Asia Pacific Journal of Molecular Biology and Biotechnology, 2003
Vol. 11 (2) : 93-100

Synthesis of a Soluble Flag-Tagged Single Chain Variable Fragment (scFv) Antibody Targeting Cucumber Mosaic Virus (CMV) Coat Protein

Chua Kek Heng[1], Tan Chon Seng[2], Norzulaani Khalid[4], Jennifer A. Harikrishna[3] and Rofina Yasmin Othman[4*]

[1]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya*
[2]*Malaysian Agricultural Research & Development Institute*
[3]*Malaysia University of Science and Technology*
[4]*Institute of Biological Sciences, Faculty of Science, University Malaya*

Received 7 October 2003 / Accepted 28 November 2003

Abstract. Cucumber mosaic virus (CMV) is a serious pathogen of many economically important commercial fruit and vegetable crops worldwide. It is a particular problem in warmer climates, where plants are not grown under cover thus necessitating undesirably high use of agrochemicals for the control of insect vectors. Efforts towards controlling of this virus would include the development of improved methods of virus detection including the ability to produce cost effective and specific reagents. In this study the production of recombinant antibodies provides one such approach. A single chain variable fragment (scFv) antibody, targeted to CMV coat protein, was constructed with mRNA from the spleen of a CMV coat protein-immunized mouse. The nucleotide sequence of the variable heavy (V_H) and variable light (V_L) framework regions of the mouse spleen cDNA were used to design and construct primers for scFv library construction via RT-PCR. Three rounds of panning of the scFv library with the coat protein of a local isolate of a chilli strain CMV resulted in the cloning of a novel soluble Flag-tagged scFv antibody that suitable for use as a diagnostic reagent with the further potential of *in situ* application in the development of transgenic plants with novel resistance.

Key words: CMV, phage, scFv

INTRODUCTION

Cucumber mosaic virus is the type member of cucumovirus group, first discovered in *Cucumis sativas* in the USA (Doolittle, 1916; Jagger, 1916). It is single-stranded RNA virus and exists as a number of allied strains. The virus particle is isometric, not enveloped and 30 nm in diameter (Kaper and Waterworth, 1981). The virus can be transmitted by mechanical inoculation as in nature, by a number of vectors commonly aphids (Smith, 1972). It infects forages, cereals, woody and herbaceous ornamentals, vegetables, fruit crops and other important agriculture crops. Systemic mosaic is the typical symptom exhibited in most of the infected plants (Kaper and Waterworth, 1981).

A cucumber mosaic virus (CMV) was identified in chilli plants in Malaysia. The viral coat protein gene of the virus was amplified using RT-PCR and cloned into a bacterial expression vector CP.pRSET. DNA sequence analysis of the cloned fragment exhibited ≈93% similarity to published CMV coat protein nucleotide sequence and ≈78% in terms of amino acid sequence (Tan, *et al.*, 1998). The recombinant CMV coat protein from the CP.pRSET construct was used as the immunizing antigen in this study.

Generation of single-chain variable fragments (scFv) is now an established technique used to produce soluble antibody in bacterial systems. An artificial peptide linker is used to join the variable heavy (V_H) and variable light (V_L) regions of an antibody molecule to form the scFv which has antigen recognition and binding affinity (Bird, *et al.*, 1988; Hutson *et al.*, 1988). The scFvs are mainly constructed from either hybridoma (Hutson *et al.*, 1988) or spleen cells of immunized mice (Clackson *et al.*, 1991). The phage display technique takes advantage of the unique features of the M13 phage life cycle as the scFv can be easily displayed as a fusion protein on the minor coat protein (PIII) of the fd phage (McCafferty *et al.*, 1990). In this study an anti-CMV scFv was constructed via a bacteriophage system using purified recombinant CMV coat protein as the immunizing antigen antigen.

MATERIALS AND METHODS

Antigen preparation and mouse immunization. *E. coli* containing the plasmid CP.pRSET harbouring the CMV coat protein was induced overnight with 0.3mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the expressed coat protein

*Author for Correspondence.
Mailing address: Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel: 603-79675824; Fax: 603-79675908; E-mail: yasmin@um.edu.my

was subjected to column purification under native conditions using Xpress® Protein Purification System (Invitrogen). Bacterial cells were harvested by centrifugation at 3000 x g for 15 min and the expressed protein purified following manufacturer's instructions. One mg/ ml of the purified CMV coat protein was emulsified with Freunds adjuvant (Sigma) and injected into a Balb/c mouse through the subcutaneous route. Booster injections were carried out using incomplete adjuvant three times at 2-week intervals. Four days after the final injection, the mouse serum was tested by enzyme-linked immunosorbent assay (ELISA) to confirm the presence of anti-CMV antibodies prior to removal of the spleen for the subsequent procedures.

Amplification of the variable heavy (V_H) and variable light (V_L) chains and construction of single chain variable fragment (scFv). Total RNA was extracted from the spleen cells of the immunized mice using the RNeasy Mini Kit (QIAGEN, USA) followed by mRNA purification using an Oligotex™ Kit (QIAGEN, USA). First strand cDNA was synthesised from the mRNA. This was followed by amplification of the V_H and V_L genes separately using universal V_H primer 1, V_H primer 2 and V_L primer mix respectively (Amersham Pharmacia) at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min for 30 cycles) in a PCR thermocycler (Eppendorf 5330). The PCR mix typically contained Taq polymerase (Roche, USA) in standard buffer mix. The amplified V_H and V_L DNA fragments were then ligated separately into pGEM®-T easy vectors (Promega) and transformed *E. coli* DH5α (*sup*E44, *hsd*R17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *rel*A1) with the construct. T7 (5'-TAA TAG ACG ACT CAC TAT AGG G-3') and SP6 (5'-CTA TTT AGG TGA CAC TAT AG-3') primers were used for PCR screening of inserted DNA in the putative transformants. Positive clones carrying either the V_H genes or V_L genes were randomly selected and the insert DNA amplified and sequenced to determine the framework region sequence for designing new primers. Based on the sequencing results (Chua, 2002), 4 primers were designed (VHA and VHB are forward and reverse primers for V_H gene amplification and VLA and VLB for V_L gene amplification) (Table 1) containing nonsense sequence, new restriction endonuclease sites, a synthetic linker ($(Gly_4Ser)_3$), Flag-tag sequences and part of the frame-work sequences. These primers were used to amplify combinatorial V_H and V_L DNA fragments. The V_H and V_L DNA fragments were cloned into the pCANTAB 5E vector through 3 fragment ligation. A schematic diagram of the full linking procedure is shown in Figure 1.

Transformation of scFv construct and rescue of recombinant phage. The pCANTAB 5E plasmid carrying the scFv construct was inserted into *E. coli* TG1 cells (K12 Δ(*lac-pro*), *supE, thi, hsd*'5/F' [*traD36, proAB' lacI^q, lacZ*ΔM15]) through electroporation at 2.5 kV, 25 μF for 0.45 msec. Transformed TG1 cells were recovered in 1 ml of 2X YT medium (0.1% yeast extract, 1.7% tryptone, 0.05% sodium chloride) with 2% glucose and incubated at 37°C with shaking at 250 rpm for 1 hour. The transformed cells were then plated on 10 SOBAG plates (0.002 w/v bacto-trypton, 0.005 w/v bacto-yeast extract, 0.008 M sodium chloride, 0.01 M magnesium chloride, 0.111 M glucose, 100μg/ ml ampicillin) and incubated overnight at 37°C. On the following day, plates containing colonies were flooded with 2X YT medium with 100 μg/ ml ampicillin and 2% glucose (2X YT-AG) and transferred into a 50 ml polypropylene tube (Falcon, USA). The culture mixture was diluted with appropriate amount of 2X YT-AG medium until an O. D. of 0.5 at A_{600}. Two membranes were duplicated from a culture plate for colony hybridisation experiments and PCR detection was also carried out on randomly selected colonies for the inserted scFv. To 10 ml of the culture mixture, approximately 4 x 10^{10} pfu of the M13KO7 helper phage was added and incubated at 37 °C with shaking at 250 rpm for 1 hour. Following that, the pellet was collected by centrifugation at 1,000 x g for 10 min and resuspended in 10 ml of 2X YT medium containing 100 μg/ ml of each ampicillin and kanamycin. The culture was incubated at 37°C with shaking at 250 rpm overnight, then centrifuged at 1,000 x g for 20 min after which 2 ml of polyethylene glycol/ sodium chloride was added to the supernatant and incubated on ice for 60 min. Centrifugation at 10,000 x g for 20 min at 4 °C was carried out and the supernatant was discarded. The phage pellet was then resuspended in 2 ml of 2X YT medium and used in subsequent biopanning experiments.

Colony hybridisation. Colony hybridization was carried out using V_H or V_L chain PCR amplified products as the probes (3 μg/μl) which were labeled using the Digoxigenin DNA Labeling and Detection Kit (Roche). The Nitrocellulose membrane with colony DNA was prepared as described in Sambrook *et al.*, 1989.

Biopanning for Selection of the Recombinant Phage Expressing the Single-Chain Variable Fragment (scFv). Wells of sterile 96 wells 'U' bottom microtiter plates (Costar) were coated with 10 μg purified and dialyzed virus coat protein in 200 μl of 0.05 M sodium carbonate (pH 9.6) as the antigen, sealed tight and incubated overnight at 4 °C. On the following day, the antigen-coated well was washed 3 times using phosphate-buffered saline (PBS) and 200 μl of blocking buffer (10% skim milk in PBS) was used to block remaining wells then the plates were sealed and incubated at 37 °C for 2 hours followed by 3 washes with PBS. Fifty microliters of the supernatant containing the recombinant phage were added to the appropriate wells and incubated at 37 °C for 2 hours. The unbound phage was then removed and the well was washed thoroughly with phosphate-buffered saline- Tween 20 (PBS-T) at least 60 times.

Table 1. Primers used for amplification of variable heavy (V_H) and light (V_L) chains

Primer 1: VHA	*Sfi* I site	5'*AAGGAAAAAA***GGCCCAGCCGGCC**ATGGTSMARCTGCAGSAGTCWGCAMCTGA3'
Primer 2: VHB	*Bsp*E I site	5'*CGGGCGGCGG***TCCGGA**TCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCCTTG3'
Primer 3: VLA	*Bsp*E I site	5'*CGGGCGGCGG***TCCGGA**GGTGGCGGTTCGSAAAWTGTKCTCACCCAGTCTCCAGCAATC3'
Primer 4: VLB	*Not* I site	5'*AAGGAAAAAA***GCGGCCGC**GTCGAC[CTTGTCATCGTCGTCCTTGTAGTC]GACCCGTTTBAKYTCCAGCTTRGTSCCCCC3'

Nonsense sequence is italicised. *Sfi* I site, *Bsp*E I sites and *Not* I site are given in bold and labeled accordingly. The start codon is boxed. The synthetic peptide linker is underlined and the Flag-tag sequence is in marked with square brackets.

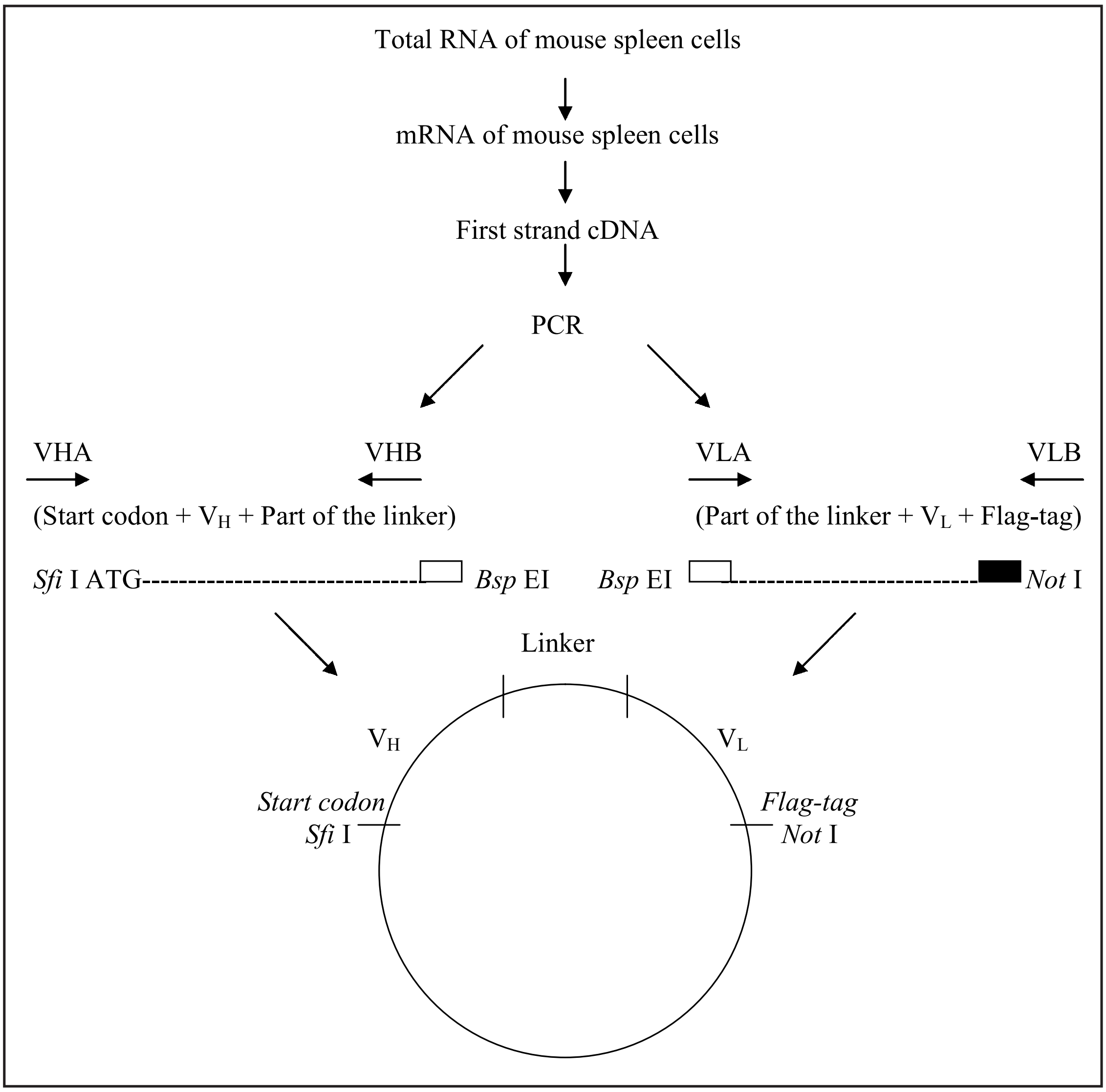

Figure 1. Schematic diagram for the scFv construction. The diagram represents the scFv construction strategy. Explanation of the strategy is as in text. The diagram is not drawn to scale. V_H - variable heavy chain of an antibody; V_L - variable light chain of an antibody; VHA and VLA - forward primers for amplification of V_H and V_L genes respectively; VHB and VLB - reverse primers for amplification of V_H and V_L genes respectively; white boxes - part of the linker; black box - Flag-tag sequence; ATG - start codon.

Reinfection of* Escherichia coli *with Recombinant Phage. To the well containing bound phage, 50 µl of trypsin (10 mg/ ml) was added and incubated at 37 °C for 30 min. Vigorous pipetting was done and the trypsin solution containing recombinant phage was used to infect 10 ml of 2X YT medium containing log phase TG1 cells. The culture was incubated at 37 °C for 1 hour with 250 shaking. Prior to second round of panning, 100 µg/ ml of ampicillin, 2% glucose and 4x 10^{10} pfu of M13KO7 were added to the TG1 cell suspension and the procedures, as described previously, repeated.

Immunodetection of Positive Recombinant Phage. The method used for immunodetection was based on the protocol provided by RPAS (Amersham Pharmacia). CMV coat protein (10 µg/ ml) in 200 µl of 0.05M sodium carbonate (pH 9.6) was dispensed into 147 separate wells in the microtiter plate. The plate was incubated overnight at 4 °C. On the following day, the contents of each well were removed, 200 µl of blocking buffer was added and the plate was incubated at 37 °C for 2 hours. One hundred µl of the supernatant containing the scFv expressing recombinant phage were added to the plates after the blocking buffer was decanted. Two rows of wells were incubated with M13KO7 helper phage in blocking buffer as the negative control. The plate was incubated at 37 °C for 2 hours and then washed 3 times with PBS-T. Sheep anti-Ml3 IgG horse radish peroxidase conjugate (Amersham Pharmacia) was diluted 1:5000 in blocking buffer and 200 µl of the solution was added to all wells. The conjugates were incubated at 37 °C for 2 hours and washed as previously described. Following the washing, 200 µl of 1X 2', 2'-azino-bis (3ethylbenzthiazoline-6-sulphonic acid) diammonium (ABTS) substrate containing 0.05% H_20_2 was added to each well and the plates incubated at room temperature for 20-60 minutes until a suitable colour (green) appeared. The absorbency was determined at 414 nm using a microplate reader, Titertek Multiskan Mcc/340 P.

Soluble scFv antibody production. The scFv clone with the highest binding affinity was chosen and the construct extracted though DNA mini-preparation then inserted into competent *E. coli* strain HB2151 (K12 "(*lac-pro*), *ara*, *nal*^{r}, thi/F'[*proAB*, *lac*I^q, *lac*ZΔM15]) through transformation technique. The transformed *E. coli* HB2151 cells were plated on SOBAG-N (0.02 w/v bacto-tryptone, 0.005 w/v bacto-yeast extract, 0.0015 w/v bacto-agar, 0.0005 w/v sodium chloride, 10 mM magnesium chloride, 0.1 M glucose, 100 µg/ ml ampicillin, 100 µg/ ml nalidixic acid) and incubated overnight at 30 °C. Selected colonies of *E. coli* HB2151 were inoculated into 5 ml of Super broth medium (0.035 w/v bacto-tryptone, 0.02 w/v bacto-yeast extract, 0.085 M sodium chloride) containing 100 µg/ ml ampicillin and 2% glucose (SB-AG) and incubated overnight at 30 °C with shaking at 250 rpm. After incubation, 5 ml of the overnight culture was used to inoculate 10 ml of SB-AG and incubated at 30 °C for 4 hours with shaking at 250 rpm. After the incubation, the bacterial pellet was collected from the culture by centrifugation at 1500 x g and resuspended in Super broth containing 100 µl/ ml ampicillin and 1 mM IPTG (SB-AI) medium. Incubation was carried out overnight at 30°C and shaking at 250 rpm. After that, the bacterial pellet was collected by centrifugation and resuspended in 0.5 ml of ice-cold 1X TES buffer (0.2 M tris hydrocloride, 0.5 mM ethylenediaminetetra-acetate acid, 0.5 M sucrose). A volume of 0.75 ml of ice-cold 1/5 X TES buffer was added and the mixture vortexed then incubated on ice for one hour. Finally, the supernatant containing scFv antibody from the bacterial periplasm was collected by centrifugation at full speed. If not used immediately, the antibody was stored at -20 °C prior to use in immuno-detection experiments.

Western Blot and ELISA Detection. Total protein of *E. coli* containing scFv antibody was separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in denaturing buffer. Fractionated protein on the gel was transferred to a nitrocellulose membrane using a trans-blotter (Bio-Rad). Blocking, incubating and washing steps were performed as described in Sambrook *et al.*, 1989. The presence of the soluble scFv antibody protein was detected using anti-Flag M2 monoclonal antibody (Sigma) and Fc specific goat anti-mouse IgG conjugated with Horseradish peroxidase (HRP) (Pierce). The soluble protein, which acts as a probe for western blot detection was added to the membrane with unpurified total bacteria including expressed CMV coat protein. Following that, anti-Flag and lastly goat anti-mouse IgG-HRP was added for detection. Direct ELISA detection using infected leaf samples and unpurified expressed coat protein was carried out as described by Michael *et al.*, 1988.

Automated DNA sequencing. Plasmid DNA was isolated from the transformed *E. coli* using Qiagen Plasmid Miniprep kit (QIAGEN) and subjected to cycle sequencing using ABI377 (Perkin Elmer). The primers used for sequencing were pCANTAB5-S1 (5'-CAA CGT GAA AAA ATT ATT ATT CGC-3') as the forward primer and pCANTAB5-S6 (5'-GTA AAT GAA TTT TCT GTA TGA GG-3') as the reverse primer.

RESULTS

The ≈35 kDa CMV coat protein used as the immunizing antigen was successfully expressed (Figure 2) and verified using anti-CMV polyclonal antibody as a probe. mRNA of mouse spleen tissue was successfully extracted and an approximate 427 bp V_H chain DNA fragment and 402 bp V_L chain DNA fragment were obtained with primers VHA/

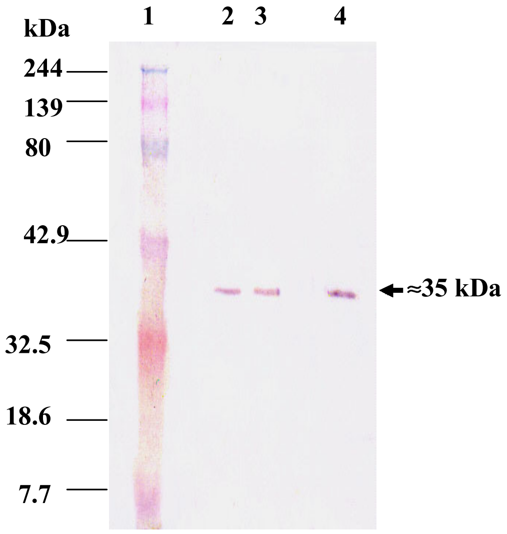

Figure 2. Western-blot immunodetection of the expressed coat protein from the CP. pRSET construct. Total protein obtained from induced *E. coli* carrying CP.pRSET construct was electrophoresed on a 12% SDS PAGE gel, transferred onto nitrocellulose membrane (Protran) and detected using anti-CMV polyclonal antibodies. Lane 1: kaleidoscope prestained protein marker (Bio-Rad); lane 2-4: Products from samples. The position of the band migration at the predicted molecular weight (≈ 35 kDa) of the CMV coat protein is indicated by the arrow.

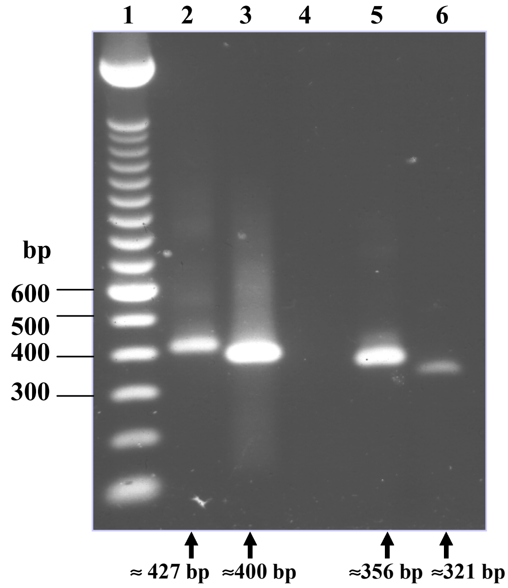

Figure 3. Amplification of the V_H and V_L chain genes using self-designed primers. Amplification of the V_H and V_L chain genes was carried out using commercial and self-designed primers. Samples were electrophoresed on a 1% agarose in 1X TBE buffer. Lane 1: 100 bp DNA ladder (Gibco BRL); Lane 2: V_H DNA fragment amplified using VHA and VHB primers; Lane 3: V_L DNA fragment amplified using VLA and VLB primers; Lane 4: negative control where PCR amplification using VHA and VHB primer and distilled water as the template; Lane 5: V_H DNA fragment amplified using V_H primer 1, V_H primer 2 (Amersham Pharmacia); Lane 6: V_L DNA fragment amplified using V_L primer mix (Amersham Pharmacia).

VHB and VLA/ VLB respectively (Figure 3). The amplified products contained additional sequences including nonsense sequence, start codon (for V_H), restriction sites, part of the linker sequence (for V_H and V_L) and Flag-tag sequence (for V_L). After restriction enzyme digestion, the V_H and V_L chain amplified fragments were then ligated into pCANTAB 5E which resulted in a pool of 3 x 10^8 independent scFv recombinants. The presence of the V_H and V_L chains in the combinatorial library was determined by colony hybridisation using DIG-labelled V_H or V_L probes with almost 100% transformation recorded for each preparation. This was further confirmed through PCR using primers VHA and VHB on randomly selected transformed cells (Figure 4). The expected 774 bp amplified fragment of the scFv indicated that the recombinant scFv was successfully constructed and transformation of *E. coli* strain TG1 with the desired construct was done. After two rounds of biopanning using ELISA plates coated with purified chilli strain CMV coat protein as the antigen, 123 colonies were selected. All of the colonies were then picked for another round of the gene rescue and biopanning to select the scFv clone with the highest affinity. The scFv clones which showed the most significant absorbance ratio (≈3.46), had the highest binding affinity, and were selected for soluble scFv production using *E. coli* strain HB2151. A clear and strong signal for putative scFv protein (≈32 kDa) was detected using anti-Flag monoclonal antibody in the induced HB2151 periplasmic samples carrying the selected scFv clones (Figure 5) after overnight incubation at 30 °C with agitation at 250 rpm. No band was observed in the uninduced sample incubated under the same conditions. Detection of the Flag peptide, which was constructed at the carboxyl terminus of the scFv, indicated that the scFv was successfully cloned and expressed. Experiments using the soluble anti-CMV scFv antibody as a probe for ELISA detection on sap extract of infected leaf samples (A_{414}= 0.819) and purified CMV coat protein (A_{414}= 0.511) showed a significant absorbance ratio of more than 2, whilst negative results were obtained when the banana streak virus was used as the negative control antigen (A_{414}= 0.046). In Western blot experiments for detection of the CMV coat protein, a 35 kDa CMV coat protein was observed in the induced *E. coli* carrying CP. pRSET construct, while no band was observed in the uninduced *E. coli* (Figure 6). Nucleotide and the deduced amino acid sequences of the heavy and light chains are shown in Figure 7.

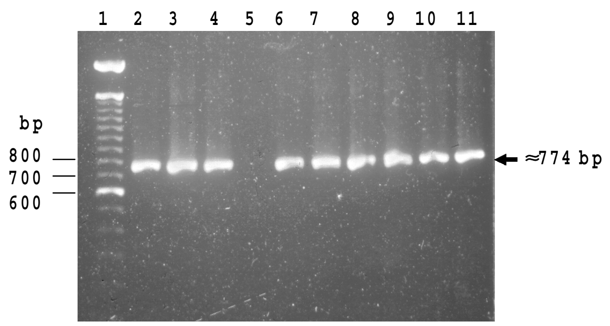

Figure 4. Detection of scFv through PCR method. Nine colonies were randomly chosen for PCR detection using VHA and VLB primers. Lane 1: 10 bp DNA ladder (Gibco BRL); Lane 2-4 and 6-11: 774 bp bands were detected in all the samples; Lane 5: Negative control where bacterial colony carrying pCANTAB 5E vector only was used for the PCR detection.

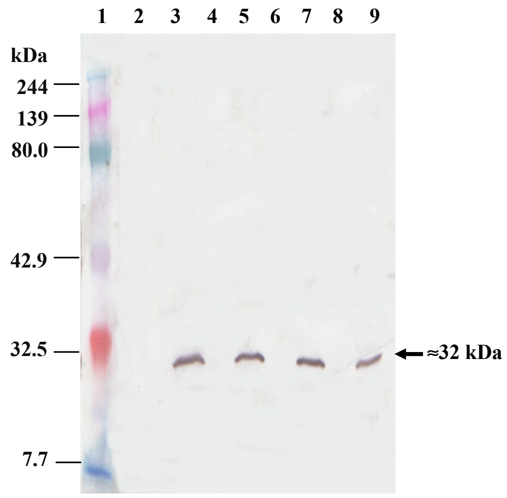

Figure 5. Immunodetection of the induced *E. coli* HB2151 carrying selected anti-CMV scFv construct. Transformed *E. coli* HB2151 carrying anti-CMV scFv clones were induced with 0.5 mM of IPTG and incubated at 37 °C for 4 hours. Total protein of the bacterial periplasm was extracted and 2 μg of the total proteins were electrophoresed on a 12% SDS PAGE gel. Fractionated proteins were then transferred onto a nitrocellulose membrane and detected using anti-FLAG monoclonal antibodies followed by Goat anti-mouse IgG-HRP. Lane 1: kaleidoscope prestained protein marker (Bio-Rad), Lane 2, 4, 6, 8: uninduced samples; Lane: 3, 5, 7, 9: induced samples. The position of the band migration at the predicted molecular weight (≈32 kDa) of the recombinant scFv is indicated by the arrow.

DISCUSSION

CMV infection affects many economically important plants and is the major viral pathogen affecting chilli in Malaysia. CMV has many isolates differing in their host range and pathogenicity as documented by Kaper and Waterworth in 1981. The isolate used as the immunizing antigen in this study was found in infected chilli plant in Malaysia. In this study, construction of a recombinant anti-CMV scFv as an alternative source of antibodies for use in CMV diagnostics was successfully carried out. The production of recombinant antibody is, in the long term, a potentially cheaper and easier approach for the production of diagnostic reagents compared to the current source of such CMV antibodies from animals or monoclonal cell lines.

In this study immunized mouse spleen tissue was used to construct the scFv as the cells are a rich source of immunoglobulin genes and repertories of V_H and V_L genes can be easily amplified from the mRNA of such cells (Hawkins *et al.*, 1992). A time-saving one-step cloning procedure modified form (Chee and Sazaly, 1998) was used in the experiment to construct an scFv combinatorial library prior to biopanning for selection of the best scFv construct. This method bypasses conventional two-step protocols using an intermediate cloning vector and potentially minimizes the possibility of mutations associated with extended protocols. The unique primers designed in this study enabled easy linking of the two chains and again bypassed the difficult PCR assembly method described by Horton et. al., 1989. As the frame-work regions of V_H and V_L chain of an immunoglobulin are relatively conserved within an animal species, sequence information could be obtained for both chains. From our results, we found that other than the highly conserved 3' end of the V_H chain framework region, some of the framework nucleotide sequences of both chains were not identical when the sequence from several clones was compared. However, since there were differences in only a few nucleotide sequences, degenerate primer design was still possible. The additional nonsense sequence introduced at the 5' end of the primers was to increase the cleavage efficiency of the amplified V_H and V_L chain DNA fragments by restriction endonucleases, and the additional tag sequence at the carboxyl terminus of the scFv was added to facilitate the detection and purification in subsequent steps (Clackson *et al.*, 1991). In addition the construct contained an E-Tag as a result of cloning into the pCANTAB 5E vector, thus allowing for both anti-E tag or Flag-tag detection in future applications. To ensure production of soluble antibodies, the non-suppressor *E. coli* strain HB2151 was used without any modification or manipulation of the selected scFv. In this *E. coli* strain, protein synthesis is aborted at the end of the scFv gene due to recognition of a stop codon and as a result, the g3p fusion protein is not made and the scFv is transported and accumulates in the periplasmic location of the bacteria up to certain maximal level, after which the soluble scFv antibody will leak into the medium (Hoogenboom *et al.*, 1991). We successfully obtained soluble anti-CMV scFv antibody not only in the periplasmic extract

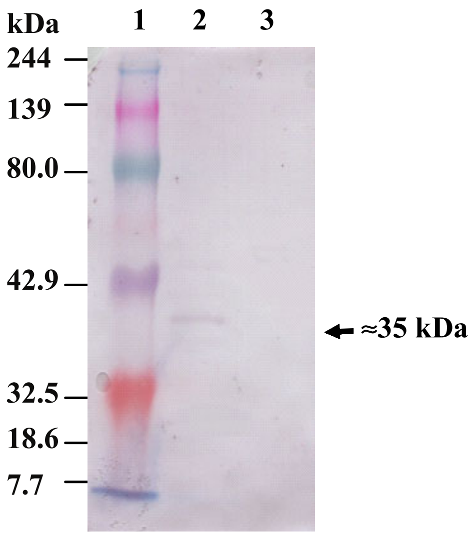

Figure 6. Determination of the binding affinity of the selected scFv antibody by Western blot. CMV coat protein was separated on a 12% SDS-PAGE gel and blotted to nitrocellulose membranes. Detection was carried out using anti-CMV scFv antibody as the primary antibody with anti-E HRP conjugate as the secondary antibody followed by anti-Flag and Fc region specific goat anti-mouse IgG-HRP conjugated antibody (Pierce). Lane 1: kaleidoscope prestained protein marker (Bio-Rad); Lane 2: coat protein of the crude CMV coat protein extracted from the induced bacteria carrying pRSET.CP construct; Lane 3: total protein of the wild type *E. coli*. The position of the band migration at the predicted molecular weight (≈35 kDa) of the CMV coat protein is indicated by the arrow.

V_H Chain Nucleotide Sequence

```
GTG CAG CTG CAG GAG TCA GCA ACT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA TCC
 V   Q   L   Q   E   S   A   T   E   L   V   K   P   G   A   S   V   K   I   S
                                 CDR-H1
TGC AAG GCT TCT GGC TAC TCC TTC ATT GTC CAC TAT ATA AAC TGG GTG AAG CAG AAG CCT
 C   K   A   S   G   Y   S   F   I   V   H   Y   I   N   W   V   K   Q   K   P
                                                     CDR-H2
GGA CAG GGA CTT GAG TGG ATT GGA TGC TTT TTT CCT GGA AGC GGT AAT AGT AAG TAC ATT
 G   Q   G   L   E   W   I   G   C   F   F   P   G   S   G   N   S   K   Y   I

GAG AAC TTC AGG GGC AAG GCC ACA TTG ACT GTA GAC ACA TCC TCC AGT ACA GCC TAC ATG
 E   N   F   R   G   K   A   T   L   T   V   D   T   S   S   S   T   A   Y   M

CAG CTC AGC AGC CTG ACA TCT GAG GAC ACT GCT GTC TAT TTC TGT GCA AGG GAT GAT TCC
 Q   L   S   S   L   T   S   E   D   T   A   V   Y   F   C   A   R   D   D   S
         CDR-H3
GAC GGA GCT ATG GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
 D   G   A   M   D   Y   W   G   Q   G   T   T   V   T   V   S   S
```

V_L Chain Nucleotide Sequence

```
GAA ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AGG GTC ACC
 E   I   V   L   T   Q   S   P   A   I   M   S   A   S   P   G   E   R   V   T
                             CDR-L1
ATG ACC TGC AGT GCC AGC TCA AGT ATA CGT TAC ATA TAT TGG TAC CAA CAG AAG CCT GGA
 M   T   C   S   A   S   S   S   I   R   Y   I   Y   W   Y   Q   Q   K   P   G
                                                 CDR-L2
TCC TCC CCC AGA CTC CTG ATT TAT GAC ACA TCC AAC GTG GCT CCT GGA GTC CCT TTT CGC
 S   S   P   R   L   L   I   Y   D   T   S   N   V   A   P   G   V   P   F   R

CTC AGT GGC AGT GGG TCT GGG ACC TCT TAT TCT CTC ACA ATC AAC CGA ACG GAG GCT GAG
 L   S   G   S   G   S   G   T   S   Y   S   L   T   I   N   R   T   E   A   E
                                                 CDR-L3
GAT GCT GCC ACT TAT TAC TGC CAG GAG TGG AGT GGT TAT CCG TAC ACG TTC GGA GGG GGC
 D   A   A   T   Y   Y   C   Q   E   W   S   G   Y   P   Y   T   F   G   G   G

ACC AAG CTG GAG CTC AAA CGG
 T   K   L   E   L   K   R
```

Figure 7. Nucleotide and the deduced amino acid sequences of the heavy and light chains. Complementarity determining regions (CDRs) shown in boldface. Heavy chain V_H (GenBank Acc. No. AY 337618). Light chain V_L (GenBank Acc. No. AY 337619)

of induced bacteria cells, but also from the culture medium making larger scale purification potentially very simple.

This study showed that the recombinant scFv had binding affinity to the immunizing antigen and can be developed for detection of chilli CMV infections. ScFvs have been developed as specific diagnostic reagents against other viruses including HIV (De Haard *et al.*, 1998). However as CMV is a highly heterogenous group we believe that a combination of specific scFvs or an scFv that would recognize a shared epitope may be further isolated form the library generated in this study and would then be applicable as a general diagnostic reagent for the group. Other than this *ex situ* application, further potential of the recombinant antibody fragment would be the development of transgenic plants with a novel *in situ* form of resistance against the targeted antigen (Tavladoraki *et al.*, 1993; Whitelam and Cockburn 1997). Initial studies using this approach (Chua, 2002) has shown promising results in tobacco plants with the final aim of producing CMV resistant chilli in the near future.

ACKNOWLEDGEMENTS

This study was funded by IRPA R&D grant ID: 09-02-03-0521, and Vote F of University of Malaya. Special thanks to Prof. Sazaly, Hui Yee and all the members of the Microbiology lab, Faculty of Medicine, UM and members from AMCAL, UM for their help and advice.

REFERENCES

Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, B. M. Kaufman, S. M. Lee, T. Lee, S. H. Pope, G. S. Riordan, and Whitlow, M. 1988. Single-chain antigen binding protein. *Science* 242: 423-426

Chee, H. R. and Sazaly, A. B. 1998. Construction of a single chain variable fragment (scFv) antibody recognizing the dengue 2 virus envelope protein. *Asia Pacific Journal of Molecular Biology and Biotechnology* 6: 79-87

Chua, K. H. 2002. Development of an anti-CMV scFv for expression in *Nicotiana tabacum* L. cv. white burley. PhD Thesis, University of Malaya.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. 1991. Making antibody fragments using phage display libraries. *Nature* 352: 624-628

De Haard, H. J. W., Kazemier, B., Koolen, M. J. M., Nijholt, L. J., Meloen, R. H., Van Gemen, B., Hoogenboom, H. R. and

Arends, J. W. 1988. Selection of recombinant, library-derived antibody fragments against p24 for human immunodeficiency virus type 1 diagnostics. *Clinical and diagnostic laboratory immunology* 5: 636-644

Doolittle, S.P. 1916. A new infectious mosaic disease of cucumber. *Phytopathology* 6: 145

Hawkins R. E., and Winter, G. 1992. Cell selection strategies for making antibodies from variable gene libraries. *European Journal of Immunology* 22: 867-870

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chriswell, D. J., Hudson, P.and Winter, G. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Research* 19: 4133-4137

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. and Pease, L. R. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77: 61-68

Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R. and Oppermann, H. 1988. Protein engineering of antibody banding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proceedings of the National Academy of Sciences, USA* 85: 5879-5883

Jagger, I. C. 1916. Experiments with the cucumber mosaic disease. *Phytopathology.* 6: 148

Kaper, J. M, and Waterworth, H. E. 1981. Cucumoviruses : Handbook of plant virus infectious and comparative diagnosis, ed. Kurstak, E., pp.257-332. The Netherland: Elsevier/ North-Holland Biomedical Press.

Michael, F. C., Richard, M. L. and Moshe, B. J. 1988. Methods for plant molecular biology: ELISA Techniques, ed. Arthur W. and Herbert, W., pp 507-530. London: Academic Press.

McCafferty, J., Griffiths, A. D., Winter, G., Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348: 552-554

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual.* 2nd edn. New York: Cold Spring Harbor laboratory.

Smith, K. M. 1972. *A textbook of plant virus disease.* (Academic Press, INC., New York, USA), pp. 234-252

Tan, C. S., Ong, C. A. and Hassan, M. D. 1998. Cloning and expression of cucumber mosaic virus coat protein in bacteria. *Proceedings of the 10th National Biotechnology Seminar,* Malaysia 40-42

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi, P. 1993. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* 366: 469-472

Whitelam, G. C. and Cockburn, W. 1997. Plantibodies: antibodies produced in transgenic plants. *The Biochemist*-February 7-9