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

Haggag Salah Zein1,2 • Kazutaka Miyatake1

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. * haggagsalaz@gmail.com, haggasalaz@yahoo.com

ABSTRACT

A mouse monoclonal antibody (mAb) prepared by hybridoma technology could recognize the Cucumber mosaic virus (CMV) coat protein. The Fab 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 Fab 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 Fab fragment revealed that the light- and heavy-chain genes belong to the Vx2 and VhL/VhS558 germline gene family with GenBank accession numbers EF672211 and EF672197, respectively. The pARA7 expression vector was designed for the expression of Fab in the periplasmic space. Recombinant Fab fragments were purified and analyzed by indirect ELISA. These results suggest that the recombinant Fab-4 antibody produced by E. coli acts as a source for the generation of Fab, with very stable and specific expression.

Keywords: CMV, light and heavy chain gene, monoclonal antibodies, recombinant Fab

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 (naive 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 (Puck and Plückthun 1992). Recombinant antibodies can be produced in 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 Fab 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 Fab, 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 CO2 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 (Burbas 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 108 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...
Virus purification and antibody production

Binant antibodies were assessed by ELISA. 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 splenocytes 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 anti-serum (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.

Production of soluble Fαb

Approximately 300 ng of the purified of heavy and light gene fragments were ligated with 500 ng of pGEM-T Easy (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 MgCl2), and 10 ml of (1 M MgSO4) in 100 ml H2O). 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 SacI-Δαhl and SpeI-Δxol sites of the expression plasmid pARA7, respectively. The soluble Fαb 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(lac)-leu7696 galE15 galK16 Δ(lac)X74 rpsL (Strr) hisd2 (rII) 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 OD600 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% bromphenol 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 cultures were harvested by centrifugation at 6,500 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), after centrifugation which contains inclusion bodies, was centrifuged at 14,000 rpm for 30 min. The resulting cell lysate 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 Fab protein was applied to a Q sepharose ff® (Amersham Biosciences) ion-exchange column and eluted with ion-exchange elution buffer. The Fab 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). Fab was then eluted with 0.1 M glycine-HCl buffer, pH 2.6 and the eluent (Fab) 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 Fdab-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 V_K 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 V_K genes were subcloned ligated into a pGEM-T Easy vector (Fig. 1C) for DNA sequences, subsequently isolated with specific restriction enzymes SacI and XhoI for the light chain and SpeI and XhoI 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_K) 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_K F_ab.

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

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

Analysis of 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 cell 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 osmotic shock 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 MgSO4 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_2ab) 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).

Purification of F_ab

A single colony was inoculated to 3 ml of Cireglegrow media (Q-BIO gene) with 100 µg/ml ampicillin according to Fujii (2004) in-cubated with shaking at 37°C until OD600 = 0.6 (~2 h). The culture was transferred to 200 ml of Cireglerow media with 100 µg/ml ampicillin and incubated with shaking at 37°C until OD600 = 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 Fab protein was applied to a Q sepharose ff® (Amersham Biosciences) ion-exchange column and eluted with ion-exchange elution buffer. The Fab fragment was further purified by applying the eluent to an affinity column (Amersham Biosciences) which is prepared by cross linking anti-mouse F(4ab)2 antibodies to a Hi-Trap (NHS-activated HP media, as per manufacturer’s instructions (Amersham Biosciences). Fab was then eluted with 0.1 M glycine-HCl buffer, pH 2.6 and the eluent (Fab) was neutralized with 1.0 M Tris-HCl buffer, pH 8.0.

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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\textsubscript{ab} or diabody fragments (Pluckthun 1991). Bacterial expression systems have the advantages of speed and abundant production. The F\textsubscript{(ab')\textsubscript{2}} fragments expressed to high levels in the periplasmic space of \textit{E. coli}, were indistinguishable from F\textsubscript{(ab')\textsubscript{2}} derived from limited proteolysis of intact antibody (Carter et al. 1992). When the rAbs expressed in bacteria accumulate as insoluble cytoplasmic inclusion bodies and even secreted antibodies form aggregates and thus require solubilization or refolding \textit{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\textsubscript{ab} constructs were subcloned into a prokaryotic expression vector and transformed into \textit{E. coli} MC1061 strains. Single colonies from freshly transformed cultures were used to initiate overnight cultures, and subcultured the following day into 2X Tryptone medium with 100 \mu g/ml ampicillin and 3% betaine. The cultures were grown at 30°C until an OD\textsubscript{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 \textit{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 \textit{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 lower in 0.0002% L-arabinose and 25°C (Fig. 3). The soluble periplasmic or culture supernatant of induction \textit{E. coli} was used directly for detection of the CMV, antigen coated plate ELISA, and significantly the recombinant F\textsubscript{ab} specifically detected CMV. No reaction was obtained with the extracts prepared from non-induced periplasmic \textit{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 periplasmic 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\textsubscript{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

### Antibody expression system

Large-scale production of recombinant antibodies is always a requirement after the therapeutically significance of the
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A

1XGY

OVQLQSGPEIVRPGASVKSISCKASGYFYTDYINNWQRPPGGLMWIGYFPRNGNTKY 60

Heavy-4

OVQLQSGPEIVRPGASVKSISCKASGYFYTDYINNWQRPPGGLMWIGYFPRNGNTAY 60

1XGY

NEKFKHATLTVDSSTAFQMLSLTSENAYVFCATTVSVMDSWQGTTTVTSASKT 120

Heavy-4

NQIFGHAKTLADSSYMQGILSSSTLSEYVSCATTVSVMDWQGTTTVTSASKT 119

1XGY

TPPSYVPLAPGSQAATNSMVLGCLVKGPEPVTWNSGSLSSGVHTFPQYIQSDLYT 180

Heavy-4

TPPSYVPLAPGSQAATNSMVLGCLVKGPEPVTWNSGSLSSGVHTFPQYIQSDLYT 179

1XGY

LSSSSTVPSSTWPSETVTCNVAHPASTKVDKIKIVPRDC 219

Heavy-4

LSSSSTVPSSTWPSETVTCNVAHPASTKVDKIKIVPRDC 218

B

1NLD

DVVTQTPTPLTSVTIGQPASICSSQSLLDSGKTLYNNWWQRFQPSRPKILLYLVSKLD 60

Light-4

DVVTQTPTPLTSVTIGQPASICSSQSLLDSGKTLYNNWWQRFQPSRPKILLYLVSKLD 60

1NLD

SGVDPRTGSGSTDTFLLKVSAWEDLGVYYCWQGTHFPPRFGGTKLKEEIKRADAPTV 120

Light-4

SGVDPRTGSGSTDTFLLKVSAWEDLGVYYCWQGTHFPPRFGGTKLKEEIKRADAPTV 120

1NLD

SIFPSEQLTSAGAVSCFLMNFYPKDINVKKIDGSEIQRGNLSWQDKDSYTSYM 180

Light-4

SIFPSEQLTSAGAVSCFLMNFYPKDINVKKIDGSEIQRGNLSWQDKDSYTSYM 180

1NLD

SSTLTLKDEYERHNSYCTETHKTSTSPIKSVFNF NEC 219

Light-4

SSTLTLKDEYERHNSYCTETHKTSTSPIKSVFNF NEC 219

Fig. 2 Alignments of the amino acid sequences of V\textsubscript{H} and V\textsubscript{L} regions of the mAb-4. (A) Alignments anti-CMV heavy chain germline, V\textsubscript{H} Variable region is most closely related antibody (1XGY). (B) Alignments anti-CMV light chain germline V\textsubscript{L}I, 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 concentration of 0.2–0.0002% 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 Fab specific was conjugated with HRP with a final dilution of 1/10000. Absorbance values were obtained after 2 h of incubation with substrate and are presented after subtraction of non-induction control values.

periplasm and in supernatant varied Fab 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 synthesized 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
of these antibodies and the determination of the variable gene usage are also described. The ability of r Fab 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.

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