Kasetsart J. (Nat. Sci.) 43 : 330 - 338 (2009)

Construction of Single-Chain Variable Fragment (scFv) Specific to *Cucumber Mosaic Virus* by Phage Display Technology

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ABSTRACT

Cucumber mosaic virus (CMV) causes serious problems in economically important crops, especially members of the Solanaceae and Cucurbitaceae families. Serological detection of this virus by specific antibodies is required as a control measure as well as for quarantine investigation to ensure any components used for agricultural propagation, especially commercial seeds, are disease free. However, the selection of recombinant antibodies by phage display nowadays presents a real challenge to provide the antibodies that are urgently needed. In this research, an anti-CMV single-chain variable fragment (scFv) was constructed using a phage display system. Both heavy (V_H) and kappa light chain variable (V_k) genes were amplified by RT-PCR from the hybridoma cell line CM2, secreting a monoclonal antibody (MAb) specific to both serogroup I and II of CMV. The V_H and V_k amplified products, approximately 400 bp in length, were joined by a PCR overlapping extension method to generate the scFv gene. A recombinant phagemid pCANTAB5E harboring the scFv gene was constructed and transformed into *Escherichia coli* TG1. The bacterial transformants were rescued by helper phage M13 to produce phage-displayd scFv and the screening for CMV-specific scFv was carried out by ELISA. Three positive, recombinant clones (2C1, 6A1 and 1D4) which gave high signal-to-noise in ELISA were utilized in order to produce soluble antibodies. Western blotting and DNA sequencing were performed to characterize the scFv products. The result showed that all clones were identical and able to bind CMV of both subgroups. DNA comparisons showed that all the V_H belonged to the J558.32 subgroup and JH2, while V_k belonged to V_k genes and JK2.

Key words: *Cucumber mosaic virus*, scFv, phage display, antibody, phagemid, construction

INTRODUCTION

Cucumber mosaic virus is a member of the genus *Cucumovirus* (Gioria *et al*., 2002). It has a very broad host range, which more than 885 known hosts have been reported worldwide (Bashir *et al*., 2006). The virus causes severe diseases leading to economic losses in vegetables, fruits, cereals and ornamentals (Hsu *et al*., 2000; Gioria *et al*., 2002). The serological techniques using polyclonal (PAb) and monoclonal (MAb) antibodies (Hsu *et al*., 2000) have been applied successfully to detect and diagnose the virus. However, PAb tends to cross-react with healthy

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Received date : 21/07/08 Accepted date : 29/01/09

plant sap, which can be a major problem in any detection assay. To overcome this problem, anti-CMV MAbs have been produced by the hybridoma technique (Koohapitagtam *et al.*, 2004). The hybridoma clone, CM2, secretes anti-CMV MAb with high specificity to both serogroup I and II of CMV, with the sensitivity of detection at 1 ng/ml being obtained in a triple antibody sandwich, enzyme-linked, immunosorbent assay (TAS-ELISA).

Maintaining hybridoma cell lines *in vitro* can be achieved by freezing below -80°C or by using liquid nitrogen. However, hybridoma cells are usually unstable in long-term storage. Moreover, the medium for culturing hybridoma cell *in vitro* is costly. In recent years, the technology for displaying an antibody on a phage particle (the so-called phage display), has provided a powerful tool for the selection of antibodies in different formats such as scFv or antigen-binding fragment (Fab) (Hoogenboom *et al.*, 1998) which has increased the opportunity to optimize the antibodies and their application**.** The phage-displayed format can be constructed by fusing the antibody genes to the coat protein gene of bacteriophage. After expression, the fusion proteins will be incorporated into the coat of the phages during phage particle formation. The antibody fragment displayed on the phage surface is physically accessible, thus enabling the selection of an antibody against the target antigens. In addition, the bacterial culture containing the antibody genes can be maintained as frozen culture and so is easy to handle. Moreover, the scFv gene can be modified to fuse with the alkaline phosphatase gene for direct ELISA (Wang *et al.*, 2006) or antibody engineering for control measures (Sanz *et al.*, 2005).

This study aimed to generate an anti-CMV scFv from a hybridoma cell line, CM2, by using the phage display system. The complementarity determining regions (CDRs) on V_H and V_κ of the scFv were also examined.

MATERIALS AND METHODS

Bacterial culture and reagents

The synthetic primers for anti-CMV MAb were synthesized at the DNA Technology Laboratory (Kasetsart University, Thailand). The mRNA extraction from the hybridoma cell line was carried out using Purescript® RNA Isolation Kits (Gentra Systems Inc. 2000, Minneapolis, USA). The Superscript™ III reverse transcriptase was purchased from Invitrogen (California, USA). The Hot Star *Taq* DNA polymerase, restriction endonucleases, T4 DNA ligase and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Promega (Madison, USA), New England Biolab (Beverly, USA), Invitrogen (California, USA) and Fermentous (Hanover, USA), respectively. DNA extraction from gel was done using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). *Escherichia coli* strains TG1 and HB2151 (Amersham Biosciences, Uppsala, Sweden) were used as hosts for phagemid manipulation and expression, respectively. The pCANTAB-5E phagemid (Amersham Biosciences, Uppsala, Sweden) was used as a vector for cloning the PCR products of the scFv (V_κ and V_H) gene. The SOBAG medium, containing 2% tryptone, 0.05% NaCl, 0.5% yeast extract, 2% glucose and 100 μg ml^{-1} of amplicillin (plus 1.5% agar in plates), was used for the selection of transformants. Anti-M13 monoclonal antibody (MAb) conjugated with horseradish peroxidase for phage library selection and anti-E-Tag MAb for scFv fragment detection were purchased from Amersham Bioscience. The anti-mouse antibody conjugated with alkaline phosphatase for anti-E-Tag detection was purchased from Sigma-Aldrich (St. Louis, USA). The substrates were 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (KPL, Maryland, USA) for peroxidase detection, with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Zymed, California,

USA) for alkaline phosphatase assay. Other reagents used in this study were at least of analytical grade.

Primer design

Two sets of degenerated primers were designed and synthesized for the amplification of the variable genes (V gene) from the hybridoma cells CM-2 (murine $IgG_{1,\kappa}$), as reported previously (Koohapitagtam *et al*., 2004), including a framework region (FR1) to joining region (J). The sequences were as follows:

the V_H forward primer 5′ CCT TTC TAT **GCG GCC CAG CCG GCC** GAG CTC SAG GTG AAA CTG CAG GAG TCT 3′;

the J_H reverse primer 5′ ACC AGA GCC GCC GCC GCC GCT ACC ACC ACC ACC ASG TTT BAK YTC CAR YYT K 3′;

the V_κ forward primer 5′ GGC GGC GGC GGC TCT GGT GGT GGT GGA TCC GAY ATT GTG MTR ACM CAR KMT CAA 3′; and

the J_κ 5′ CAT CGG CAC CGG CGC ACC T**GC GGC CGC** GMR GAR ACR GTG ACC RKR GTC CCT K 3′.

where S, B, K, Y and M represents G/C, C/G/T, G/T, C/T, A/G and A/C, respectively. The heavy chain 5′ and the light chain 3′ primers were modified to include a *Sfi*I site (underlined) and a *Not*I site (underlined), respectively. In addition, $(Gly_4Ser)_3$ linkers were also added to the light chain 5′ primer and to the heavy chain 3′ primer for the assembly of V_H and V_κ polypeptides into scFv format (Okamoto *et al*., 2004)

cDNA synthesis, PCR amplification and construction of CMV-scFv gene

The mRNA of 10^7 hybridoma cells (Lai *et al*., 2002) was isolated using a Purescript® RNA Purification System. Subsequenly, cDNA was generated using oligo (dT) primers and Superscript™ III reverse transcriptase which was the first strand cDNA synthesis system for reverse transcription (RT). From each cDNA, heavy-and light-chain genes were amplified separately and recombined by third subsequent PCRs. In the first-step PCR, the V_H and V_κ genes were amplified by PCR containing 500 pg cDNA and 70 pmole of each primer, using the Hot Star *Taq* DNA polymerase. The samples were cycled 35 times at 95°C for 15 min, 94°C for 45 s, 55°C for 45 s and 72°C for 1 min on a programmable heating block (MJ Research, USA). The samples were purified from the 2% agarose gel using a QIAquick Gel Extraction Kit. In the second-step PCR, V_H and V_κ DNA were assembled using equal volumes of the products without primer. Each 50 μl of this PCR step contained 2.5 μl 10 mM dNTPs, 5 ml 10XPCR buffer, 250 ng of each V_H and V_κ and 5U Hot Star *Taq* DNA polymerase. The reaction mixture was preheated at 95°C for 15 min and 30 cycles of the amplification were carried out at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The scFv DNA was analyzed in 2% agarose gel and purified by a QIAquick Gel Extraction Kit.

Cloning and expression of the recombinant phage

The PCR-amplified scFv DNA and pCANTAB5E phagemid vector were digested with *Sfi*I and *Not*I. The resulting scFv fragments were inserted into pCANTAB5E to generate scFv-gene III fusion, using T4 ligase. The reaction was incubated at room temperature for 2 h. Ligated DNA populations were introduced into *E. coli* TG1 by electroporation at 2.5 kV for 4.5 msec. The transformants were grown by culturing in 2YT supplemented with amplicillin (100 μg ml^{-1}) and 2% glucose (w/v) at 37°C. The scFv expressing phages were rescued by following the manufacturer's instructions for the Recombinant Phage Antibody System (Amersham Biosciences, Uppsala, Sweden). The phages were then purified and concentrated using 2% polyethylene glycol (PEG) and sodium chloride. The phage pellet was diluted in 50 μl of 2% skim milk in PBS per 1.5

ml original volume and used in the following panning step.

Selection of anti-CMV scFv

Three rounds of panning assay were carried out for the selection of anti-CMV scFv according to Saldarelli *et al*. (2005). Purified CMV-coat protein (CP) was used as an antigen.

Screening for antigen-specific scFv-displayed phage by ELISA

After the panning process, individual TG1 colonies were picked, grown at 37°C in a 96-well tissue culture plate (Corning, New York, USA) for 3 h and rescued with M13KO7 helper phage. The amplified phages were diluted with 4% skim milk in PBS with a 1:1 (v/v) ratio and incubated at room temperature for 1 h to block any non-specific protein-protein interactions that might have occurred between recombinant antibodies and surrounding proteins. A concentration of 5 μg ml^{-1} of expressed CMV-CP or purified CMV was coated into the microtiter plate (Corning, New York, USA) and incubated at 37°C for 1.30 h. Subsequently, the plate was washed three times with 200 μl of PBST and 150 μl of 2% skim milk was added to block non-specific binding. A 50 μl quantity of each of the amplified phages was then added to the plate and incubated for 2 h at 37°C. Plates were washed six times with PBST and 50 μl of mouse anti-M13 phage-conjugated with HRP (1:5000) was added into each well. The plate was washed three times and 50 μl of the TMB substrate was added and incubated at 37°C for 30 min. To stop the reaction, an equal volume of 1 N HCl was added and the absorbance at 450 nm was recorded by an ELISA reader (MultiscanEX, Labsystems, Finland).

Production of soluble scFv in *E. coli*. HB2151 cells

Soluble, anti-CMV scFv, three positive, recombinant phage clones, which were all very different in their ELISA selection, were used to infect log-phase *E. coli* HB2151 (Amersham Pharmacia Biotech, Sweden). Expression of soluble scFv was induced by adding IPTG to a final concentration of 1 mmol l^{-1} and the cultures were grown overnight at 30°C. The induced culture was centrifuged at 1,500 rpm for 20 min. Cell pellets were resuspended in 2% of culture volume of ice-cold 1×TES. Subsequently, 3% of culture volume of ice-cold 0.2×TES was added and the mixture was incubated on ice for 30 min to induce a mild osmotic shock. The contents were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant, containing the soluble antibodies from the periplasm, was transferred to clean tubes and stored at -20°C.

Detection of antigen-binding affinity of the soluble CMV scFv

Each of the soluble scFv clones was characterized for binding to CMV particles by using indirect ELISA. Briefly, 96-well microtitre plates were coated with 5 μg ml^{-1} (50 μl) of purified CMV in a carbonate coating buffer and incubated at 37°C for 1.30 h. Subsequently, the plate was washed three times with 200 μl of PBST. Then 150 μl of 2% skim milk was added to block non-specific binding and 50 μl of each scFv periplasmic preparation was added to the plate and incubated for 1.30 h at 37°C. The plate was washed three times with PBST. After 50 μl of a 8 μg ml^{-1} concentration of mouse anti-E tag was added into each well, they were incubated for 1.30 h at 37°C. Each plate was washed three times with PBST and 50 μl of 1: 5,000 dilution of rabbit anti-mouse conjugated with alkaline phosphatase was added. The plate was incubated for 1.30 h at 37°C. The plate was then washed with PBST three times, after which the *p*-nitrophenyl phosphate substrate was added and incubated at 37°C for 30-60 min. The reaction was read at an absorbance of 405 nm by an ELISA reader.

Automated DNA sequencing

Three CMV-specific clones, from infected *E. coli* HB2151, were analyzed by sequencing. Two sequencing primers were used: pCANTAB5-S1 (5′-CAA CGT GAA AAA ATT ATT ATT CGC-3′) as the forward primer; and pCANTAB5-S6 (5′-GTA ATT GAA TTT TCT GTA TGA GG-3′) as the reverse primer. Nucleic acid sequencing was carried out on the ABI PRISM 377 DNA sequencer (AME Bioscience, Bedfordshire, UK) by the method of dideoxynucleotide sequencing.

RESULTS AND DISCUSSION

Construction of anti-CMV scFv gene

To construct an anti-CMV scFv encoding gene, the V_H and V_κ genes were amplified from the mRNA of a hybridoma cell, CM2, which secreted anti-CMV MAb specific to both subgroup I and II, by RT-PCR method. As shown in Figure 1, the DNA bands of V_H and V_κ, at approximately 400 bp, were clearly observed after gel electrophoresis. Five-hundred picograms each of V_H and V_κ were then joined to form the scFv gene by a PCR overlapping extension method without primer. The scFv encoding gene, 800 bp, was generated successfully as shown in Figure 1. The resulting DNA fragment encoding the scFv was purified, digested with *Sfi*I and *Not*I, and ligated into the phagemid vector pCANTAB5E.

Recombinant phage expressing CMV-specific scFv on the surface

Recombinant DNA was electroporated into *E. coli* TG1, which produced an amber suppressor tRNA to allow read through (suppression) of the amber stop codon located between the cloned scFv and gene III sequences of pCANTAB5E. The bacterial transformants were co-infected with helper phage to rescue phage particles displaying scFv on their tips. Subsequently, phages displaying scFv were panned against purified CMV coated on an immunotube. After three rounds of selective panning, 176 clones of recombinant phages were randomly picked and analyzed by plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA). Of the analyzed clones, 154 showed positive interactions, which gave absorbances (A_{450nm}) between 0.4-0.9 with a background absorbance of 0.1. These results showed the efficiency of panning, which eliminated clones with a non-specific interaction.

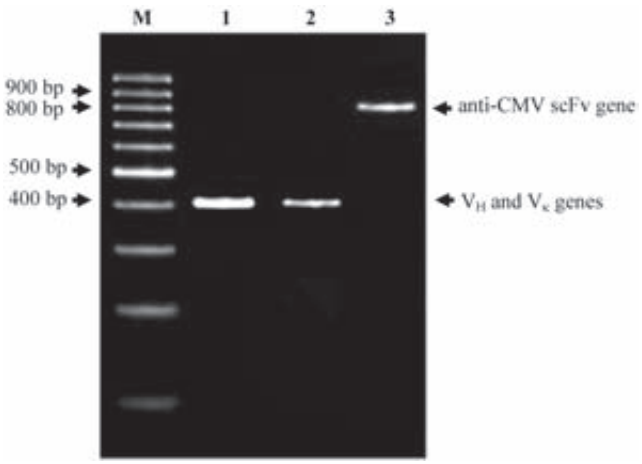

Figure 1 RT-PCR amplification. M=GeneRuler™ 100 bp DNA ladders plus, lane 1-3=V_H, V_κ and anti-CMV scFv genes, respectively.

However, the difference in absorbance among the tested clones, which were supposed to be identical, was due to the variation in cell growth and the production of recombinant phages (Anamaria *et a*l., 2000).

Characterization of CMV-specific scFv antibody

To produce soluble, anti-CMV scFv, three positive, recombinant, phage clones 2C1, 6A1, and 1D4, which gave a high signal to noise ratio in ELISA, were chosen to infect *E. coli,* HB2151. After induction for 8 h (Chua *et al*, 2003), each anti-CMV scFv was extracted from the bacterial periplasm by osmotic shock (Ming-Yan *et al*., 2004). The molecular weight of each anti-CMV scFv was approximately 26.3 kDa as determined by 12% SDS-PAGE (Laemmli, 1970) (Figure 2). Periplasmic extracts of all three clones were tested by PTA-ELISA and the results showed strong positive signals, while they did not interact with the healthy plant sap. In addition, the ability of the soluble scFv from the periplasmic extraction to detect CP-CMV using purified CMV, healthy and diseased plant sap was also investigated by Western blotting (Towbin *et al*., 1979). The results not only demonstrated strong interactions with both the monomer and dimer forms of coat protein (Figure 3) but also clearly showed that the periplasmic expression yielded functional scFv (Padiolleau-Lefevre *et al*., 2007). Therefore, this study successfully obtained soluble anti-CMV scFv from the periplasmic extract, which was amenable for large scale production and purification (Padiolleau-Lefevre *et al*., 2007).

DNA sequencing and molecular modeling of anti-CMV scFv antibody

Three CMV-specific clones (2C1, 6A1 and 1D4) from *E. coli* HB2151 were chosen for DNA sequence analysis. After the sequences were aligned by ClustalW Multiple Sequence Alignment Programs, Version 1.83 (European Bioinformatics Institute,EBI), the result not only demonstrated that all three clones were identical (data not shown), but also clearly showed no evidence of error from synthesis by *Taq* polymerase during PCR. When the V_H and V_κ of the anti-CMV gene

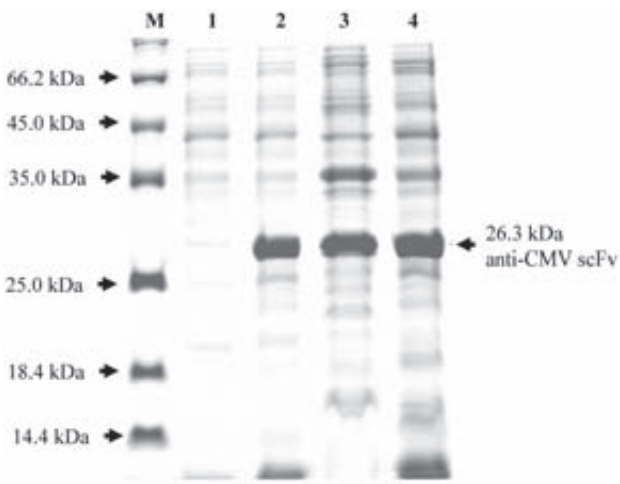

Figure 2 Coomassie brilliant blue-stained of 12% SDS-PAGE showing the anti-CMV scFv were extracted from *E.coli* HB2151 by osmotic shocked extraction. M=prestained protein marker ladders; lane 1=total proteins of the wild type *E.coli* HB2151; lanes 2-4=anti-CMV scFv from clones 2C1, 6A1 and 1D4, respectively. The arrow indicates the 26.3 kDa scFv band.

were compared with the murine Ig germline using IgBlast program, the result showed that the amplified V_H belonged to the J558.32 subgroup and JH2, while V_κ belonged to the immunoglobulin V_κ genes and JK2. In addition, the distribution of complementarity determining regions (CDRs) of V_H and V_κ were indicated as shown in Figure 4.

For 3D structure modeling, the nucleotide sequence of 6A1 was translated to an amino acid sequence using the Translate Program (Expert Protein Analysis System, Expasy).

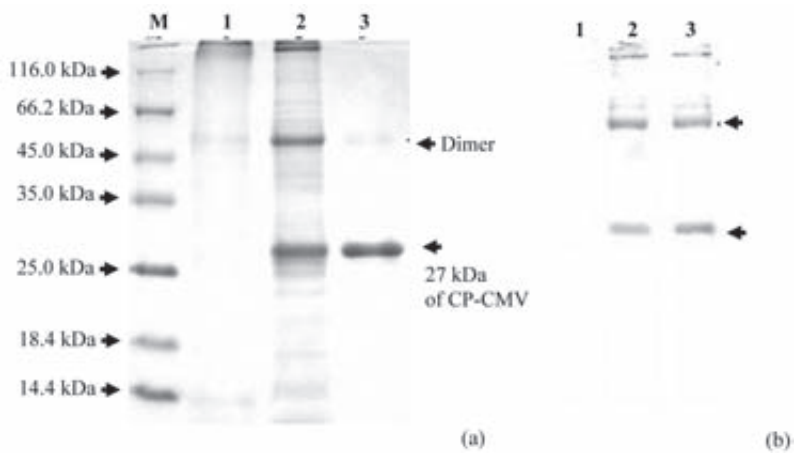

Figure 3 Analysis of molecular weight of CMV-coat protein by (a) 12% SDS-PAGE and (b) determination of binding specificity of scFv molecule by Western blotting. M=prestained protein marker ladders, lane 1=healthy plant, lane 2=diseased plant and lane 3= purified CMV. Arrows indicate the scFv band at 27 kDa (monomer form) and 54 kDa (dimer form) of CMV major coat protein.

V_H amino acid sequence

FR1 CDR1 FR2 CDR2
QVKLQQSGTEVVKPGASVKLSCKASCYIFT**SYDID**WVRQTPEQGLEWIG**WIFPGEGST**EY 60

FR3 CDR3 FR4
NEKFKGRATLSVDKSSSTAY MELTRLTSESAVYFCAR**GDYYRRYF**DLWGQGTTVTVSS 119

V_κ amino acid sequence

FR1 CDR1 FR2 CDR2
DIELTQSPAIMSASPGRVTMTC**SASSSIRYIY**WYQQKPGSSPRLLIY**DTSNVAP**GVPFRF 60

FR3 CDR3 FR4
SGSFGWGPSYFLTINRMEAEDAATYYC**QEWSGYPYT**FGGGTKLELKR 107

Figure 4 Deduced amino acid sequences of the anti-CMV scFv gene (GenBank Acc. No. EU589245). A total of six CDRs are identified using the IgBlast Program and are shown in boldface and underlined.

Suitable templates for homology modeling were searched for in the PDB database using the MATRAS program (Kawabata, 2003). The 6A1 showed 82 % similarity to anti-carcinoembryonic scFv in the the PDB, accession number 1qok (Boehm *et al*., 2000). The 3D structure model of the anti-CMV scFv could be visualized with the Jmol viewer as shown in Figure 5.

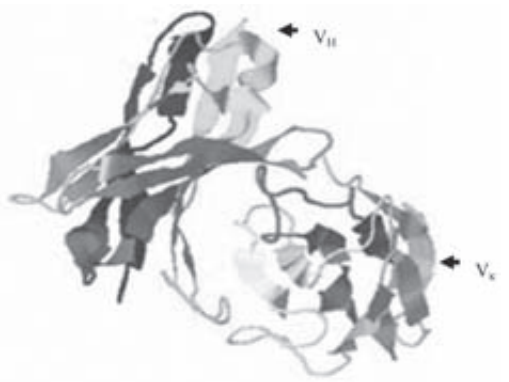

Figure 5 Three-dimensional structure of the anti-CMV scFv; V_H is left and V_k is right.

ACKNOWLEDGEMENTS

The authors thank the Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom and the Graduate School, Kasetsart University for their financial support.

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