Plant Viruses ©2007 Global Science Books

Cloning, Sequencing and Expression of Immunoglobulin Variable Regions of Murine Monoclonal Antibodies Specific to *Cucumber mosaic virus* coat protein

Haggag Salah Zein[1,2*] • Kazutaka Miyatake[1]

[1] Department of Applied Biological Chemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, 1-1, Gakuen-Cho, Sakai, Osaka, 599-8531, Japan

[2] Present address: Department of Genetics, College of Agriculture, Cairo University, 12613, Egypt, 12 Gamaa Street, Giza, 12613, Egypt

Corresponding author: * **haggagsalah@gmail.com**, **hagzein8@yahoo.com**

ABSTRACT

A mouse monoclonal antibody (mAb) prepared by hybridoma technology could recognize the *Cucumber mosaic virus* (CMV) coat protein. The F_{ab} fragments (antigen-binding site, one complete light chain, and part of one heavy chain) genes encoding the light chain and the Fd (a monovalent antigen-binding fragment consisting of a complete light chain paired with the variable region and the first constant domain of the heavy chain) region of the heavy chain of the mAb were cloned and expressed in *Escherichia coli*. The F_{ab} fragment was produced by subjecting the heavy and light chain antibody genes of the pepo-4 cell line to reverse transcription-polymerase chain reaction, then subcloning the products in the pGEM-T easy vector. Sequence analyses of the F_{ab} fragment revealed that the light and heavy chains belong to the Vκ2 and V_H1/V_HJ558 germline gene family with GenBank accession numbers EF672211 and EF672197, respectively. The pARA7 expression vector was designed for the expression of F_{ab} in the periplasmic space. Recombinant F_{ab} fragments were purified and analyzed by indirect ELISA. These results suggest that the recombinant F_{ab}-4 antibody produced by *E. coli* acts as a source for the generation of F_{ab} with very stable and specific expression.

Keywords: CMV, light and heavy chain gene, monoclonal antibodies, recombinant F_{ab}

INTRODUCTION

Antibodies (both polyclonal and monoclonal) have been used extensively in tests to detect a wide range of plant pathogens, including plant viruses (Huse *et al*. 1989). Monoclonal antibodies (mAbs) are superior to polyclonal antibodies because they can provide a constant supply of specific diagnostic reagents. However, mAbs are expensive to produce and to maintain, because specialized cell cultures and costly low-temperature storage facilities are required. Moreover, during storage, some hybridoma cell lines can die and others can lose their capacity to secrete specific antibodies. Recombinant antibodies constructed from immunoglobulin genes obtained from immunized or nonimmunized donors (naïve libraries) have provided a source of antibody fragments (single-chain variable fragments [scFv]) that have been shown to have high affinities for antigens and to have binding properties equivalent to those of antibodies produced by immunized animals (Pack and Plückthun 1992). Recombinant antibodies can be produced from libraries of such antibody fragments quickly (2 to 4 weeks) by methods that do not require the use of animals. DNA from clones selected from libraries can be stored indefinitely and is readily propagated. Thus, it can provide an unlimited source of reagent. Monitoring plants for virus infection is essential to detect and eliminate viruses from germplasm collections or propagation material. Such tests are done routinely by enzyme-linked immunosorbent assays (ELISA) that use either polyclonal or monoclonal antibodies (Zein *et al*. 2007). In recent years, the use of genetic engineering techniques has stimulated the development of antibody-like molecules for therapeutic and diagnostic uses (Winter *et al*. 1994). scFv proteins are expressed in fusion with bacteriophage coat proteins and maintain the original antibody binding properties (Marks *et al*. 1991; Hoogenboom *et al*. 1998). Unlike glycosylated whole antibodies, fragments such as F_{ab} and scFv can be easily produced in bacterial cells as functional antigen binding molecules; the efficient expression of active antibody fragments in bacteria is clearly of great technological importance (Better *et al*. 1988; Skerra and Plückthun 1988). The scFv genes can be easily manipulated and applied for further genetic modification for rapid detection of virus particles in plant saps, or even for the generation of transgenic plants resistant to a certain pathogenic virus (Tavladoraki *et al*. 1993). However, isolations of several recombinant human scFv antibodies that are specific for CMV (Ziegler *et al*. 1995; Zein *et al*. 2007b), *Tomato spotted wilt virus* (Griep *et al*. 1999), *Beet necrotic yellow vein virus* (Fecker *et al*. 1996), and *Potato leafroll virus* (Legorburu *et al*. 1998) have been reported. The bacterial expression of F_{ab} fragments ensures a reliable supply of such a useful antibody. This technique may be applicable in the cloning and expression of genes from hybridomas showing low levels of antibody production. Since fetal bovine serum, a CO_2 incubator, and mice are not required in this expression system, it is also economically beneficial. Another advantage of this system seems to be a possible modification of the original antibody gene, thereby increasing the affinity of the antibody (Barbas *et al*. 1993, 1994). However, it is known that some plant viruses, including CMV, are poor immunogens for the preparation of antisera by conventional means (Palukaitis *et al*. 1992). Virus-specific recombinant antibody can be either obtained from cloned antibody genes derived from selected hybridomas, or selected from libraries containing up to 10^8 different antibody genes (Griffiths *et al*. 1993). Once selected, antibody genes could be maintained stably in and expressed from bacterial plasmids, allowing the production of large quantities of

Received: 11 November, 2007. Accepted: 8 December, 2007.

Original Research Paper

synthetic antibodies. Thus this technology has the potential for developing a fully recombinant ELISA kit for plant virus diagnosis (Griep *et al*. 1999).

In this study, we attempted to prepare anti-CMV monoclonal antibodies (mAbs) with high affinity and to develop a highly sensitive ELISA based on mAbs. Furthermore, isolation of cDNA clones encoding anti-CMV mAbs and expression of these cDNA clones in *Escherichia coli* were also attempted to produce recombinant antibodies. The molecular characteristics of these mAbs and corresponding recombinant antibodies were assessed by ELISA.

MATERIALS AND METHODS

Virus purification and antibody production

Japanese strains and isolates of CMV were propagated in tobacco plants (*Nicotiana tabacum* cv. 'Xanthi') in a greenhouse. Virus purifications were performed in a long linear sucrose gradient as described previously (Osaki *et al*. 1973). Production of mAbs, Eight-weeks-old BALB/c female mice (Nippon SLC Co., Japan) was immunized with 100 μg of CMV coat protein as an immunogen. Their splenoccytes were fused with P3-X63-Ag8.653 myeloma cells and screened for their binding ability towards CMV in ELISA as previously described Zein *et al*. (2006). The isotype of the mAbs was identified with anti-mouse subclass specific antiserum (Bio-Rad, USA) according to the manufacturer's instructions. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

cDNA preparation and cloning of PCR amplification

Standard methods of molecular cloning procedures were performed according to Sambrook *et al*. (1989). The mRNA was purified from approximately 10^6 hybridoma cells using an mRNA purification kit (Amersham Pharmacia, Uppsala). First-strand cDNA was synthesized from a mRNA template with the M-MLV kit (Takara, Kyoto, Japan) using oligo-dT_{20}-TATGCAAGGCTTA CAACCACA specific to heavy chain and oligo-dT_{20}-CTCATTC CTGTTGAAGC-TCTTGAC specific to light chain kappa (Pharmacia Biotech). The polymerase chain reaction for the V-regions of the mAbs were amplified by KOD DNA polymerase (Toyobo, Japan) using the two primer pairs. The primer sequences used for V_H and V_L domain amplifications are defined in **Table 1**. The constituents and proportions of the reaction {30 cycles of PCR (1 cycle is 30 min at 94°C, 30 min at 55°C, and 1 min at 72°C)}. The PCR products were analyzed on 2% agarose gel. V_H and $V_{L\ (\kappa)}$ DNA fragments were purified from bands using a QIAGEN gel extraction kit. The heavy and light chain fragment was first subcloned into pGEM-T Easy vector according to the manufacturer's instructions.

Table 1 Primers used to amplify the mouse heavy and light chain.

Primer №	The nucleotide sequences.
H1[a]	5′-AGGTCCAGCTG**CTCGAG**TCTGG-3′
H2	5′-AGGTCCAGCTG**CTCGAG**TCAGG-3′
H3	5′-AGGTCCAGCTT**CTCGAG**TCTGG-3′
H4	5′-AGGTCCAGCTT**CTCGAG**TCAGG-3′
H5	5′-AGGTCCAACTG**CTCGAG**TCTGG-3′
H6	5′-AGGTCCAACTG**CTCGAG**TCAGG-3′
H7	5′-AGGTCCAACTT**CTCGAG**TCTGG-3′
H8	5′-AGGTCCAACTT**CTCGAG**TCAGG-3′
H9	5′-AGGCTT**ACTAGT**ACAATCCCTGGGCACAAT-3′
L1[b]	5′-CCAGTTCC**GAGCTC**GTTGTGACTCAGGAATCT-3′
L2	5′-CCAGTTCC**GAGCTC**GTGTTGACGCAGCCGCCC-3′
L3	5′-CCAGTTCC**GAGCTC**GTGCTCACCCAGTCTCCA-3′
L4	5′-CCAGTTCC**GAGCTC**CAGATGACCCAGTCTCCA-3′
L5	5′-CCAGATGT**GAGCTC**GTGATGACCCAGACTCCA-3′
L6	5′-CCAGATGT**GAGCTC**GTCATGACCCAGTCTCCA-3′
L7	5′-CCAGTTCC**GAGCTC**GTGATGACACAGTCTCCA-3′
L8	5′-GCGCCG**TCTAGA**ATTAACACTCATTCCTGTTGAA -3′

[a] The underlined portion of the 5′ primers ofγ heavy chain incorporates an *Xho*I site (H1-H8) and the 3′ primer on *Spe*I restriction site (H9).
[b] The underlined portion of the 5′ primers of κ light chain incorporate a *Sac*I restriction site (L1-L7) and that of the 3′ primers an *Xba*I restriction site (L8).

Quantitation of nucleic acid

The amount of RNA in the sample was estimated at OD_{260} nm. An OD_{260} of 1 is ~50 μg/ml of double strand DNA, ~40 μg/ml of single strand DNA and RNA and ~20 μg/ml of single strand oligonucleotide. The purity of the nucleic acid was ascertained by the OD_{260}/OD_{280} ratio, with purity defined as OD_{260}/OD_{280} = 1.8 for DNA and 2.0 for RNA. The quantity of DNA in the sample was estimated by comparing the fluorescent intensity of the sample DNA with the intensity and concentration of λ-DNA (Invitrogen, Japan) bands.

DNA sequence of variable region of the heavy and light chain genes

Direct sequencing of the treated DNA fragment was made using M13 primer an ABI PRISM BigDye Primer Cycle Sequencing Kit reagent following the manufacturer's instructions (Applied Biosystems) and run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using ABI Prism Sequencing Analysis 3.7 software for data analysis. The PCR product was analyzed and sequenced using M13 primer sequencing of V regions. Cyclic sequencing of these DNAs was performed in both directions using a commercial kit (Thermo Sequence kit, Amersham Pharmacia Biotech), M13 forward (5′-CACGACGTTGTAAAACGAC-3′) and reverse (5′-GGATAACAATTTCACACAGG-3′) primers set (Pharmacia Biotech). Fd or Lc sequences of the variable region was compared to the closest germline sequence using the International database, which "blasted" against the publicly accessible "Ig-Blast" database of mouse Ig sequences at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/ig-blast) to determine the closest germline gene of origin, and to identify potential mutations.

Base differences between the immunoglobulin genes sequenced and the corresponding germline genes were scored as mutations. The CDR position and numbering scheme adopted matched according to The Kabat numbering (Kabat *et al*. 1991) and CDR definition were adopted according to Martin (1996) from Andrew's web site (www.bioinf.org.uk/abs/).

Production of soluble F_{ab}

Approximately 300 ng of the purified of heavy and light gene fragments were ligated with 500 ng of pGEM-T Easy (Promega) using ligation high T4 DNA-ligase (Promega) following the manufacturer's protocol. However, a vector-insert ratio of 1:5 was used and ligation was carried out for 4 hr at 16°C, a half volume of ligation mixtures were transformed into competent bacterial (*E. coli* strain JM109 or DH5α) cells the transformed cells were incubated shaking at 37°C for 30~60 min, and cells were centrifuged at 800 × *g* for 2 min at 4°C. The supernatant was then discarded and resuspended in 100 μl of SOB media (20 g bactotryptone, 5 g yeast extract, 2.5 ml of (1 M KCl), 2 ml of (5 M NaCl), 10 ml of (1 M $MgCl_2$), and 10 ml of (1 M $MgSO_4$) in 1000 ml H_2O). Selection for transformants was on LB/ampicillin/IPTG/X-Gal plates. Individual white colonies were transferred into 3 ml of LB medium containing 100 μg/ml ampicillin, with occasional shaking 100~200 rpm/min overnight. The gene encoding the light chain and heavy chain of the selected pGEM-T Easy vector was subcloned into the *Sac*I-*Xba*I and *Spe*I-*Xho*I sites of the expression plasmid pARA7, respectively. The soluble F_{ab} fragment of the selected clone was expressed in *E. coli* MC1061 cultures with L-arabinose induction.

Recombinant protein expression

The *E. coli* strain MC1061 (*F⁻ araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (Str^r) hsdR2 ($r_k^-m_k^+$) mcrA mcrB1*) was selected on LB agar plate containing 100 μg/ml of ampicillin. The transformants were inoculated into a 5 ml test tube culture and allowed to grow at 37°C in a shaker at 220 rpm. Cultures in a loga-

rithmic phase (at OD_{600} of ~0.5-0.6) were induced for 3 h with 0.2% L-arabinose at 28°C. After induction, cells were lysed in 5 × sample buffer (0.313 M Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue, with 100 mM DTT) and analyzed by 12% SDS–PAGE (Laemmli 1970). Uninduced control culture was analyzed in parallel. For initial experiments designed to determine the solubility of the recombinant protein, log phase culture was induced with 0.2% L-arabinose at 28°C. The cells were harvested by centrifugation at 6500 rpm for 10 min. The cell pellet was resuspended in 1 ml of buffer A (50 mM Tris–HCl and 200 mM NaCl, pH 7.5). The cell suspension was sonicated on ice for 10 cycles at 20 sec/cycle. The resulting cell lysate was centrifuged at 14,000 rpm for 30 min. The clear supernatant (soluble fraction) was collected and the remaining pellet (insoluble fraction), after centrifugation which contains inclusion bodies, was also resuspended in 1 ml of buffer A. Soluble and insoluble fractions were then analyzed in parallel on 12% SDS–PAGE. To increase the solubility of the protein, L-arabinose at different concentrations (0.2, 0.02, 0.002, 0.0002, and 0.00002%), and different temperatures (23, 25, and 28°C) were tested.

Periplasmic preparation

Cells pellets were resuspended in minimal volume (~5 ml) of cold Tris sucrose buffer (30 mM Tris-HCl, 20% Sucrose, pH 8.0 and 1 mM EDTA). The suspension was incubated on ice for 15 min and centrifuged at 8000 × *g* for 20 min at 4°C. The cell pellet was resuspended in a similar volume of cold 5 mM $MgSO_4$ and 1 mM EDTA. The suspension was again incubated on ice for 15 min and centrifuged as before. The supernatants from the two fractions were mixed for use in ELISA or dialyzed against PBS for purification.

ELISA

The ELISA plate wells were coated with CMV coat protein diluted to 1 µg/ml in PBS overnight at 4°C. Periplasmic extraction, diluted in PBST, was applied to the wells. Detection of the bound light and heavy chains was carried out by using anti-mouse $(F_{ab})_2$ alkaline phosphatase (AP) conjugated antibody. The substrate used for the AP label was 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in substrate buffer. The absorbance was measured at 450 nm using an ELISA reader (Bio-Rad, USA).

Analysis of r F_{ab} proteins with SDS-PAGE and Western blotting

The periplasmic osmotic shock fractions of *E. coli* cells were obtained by a method described previously by Dübel *et al*. (1992) The cells were pelleted at 6,200 × *g* for 10 min at 4°C, the pellet was resuspended in 1/10 volume of the original culture in a buffer containing 50 mM Tris-HCl (pH 8.0), 20% (wt/vol) sucrose, and 1 mM EDTA and left for 30 min on ice with occasional shaking. After centrifugation, the supernatant representing the enriched periplasmic fraction was stored at 4°C. The bacterial pellet was resuspended by vortexing in 1/10 of the original culture volume in a buffer containing 5 mM $MgSO_4$ and incubated for 30 min on ice with occasional shaking. After centrifugation at 6,200 × *g* for 10 min at 4°C, the supernatant representing the osmotic shock fraction was stored at 4°C. The samples were then mixed with gel-loading buffer (0.06 M Tris-HCl, pH 6.8, containing 20 g/l of SDS, 5 ml/liter of β-mercaptoethanol, 1 ml/l of glycerol, and 0.2 g/l of bromophenol blue) for SDS-PAGE and immunoblotting. The *E. coli* periplasmic and osmotic shock fractions were analyzed by 12% SDS-PAGE. After separation, the protein bands were transferred to a nitrocellulose membrane (Amersham, Biotech) with a horizontal electrophoretic transfer system. The transblotted membrane was probed with goat anti-$(F_{ab})_2$ mouse (HRP) conjugated antibody, washed, and as specified by the manufacturer. SuperSignal West Pico Chemiluminscent Substrate (PIERCE).

Purification of rF_{ab}

A single colony was inoculated to 3 ml of Circlegrow media (Q-BIO gene) with 100 µg/ml ampicillin according to Fujii (2004) incubated with shaking at 37°C until OD_{600} = 0.6 (~2 h). The culture was transferred to 200 ml of Circlegrow media with 100 µg/ml ampicillin and incubated with shaking at 37°C until OD_{600} = 0.2 (~ 1 h). Two ml of 0.2% L-arabinose was added to sterile distilled water to the culture and incubated with shaking at 100-200 rpm at 28°C for 3 days. The 200 ml of culture was centrifuged at 8,500 × *g* for 30 min. The supernatant was filtered through a 0.22 µm filter (Millipore, USA). Ammonium sulfate (96.4 g) was added to culture supernatants and stirred for 1 h on ice, then centrifuged at 30,000 × *g* for 30 min, after which the supernatant was discarded. The precipitate was resuspended in 20 ml of dialysis buffer and dialyzed (MW cut-off 12,000-14,000 Da) at 4°C overnight. The F_{ab} protein was applied to a Q sepharose ff® (Amersham Biosciences) ion-exchange column and eluted with ion-exchange elution buffer. The F_{ab} fragment was further purified by applying the eluent to an affinity column (Amersham Biosciences) which is prepared by cross linking anti-mouse $F(_{ab}')_2$ antibodies to a Hi-Trap (NHS-activated HP media, as per manufacturer's instructions (Amersham Biosciences). F_{ab} was then eluted with 0.1 M glycine-HCl buffer, pH 2.6 and the eluent ($F_{ab)}$ was neutralized with 1.0 M Tris-HCl buffer, pH 8.0.

Statistical treatments

Results presented in this study are qualitative and were thus not subjected to statistical analysis. The over expression obtained for clone F_{ab}-4 was, however expressed as means ± SEM.

RESULTS AND DISCUSSION

Monoclonal antibody production

Nine stable hybridoma cell lines secreting MAbs specific to CMV coat protein were obtained from five fusion experiments, and the immunoglobulin classes and subclasses for each were determined in our previously reports (Zein *et al*. 2006, 2007a, 2007b).

PCR Amplification of F_{ab} genes from hybridoma cells

The complementary DNA (cDNA) synthesized from the mRNA of hybridoma cells was successfully amplified by two sets of primers (**Table 1**), for the Fd region of the heavy chain and the other for the light chain. PCR amplification of the V_H and Vκ genes generated a major DNA fragment which was analyzed by electrophoresis with an expected length of about 650 bp (**Fig. 1A**). The PCR product of the Fd and Vκ genes were subcloned ligated into a pGEM-T Easy vector (**Fig. 1C**) for DNA sequences, subsequently isolated with specific restriction enzymes *Sac*I and *Xba*I for the light chain and *Spe*I and *Xho*I for the heavy chain (**Fig. 1B**). It is clearly revealed by Huse *et al*. (1989) that combinatorial libraries created by RT-PCR of pooled lymphocytes or tissues provide a wealth of immunoglobulin sequences, but cannot be relied upon to provide correct *in vivo* pairings of heavy (V_H) and light (V_L) chain V regions. Even on panning in phage display or other format against an identified antigen source, which is both laborious and assumes the availability of an antigen (Kang *et al*. 1991). A single cell-based methodology (hybridoma) is necessary to yield correctly paired $V_H + V_L$ F_{ab}.

DNA sequence of variable region of the heavy and light chain genes

Cyclic sequencing of these DNAs was performed in both directions using a commercial Thermo Sequence kit (Amersham Pharmacia Biotech), and M13 forward and reverse primers set (Pharmacia, Biotech). Fd or Lc sequences of the variable region was compared to the closest germline sequence using the International database, which "blasted" against the publicly accessible "Ig-Blast" database of mouse Ig sequences at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/ig-blast) to

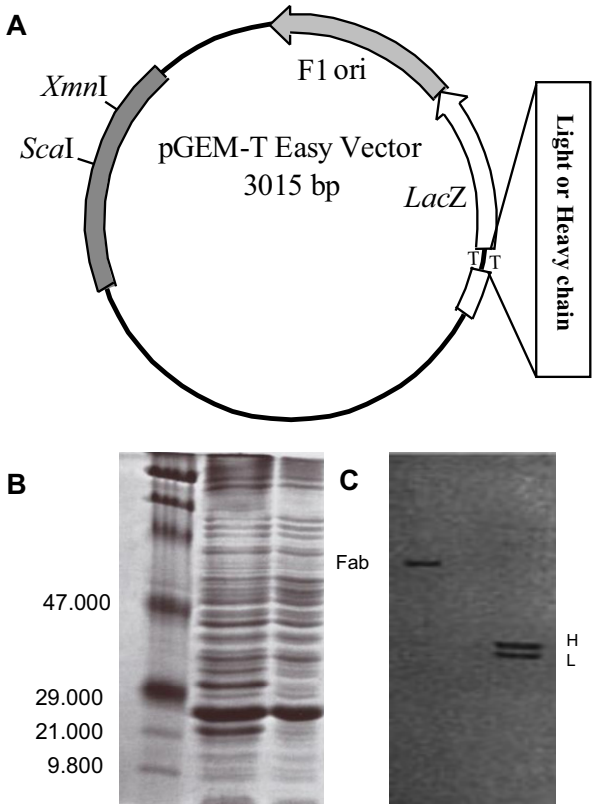

Fig. 1 Construction, expression and purification of F_{ab} fragments specific to CMV coat protein. (**A**) The construction of pARA7 with heavy chain and light chain fragment under *ara*C promoter. (**B**) SDS–PAGE analysis under reducing condition of recombinant F_{ab} fragments lane-1 induced and lane-2 non-induced condition. Western blotting analysis of the periplasmic extraction of induced and non-induced condition lanes 3 and 4 respectively. (**C**) Purification of F_{ab} from periplasm of *E. coli* cells shows homogeneously purified recombinant F_{ab} fragments were expressed. Samples were electrophoresed under either reducing or nonreducing conditions and the gel was then stained with Coomassie blue.

determine the closest germline gene of origin, and to identify potential mutations.

Base differences between the immunoglobulin genes sequenced and the corresponding germline genes were scored as mutations. The V_H and Vκ regions of mAb-4 CMV coat protein specifically generated from fusions of BALB/c mice were sequenced and when these sequences were analyzed by nucleotide databases, a high degree of homology with known murine κ and γ1 genes was demonstrated. The identity of the V genes used was determined by searching the GenBank database for homologies to known V genes using the BLAST protocol Altschul *et al*. (1997). The nucleotide and deduced amino acid sequences of the expressed light chain germline gene segment assignments confidently belonging to germline family Vκ2, gene *bd2*, and GenBank accession number EF672211 while V_H genes belonged to V_H1/V_HJ558 GenBank accession number EF672197. The deduced amino acid sequences of the genes coding the variable region of the light and heavy chains are shown in **Fig. 2**. Previous studies have shown scFv to bind to plant viruses, viz. *Black currant reversion associated virus* (Susi *et al*. 1998), *Cucumber mosaic cucumovirus* (Ziegler *et al*. 1995), and *Potato leafroll virus* (PLRV) (Harper *et al*. 1997) can be obtained from a semisynthetic phage display library. However, results of tests using these scFvs were not as good as those using antibodies produced by immunization and hybridoma cell lines.

Antibody expression system

Large-scale production of recombinant antibodies is always a requirement after the therapeutically significance of the antibodies has been defined. Since the earliest reports on the construction of recombinant antibody fragments, bacterial cells have been routinely used for expression of scFv, F_{ab} or diabody fragments (Pluckthun 1991). Bacterial expression systems have the advantages of speed and abundant production. The $F(_{ab}')2$ fragments expressed to high levels in the periplasmic space of *E. coli*, were indistinguishable from $F(_{ab}')2$ derived from limited proteolysis of intact antibody (Carter *et al*. 1992). Often the rAbs expressed in bacteria accumulate as insoluble cytoplasmic inclusion bodies and even secreted antibodies form aggregates and thus require solubilization or refolding *in vitro* (Sanchez *et al*. 1999; Hashimoto *et al*. 2000). But the expression of full size multimeric glycosylated antibodies in bacterial systems is not feasible.

Bacterial expression for purification of recombinant fragments

For bacterial expression and purification, F_{ab} constructs were subcloned into a prokaryotic expression vector and transformed into *E. coli* MC1061 strains. Single colonies from freshly transformed cultures were used to initiate overnight cultures, and subcultured the following day into 2X TY medium with 100 µg/ml ampicillin and 0.1% glucose. The cultures were grown at 30°C until an OD_{600} of 0.7-0.9 was achieved and then induced with the final concentrations of L-arabinose varying from 0.2%, 0.02%, 0.002%, 0.0002% and 0.00002%. For expression of rAb fragments, the induced culture was grown at 23°C, 25°C, and 28°C overnight or for 3 h (**Fig. 3**). The overnight-induced culture supernatant was used in ELISA or the cells harvested from the 3 h-induced culture for periplasmic extraction. The cell pellet was either directly processed or frozen at -20°C until use. The L-arabinose-inducible gene expression system could be regulated, and had a consistent control in all culture cells which would vary depending on the level of inducer and temperature of incubation. Engineered plasmid vectors carrying the *ara* operon have been used successfully in *E. coli* (Fujii 2004). This self-regulating system provides fine control of expression, tight repression in the absence of an inducer, and induction over a 1,000-fold range in the presence of an inducer. The optimization of protein expression in *E. coli* was performed to obtain a maximum level of protein induction by L-arabinose. Two factors varied, namely the concentration of L-arabinose and duration of L-induction. The ELISA results showed the difference in level of protein expression among these conditions was highest in 0.002% L-arabinose and 28°C and lowest in 0.00002% L-arabinose and 25°C (**Fig. 3**). The soluble periplasmic extraction or culture supernatant of induction *E. coli* was used directly for detection of the CMV, antigen coated plate ELISA, and significantly the recombinant F_{ab} specifically detected CMV. No reaction was obtained with the extracts prepared from non-induced periplasmic *E. coli*.

The signal sequences of the Fd and L chains were correctly processed, and the fragments were secreted into the periplasmic space and released into the culture medium upon prolonged cultivation. However the degree of successful folding in the periplasm appears to depend to a large extent on the primary sequence of the individual variable domains. Nevertheless, modifying the conditions of bacterial growth can increase the proportion of correctly folded soluble antibody fragments. For example, lower temperatures and the addition of nonmetabolized additives that induce osmotic stress, such as sucrose or sorbitol and glycine betaine, can increase yields (Little *et al*. 2000). The PelB secretion signal directs the synthesized foreign protein through the periplasmic extraction membrane. A mouse F_{ab} fragment was synthesized efficiently by pARA7 vector (**Fig. 4A**) and accumulated with the cell membranes (not as inclusion bodies), so it would seem that the periplasm is an ideal place to fold mammalian proteins because it already has the ability to form and isomerise disulfide bonds. Indeed, a number of secreted, disulfide bond containing pro-

A

```
1XGY      QVQLQQSGPELVRPGASVKISCKASGYTFTDYYINWVKQRPGQGLEWIGWIFPRNGNTKY 60
Heavy-4   QVQLQQSGAELVRPGASVKLSCRALIYTFTDYEVHWVKQTPVHGLEWIGAILPGNGNTAY 60
          ********.**********:**:*  ****** ::**** * :****** *:* **** *

1XGY      NEKFKGKATLTVDKSSSTAFMQLSSLTSEDSAVYFCATTVSYVMDYWGQGTTVTVSSAKT 120
Heavy-4   NQIFKGKATLTADKSSSTAYMELSSLTSEDSAVYYCTNPY-YRFDYWGQGTTLTVSSAKT 119
          *: ********.*******:*:************:*:..  * :********:*******

1XGY      TPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYT 180
Heavy-4   TPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYT 179
          ************************************************************

1XGY      LSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDC 219
Heavy-4   LSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDC 218
          ***************************************
```

B

```
1NLD      DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLD 60
Light-4   DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSKLD 60
          ************************************************************

1NLD      SGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPRTFGGGTKLEIKRADAAPTV 120
Light-4   SGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIKRADAAPTV 120
          ************************************:***********************

1NLD      SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSM 180
Light-4   SIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSM 180
          ************************************************************

1NLD      SSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC 219
Light-4   SSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC 219
```

Fig. 2 Alignments of the amino acid sequences of V_H and V_L regions of the mAb-4. (**A**) Alignments anti-CMV heavy chain germline, V_H Variable region is most closely related antibody (1XGY). (**B**) Alignments anti-CMV light chain germline VκII, bd2 gene with database light chains (1NLD_L).

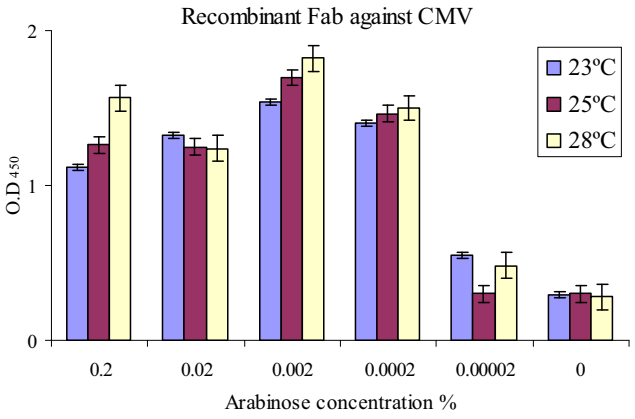

Fig. 3 The binding reactivity of the recombinant antibodies against CMV coat protein with antigen coated plates ELISA. The periplasmic extractions of MC1061 *E. coli* induced with L-arabinose a final concentrations (0.2~0.00002) at 23, 25, and 28°C for overnight incubation, ELISA plates were coated with CMV coat protein (1 µg/ml) followed with incubation with periplasmic extraction. Goat anti-mouse F_{ab} specific was conjugated with HRP with a final dilution of 1/10000. Absorbance values (A_{450nm}) were obtained after 2 h of incubation with substrate and are presented after subtraction of non-induction control values.

teins fold readily in the periplasm, including human growth hormones and a very large number of single-chain Fv antibody fragments (Power *et al*. 1992). Active F_{ab} of 50 kDa with an inter-chain disulfide bond has been isolated from the periplasm of *E. coli* in two-steps affinity purification in high yield (**Fig. 4B**). Under the *ara*C promoter and using the pelB signal sequences the production levels of the soluble heavy and light chain F_{ab} fragment combinations in periplasm and in supernatant varied F_{ab} fragment of the antibody was prepared. They have similar properties to MAbs but have the potential advantages of cheaper, large-scale production in bacteria and cheaper storage costs. Also, since the antibody genes are cloned, it is possible to manipulate them by making genetic modifications to produce fusions to reporter molecules (Kerschbaumer *et al*. 1997) or bivalent molecules (Pack and Plückthun 1992) to facilitate assay development. Enlightening the molecular structure of immunoglobulins and sequence data have made it possible to develop immunoglobulin-specific oligonucleotide primers and to use them in conjunction with polymerase chain reaction (PCR) techniques to clone antibody fragments for generating recombinant antibodies. The expression of these antigen binding proteins in bacterial cultures provides standardized diagnostic reagents that are theoretically able to replace conventional monoclonal or polyclonal antibodies and conjugates, providing significant advantages in time and cost. However, their applications for diagnostic purposes are scarce. In plant pathology, serological detection is widely used, but although some recombinant constructs have been produced (Harper *et al*. 1997; Kerschbaumer *et al*. 1997; Boonham and Baker 1998; Griep *et al*. 1999; Remko *et al*. 1998; Susi *et al*. 1998) and applied for routine ELISA, only a few of them have been applied for routine ELISA tests.

We successfully produced and characterized the Fab fragments in *E. coli* isolated from hybridoma cells, the recombinant Fab specifically bind the corresponding antigens and are suitable for a variety of applications, for example, the treatment of viral infections with so-called intrabodies, which are intracellular antibodies synthesised by the cell and targeted to inactivate specific proteins within the cell (Marasco 1995). Another application of antibody fragments is to apply the potential both in immunomodulations and in virus disease diagnoses (Zein *et al*. 2007b). The V-domains

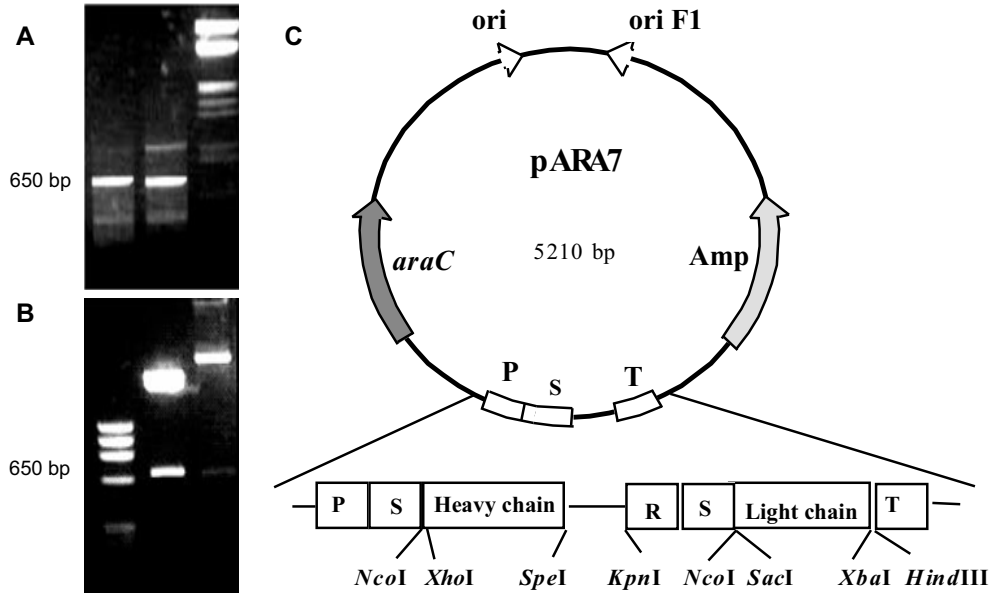

Fig. 4 Isolation and amplification of the Fd of heavy and light chain fragments from hybridoma cell lines specific to *CMV* coat protein. (**A**) Agarose gel electrophoresis of the PCR amplification of the heavy and light gene. (**B**) Subcloning of the light chain pGEM-T easy vector lane1 and pARA7 expression vector lane 2 digested with restriction enzymes *Sac*I and *Xba*I respectively. (**C**) Construction of the pGEM-T easy vector with light or heavy chain M1 = Invitrogen molecular mass markers: λ DNA digested with *Hind*III-*Eco*RI. M2; Molecular weight φX174 digested with *Hae*III.

of these antibodies and the determination of the variable gene usage are also described. The ability of r F_{ab} to detect CMV under routine assay conditions was the same as with whole mAbs. The success of the developed engineered reagents in binding and detecting CMV in extracts and dot blot assay have opened new possibilities for easy and fast production of detecting agents.

ACKNOWLEDGEMENTS

The first author is a recipient of the MONBUSHO Scholarship from the Ministry of Education, Science, Sports and Culture of Japan. The authors are grateful to Dr. Ikuo Fujii, Osaka Prefecture University Graduate School of Science Department of Biological Science, for the gift of the pARA-7 expression vector.

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402

Barbas CF III, Rosenblum JS, Lerner RA (1993) Direct selection of antibodies that coordinate metals from semisynthetic combinatorial libraries. *Proceedings of the National Academy of Sciences USA* **90**, 6385-6389

Barbas CF, Hu D, Dunlop N, Sawyer L, Cababa D, Hendry RM, Nara PL, Burton DR (1994) *In vitro* evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross reactivity. *Proceedings of the National Academy of Sciences USA* **91**, 3809-3813

Better M, Chang CP, Robinson RR, Horwitz AH (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043

Dübel S, Breitling F, Klewinghaus I, Little M (1992) Regulated secretion and purification of recombinant antibodies in *E. coli*. *Cell Biophysics* **21 (1-3)**, 69-79

Fecker LF, Kaufmann A, Commandeur U, Commandeur J, Koenig R, Burgermeister W (1996) Expression of single-chain antibody fragments (scFv) specific for beet necrotic yellow vein virus coat protein or 25 kDa protein in *Escherichia coli* and *Nicotiana benthamiana*. *Plant Molecular Biology* **32**, 979-986

Fujii I (2004) Antibody affinity maturation by random mutagenesis. In: Lo BKC (Ed) *Antibody Engineering: Methods and Protocols*. *Methods in Molecular Biology* (Vol 248), Humana Press, NY, pp 345-360

Griep RA, van Twisk C, Kerschbaumer RJ, Harper K, Torrance L, Himmler G, van der Wolf JM, Schots A (1999) pSKAP/S: An expression vector for the production of single-chain Fv alkaline phosphatase fusion proteins. *Protein Expression and Purification* **16**, 63-69

Griffiths AD, Malmqvist M, Marks JD, Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC, Hoogenboom HR, Winter G (1993) Human anti-self antibodies with high specificity from phage display libraries. *The EMBO Journal* **12**, 725-734

Harlow E, Lane D (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 726 pp

Harper K, Kerschbaumer RJ, Ziegler A, Macintosh SM, Cowan GH, Himmler G, Mayo MA, Torrance L (1997) A scFv-alkaline phosphatase fusion protein which detects potato leafroll luteovirus in plant extracts by ELISA. *Journal of Virological Methods* **63**, 237-242

Hoogenboom HR, de Brine AP, Hufton SE, Hoet RM, Arends J, Roovers RC (1998) Antibody phage display technology and its applications. *Immunotechnology* **4**, 1-20

Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**, 1273-1278

Kabat EA, Wu TT, Reid-Miller M, Perry HM, Gottesman KS, Foeller C (1991) *Sequences of Proteins of Immunological Interest* (4th Edn), United States Department of Health and Human Services, Washington, DC. Public Service, NIH. A computerized database is compiled with the DNA sequences tabulated in this issue (Kabat N database).

Kang AS, Jones TM, Burton DR (1991) Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proceedings of the National Academy of Sciences USA* **88**, 11120-11123

Kerschbaumer RJ, Hirschl S, Kaufmann A, Ibl M, Koenig R, Himmler G (1997) Single-chain Fv fusion proteins suitable as coating and detection reagents in a double antibody sandwich enzyme-linked immunosorbent assay. *Analytical Biochemistry* **249**, 219-227

Legorburu FJ, van den Bovenkamp GW, Browing I, Darling M, de Haan EG, Parper K, Pereira LG, Serra C, Toth R, Torrance L (1998) Routine detection of PLRV by recombinant antibodies: prospects for inter-laboratory standarization. *The 10th EAPR Virology Section Meeting*, p 71-72 (Abstract)

Little M, Kipriyanov SM, Le Gall F, Moldenhauer G (2000) Moldenhauer of mice and men: Hybridoma and recombinant antibodies *Immunology Today* **21**, 364-370

Marasco WA (1995) Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy. *Immunotechnology* **1**, 1-19

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *Journal of Molecular Biology* **222**, 581-597

Martin ACR (1996) Accessing the Kabat antibody sequence database by computer PROTEINS. *Structure, Function and Genetics* **25**, 130-133

Osaki T, Shobara K, Takahashi T (1973) A strain of *Cucumber mosaic virus* isolated from *Cucurbita pepo*. *Proceedings of the Kansai Plant Protection* **15**, 135-136 (in Japanese)

Pack P, Plückthun A (1992) Miniantibodies: Use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* **31**, 1579-1584

Palukaitis P, Roossinck MJ, Dietzgen R G, Francki RIB (1992) *Cucumber mosaic virus*. *Advances in Virus Research* **41**, 281-348

Power BE, Ivancic N, Harley VR, Webster RG, Kortt AA, Irving RA, Hudson PJ (1992) High-level temperature-induced synthesis of an antibody VH-domain in *Escherichia coli* using the PelB secretion signal. *Gene* **113**, 95-99

Remko AG, Marcel P, Charlotte T, Hans JK, Randolf JK, Richard K, Rob WG, Arjen S (1998) Application of phage display in selecting *Tomato spotted wilt virus*-specific single-chain antibodies (scFvs) for sensitive diagnosis in ELISA. *Phytopathology* **90**, 183-190

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, (2nd Edn), Cold Spring Harbor Laboratory Press, New York, 1659 pp

Skerra A, Pluckthun A (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038-1041

Susi P, Ziegler A, Torrance L (1998) Selection of single-chain variable fragment antibodies to black currant reversion associated virus from a synthetic phage display library. *Phytopathology* **88**, 230-233

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

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR (1994) Making antibodies by phage display technology. *Annual Review Immunology* **12**, 433-455

Zein HS, Nakazawa M, Ueda M, Ohki ST, Takashima Y, Miyatake K (2006) Detection and diagnosis of *Cucumber mosaic virus* infected plants using monoclonal antibodies by enzyme-linked immunosorbent assays. *Eco-Engineering* **18**, 15-20

Zein HS, Nakazawa M, Ueda M, Miyatake K (2007a) Development of serological procedure for rapid and reliable detection of *Cucumber mosaic virus* with Dot-immunobinding assay. *World Journal of Agricultural Sciences* **3**, 430-439

Zein HS, Mona HH, Miyatake K (2007b) Characterization of *Cucumber mosaic virus* monoclonal antibodies (mAbs): Specificity, neutralization of infectivity and gene sequence. *Plant Viruses* **1**, 193-199

Ziegler A, Torrance L, Macintosh SM, Cowan GH, Mayo MA (1995) *Cucumber mosaic cucumovirus* antibodies from a synthetic phage display library. *Virology* **214**, 235-238