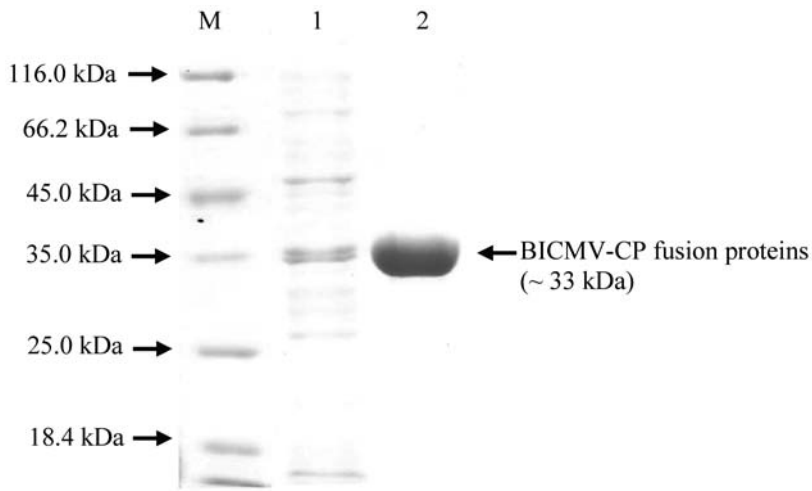

Figure 4 Coomassie brilliant blue-stained 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the *Blackeye cowpea mosaic virus* coat protein gene (BICMV-CP) fusion proteins were expressed (lane 1) and purified from *E. coli* DH5α (lane 2); M is molecular weight marker (left) and the arrow indicates the 33.0 kDa BICMV-CP fusion proteins band (right).

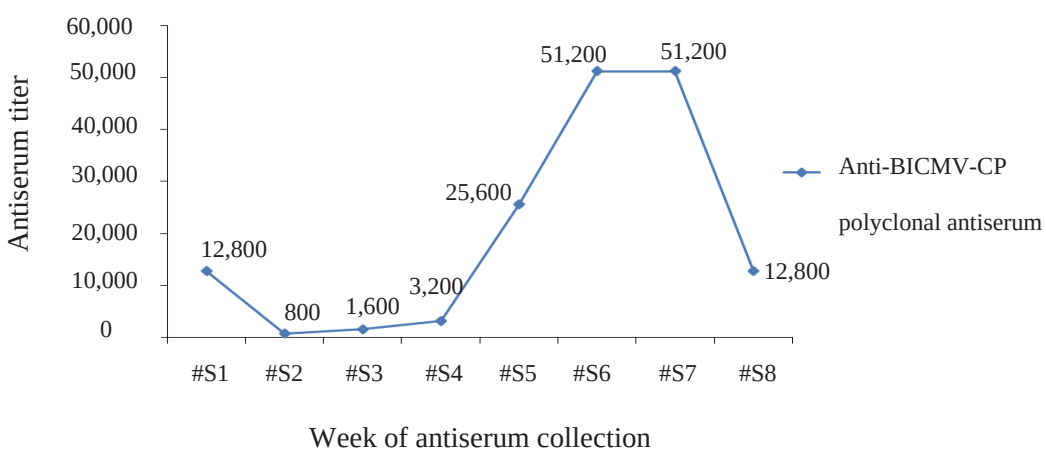

Figure 5 Antibody titer of polyclonal antisera against the *Blackeye cowpea mosaic virus* coat protein gene (BICMV-CP) recombinant proteins for the eight antiserum fractions.

Table 1 Indirect enzyme-linked immunosorbent assay values showing efficiency of anti- *Blackeye cowpea mosaic virus* (BICMV) recombinant coat protein polyclonal antiserum (#S7) in disease detection. Anti-BICMV recombinant coat protein polyclonal antiserum dilution: 1: 200 and 1: 500 reactions with healthy yardlong bean were used as the negative control.

Sample	Crude polyclonal antiserum #S7 (1:200)						Crude polyclonal antiserum #S7 (1:500)					
	Sap dilution						Sap dilution					
	1:10	1: 20	1: 40	1: 80	1:160	1:320	1: 10	1: 20	1: 40	1: 80	1: 160	1:320
Diseased	1.72	2.19	1.60	1.36	1.21	1.14	1.64	2.17	1.45	1.31	1.20	1.00
Healthy	0.82	0.79	0.60	0.34	0.28	0.19	0.83	0.70	0.61	0.44	0.33	0.25
S/H[a]	2.1	2.77	2.67	4.00	4.32	6.00	2.00	3.10	2.40	2.98	3.63	4.00

[a] = This ratio represents the absorbance value of sample OD405 / control OD405 measured 30 min after substrate was added.

Table 2 Specificity test of polyclonal antiserum against *Blackeye cowpea mosaic virus* (BICMV) recombinant coat protein as determined by indirect enzyme-linked immunosorbent assay. Polyclonal antiserum dilution: 1:200 reacted with healthy yardlong bean was used as a negative control.

Sample	Genus	Crude polyclonal antiserum #S7
Purified BICMV-CP recombinant protein	-	1.56
Yardlong bean infected with BICMV	*Potyvirus*	2.05
Bean common mosaic virus	*Potyvirus*	0.69
Cowpea mosaic virus	*Comovirus*	0.14
Cucumber mosaic virus subgroup I	*Cucumovirus*	0.09
Cucumber mosaic virus subgroup II	*Cucumovirus*	0.12
Healthy yardlong bean (×2)	-	0.239

CONCLUSION

Blackeye cowpea mosaic virus (BICMV) can have economically important repercussions and can be a major limiting factor for cowpea and yardlong bean production. To develop serological diagnostic tools for the detection and screening of the resistance of crop species to BICMV, the production of an antibody is a necessity. The coat protein gene (*CP* gene) of BICMV was amplified and cloned into a bacterial expression vector (pQE-80L). The recombinant BICMV-CP was expressed as fusion proteins containing a fragment of 6×His-tagged protein. Expressed BICMV-CP was purified under denaturing conditions by affinity chromatography with Ni-NTA Agarose and used as an antigen for raising BICMV-CP antisera in rabbit. The antiserum which gave the highest antibody titer was chosen to investigate its ability to detect BICMV-infected yardlong bean by indirect ELISA. Efficiency testing of the antiserum revealed it could detect BICMV at a dilution of up to 1:320. The produced antiserum demonstrated specificity with a BICMV-infected plant without cross reaction with other virus species tested, including *Cucumber mosaic virus* (subgroup I and II) and *Cowpea mosaic virus*. Hence, the antiserum against the BICMV-CP recombinant protein can be used to detect the presence of the virus in cowpea and yardlong bean breeding programs.

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