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Production and Characterization of Monoclonal Antibody against Recombinant Virus Coat Protein CP42

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ABSTRACT

There are many studies related to the production of a ELISA kit for diagnosing virus infections. However, production of most kits depends on purification of whole virus particles, which involves the use of costly equipment and reagents. The purpose of this study was to check out if the anti-CP42 antibodies could be used as a diagnostic assay for detection of *Grapevine fanleaf* Virus (GFLV).

In this study, recombinant GFLV coat protein gene related to selected antigenic determinants was inserted into pET-28a bacterial expression vector and the construct (pET-28a *CP42*) was cloned into *E. coli* strain (DE3). Expressed protein was verified with western blotting assay by the use of commercially available anti-GFLV antibody. The recombinant protein was purified using nickel–nitrilotriacetic acid (Ni–NTA) resin. Balb/c mice were immunized with purified protein and splenocytes of hyperimmunized mice were fused with murine myeloma Sp2/0 cells. Positive hybridomas were selected by ELISA using CP42 as coating antigen.

The results showed that monoclonal antibody (MAb) specific to CP42 has been successfully generated. Efficiency of produced antibody was analyzed by ELISA and western blotting assay using some confirmed grapevine samples. The infection was confirmed previously based on morphological features and ELISA assay, performed using commercial anti-GFLV antibody. The monoclonal antibody reacted with antigen in ELISA and immunoblot method.

Our results demonstrated that anti recombinant CP42 monoclonal antibodies are able to diagnose whole virus in infected grapevine sample using ELISA test.

Keywords: Capsid protein; Epitopes; Enzyme-linked immunosorbent assay; *Grapevine fanleaf* virus (GFLV); Monoclonal antibodies

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INTRODUCTION

Monoclonal antibodies (MAb) can be generated through some techniques, among which hybridoma technique is the most common one. The production of monoclonal antibodies allows the isolation of reagents with a unique, selected specificity. This is due to identical identity of antibodies related to descendent of one hybridoma cell. Monoclonal antibodies are vigorous reagents for development of diagnostic kits. Hybridoma cell lines provide an unlimited supply of antibodies and thereby, they are permanent sources of kit production.[1]

Production of recombinant protein as an antigen has several applications such as the preparation of antibodies and candidate vaccine development.[2,3] Expression of viral coat protein gene (*CP*) in bacteria as antigen has been used commonly to prepare antibodies against viruses. It has been strengthened by the advent of recombinant DNA technology in recent years.[4] In this method, there is no need for virus purification as a crucial step in conventional antibody preparation, which is the significant advantage of the study. In addition, the cloned *CP* gene can be maintained safely in deep freezer for future uses.

Grapevine fanleaf Virus (GFLV) is one of the most devastative viral diseases of grapevine, which contributes to yield losses of up to 80% in the case of susceptible cultivars.[5] The icosahedral GFLV capsid is organized by 60 copies of the CP arranged according to a pseudo T=3 symmetry and has a predicted molecular mass of approximately, 56 kDa. The GFLV exists in vineyards of Iran.[6-11] There are many different ways for GFLV transmission and disease spreading, including transmission by infected pollen, seeds, propagative materials, and also through the longidorid nematode *Xiphinema index*.[12]

It seems that the widespread occurrence of GFLV in the vineyards of Iran originates from the absence of an efficient certification program for virus free grapes propagation materials. To detect GFLV in grapevine biological index, serological diagnostic techniques such as enzyme-linked immunosorbent assay (ELISA), and gene diagnostic techniques such as reverse transcription-polymerase chain reaction (RT-PCR)[11] and immunocapture reverse transcription-PCR (IC-RT-PCR)[13] have been developed. Biological indexing is time-consuming and it is not a common screening method of infected materials.[14] Molecular methods such as RT-PCR or IC-RT-PCR are generally not suitable tests for indexing large numbers of samples and also require specialized equipments and trained personnel.[15] Therefore, serological tests particularly ELISA has been widely used for low technical skill requirement, cost effectiveness, and the ability of screening a large number of samples.[16] Therefore, ELISA is a frequently preferred diagnostic technique because of its speed, specificity, and simplicity.[17]

Huss et al. in 1987 was the first scientist to produce GFLV monoclonal antibody and used it in ELISA distinguishing different GFLV variant from five countries. After him, different monoclonal and polyclonal antibodies were produced by other scientists.[18] In spite of all benefits of present commercial antibodies, they are not able to detect all infected grapevine in the area. Since Iranian GFLV strain is in distinct cluster of parsimonious tree and they have independent evolution[11], the inefficacy of present commercial antibodies is predictable. Thus, the limitation of current anti-GFLV MAbs in immunodiagnostic test of GFLV was the main reason for researchers in this study to perform further investigation for development of another anti-GFLV monoclonal antibody. However, the efficiency of monoclonal antibody production is directly related to the quality of immunogen. Regarding this, we have previously designed and expressed the new chimeric GFLV coat protein (CP42), which is composed of some of antigenic determinants of whole protein (GenBank KU640965). The new chimeric coat protein consists of 377 amino acids which are less than that of native antigen. In this study, a new anti-GFLV monoclonal antibody, 3B7 MAb, was developed and characterized based on novel 42 KDa chimeric coat protein antigen for diagnostic purposes.

MATERIALS AND METHODS

Selection of Antigenic Determinants

Continuous B cell epitopes were predicted using BcePred (http://www.imtech.res.in/raghava/bcepred),[19-21] ABCpred (http://www.imtech.res.in/raghava/abcpred/),[22] BCPREDS server (http://ailab.ist.psu.edu/bcpred/predict.html)[23] and protein structure prediction and annotation Protean software (PROTEAN subroutine in the DNASTAR 5.0 software package from DNASTAR company, USA).[24] Tertiary structure of different constructs was predicted by

two different modeling website such as SWISS-MODEL Workspace (http://swissmodel.expasy.org/repository/),[25] and I-TASSER (http://zhanglab.ccmb.med.umich.edu/ I-TASSER/).[26] For conformational B cell epitope prediction, the predictive models were uploaded to Disco Tope 1.2 server (http://www.cbs.dtu.dk/services/DiscoTope/).[27]

Synthesis of Chimeric Construct and Expression of Recombinant Protein in the Prokaryotic Host

A synthetic DNA coding for potential epitopes of GFLV CP (GenBank KU640965) was chemically synthesized by ShineGene Molecular Biotech (Shanghai, China). The synthetic gene was sub-cloned into pET-28a expression vector (Novagen, USA), and the recombinant vector containing the antigenic determinants named as CP42 was transformed into *E. coli* strain BL21 (DE3). The overnight culture of recombinant cells was used to inoculate 10 mL of Luria–Bertani medium for recombinant protein expression. The culture was grown at 37 ◦C up to an OD_{600} of 0.9 (OD, optical density). The protein expression was induced by adding 1 mM IPTG (isopropyl-β -d-thiogalactoside) (Sigma, St. Louis, MO, USA) and grow the culture for 7 h of induction. Samples from the cultures were taken prior to the induction and 7 h of the induction. Cells were harvested by centrifugation at 3,000 g for 10 min at 4°C. Pellet was re-suspended in 50 µL of homemade 1X SDS-PAGE sample buffer and was analyzed by SDS-PAGE.[28]

Purification of the Recombinant Protein and Confirmation by Western Blot Analysis

The recombinant protein was purified using nickel-nitrilotriacetic acid (Ni–NTA) (Qiagen, Valencia, CA, USA) resin under denaturing conditions. The pellet of 200-mL induced culture was re suspended in 8 mL lysis buffer (50 mM NaH2PO4 pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.2 mg/mL lysozyme). The bacterial suspension was lysed at room temperature for 1 h, and the lysate was then centrifuged at 16,000g for 20 min. The supernatant was poured into the Ni–NTA affinity column, and the low-through fractions were collected. The column was washed with washing buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, pH 6.8), and the bound protein was eluted with 2 mL of elution buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, pH 4.5). Collected fractions were analyzed on 10% SDS-PAGE, and the fractions containing purified protein were pooled and diluted (1:10) into refolding buffer (100 mM Tris, 0.1 M NaCl, 5 mM 2-ME, 0.1glycine 2.5% Glycerol and 0.5% triron X-100, pH 8.5) on a gently rotating magnetic stirring apparatus. Sample was dialyzed several times using buffers with decreasing concentration of 100 mM Tris (pH 8.5), 0.1 M NaCl, and the last dialysis was performed against phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.3). The protein concentration was estimated by Bradford assay.[29] The purified protein was transferred from SDS-PAGE gel to polyvinylidene fluoride (PVDF) membrane (Biocom Semi Dry Blotters, UK) using transfer buffer (192 mM glycine, 25 mM Tris base and 20% methanol). The membrane was incubated in the blocking buffer composed of 3% skim milk in PBS for 2 h at room temperature. After three times of washing, the membrane was incubated for 2 h at 37 °C with the commercial anti-GFLV antibody (virus research center, Turin, Italy). Again, after five times of washing, it was incubated for 2 h at 37 °C with Goat Anti- Rabbit IgG HRP conjugated antibody (1/2000 dilution). The membrane was washed and exposed to ECL (Amersham Phamacia Biotech Inc, USA) hyperfilm for 5 min.

Ethical Approval

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee (IR.NIGEB.EC.1395.11.10.A).

Statistical Analysis

All results in this study are presented as mean±standard deviation (SD). Statistical significance of differences between groups was explored by One-way ANOVA and two way ANOVA followed by Bonferroni using GraphPad Prism 6.01 (software GraphPad Software, Inc., USA, http://www.graphpad.com). A *p* value less than 0.05 was considered significant.

Immunization Procedure and Screening of Immunized Animals

Four female Balb/c (6-8 weeks old) mice were used for immunization. Each mouse was immunized 4 times with an interval of 2-3 weeks subcutaneously. The first Immunization was performed using Freund's complete

adjuvant (Sigma-Aldrich Co. St. Louis, MO, USA). Incomplete Freund's adjuvant (Sigma-Aldrich Co. St. Louis, MO, USA) was used for subsequent immunization (50 µg protein/Immunization/mouse). One week after each immunization, blood samples were collected by a vertical incision of the tail vein of mice for determination of antibody titers by plate trapped antibody-enzyme linked immuno sorbent assay (PTA-ELISA) method using 96 wells plates coated with 20 µg/mL of CP42 and HRP goat anti mouse antibody as second antibody.[30] The mouse with the highest serum antibody titer was selected as the spleen donor. The last injection of 50µg of antigen (without any adjuvant) was performed intravenously three days before the cell fusion. Sera collected from non-immunized and immunized mice served as negative and positive controls.

Cell Fusion and Hybridoma Production

Three days after final immunization, spleen of the immunized mouse was aseptically removed and fused with SP2/0 myeloma cell line at a ratio of 1:5 (1 SP2/0 and 5 spleen cells) by polyethyleneglycol (PEG , MW 1450, Sigma, UK) as fusogen. Selective hypoxanthine, aminopterin, Thymidine (HAT) medium (Gibco, Thermofisher, USA) was added to the fused cells and cells were seeded into five 96-well microtitre plates (Nunc, Thermofisher, USA) containing feeder layer. The cells were incubated at 37 °C with 5% CO2 for 2-3 days. Cell growth and colony formations were examined daily. Colonies were appeared after 5-7 days. Once the colony diameter reached to 1 mm the presence of antibody against the immunized antigen was determined by ELISA method[31]. The reactivity of hybridoma supernatants were determined using 96-wells pre-coated ELISA plates with 20 µg mL−1 of PBS dissolved CP42.

Cloning of Hybridoma Cells by Limiting Dilution (LD) Assay

After screening, the clones with high absorbance were selected for cloning by LD method. The cells were diluted so that contained only one cell in each 10 µL. Twelve days after LD, the supernatants of monoclones were screened for production of antibody. Suitable monoclones possessing high absorbance were selected for further characterization and were considered for mass production.

Assessments of Monoclonal Supernatant for GFLV Detection by ELISA and Western Blotting

In order to determine efficiency of the new monoclonal antibody for detection of GFLV disease in vineyard, virus infected symptomatic grape samples were collected from vineyards of Maragheh, Bonab and Urmia (all cities located in Iran). For more reliability, sample infection confirmation was done using ELISA test with commercial polyclonal antibody (virus research center Turin, Italy). For this aim, Phloem tissue was scraped from shoot and ground (1:7 ratio) in Extraction buffer «General» containing 2.40 g Tris-base, 8 g NaCl, 20g PVP K25 (MW 24000), 0.50% Tween20, 0.20 g KCl and 0.20 g NaN3 (pH 7.4; for 1000 mL). Each step was incubated overnight at -4◦c and washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) for 5 min. Last step was carried out using a 1:2000 dilution of polyclonal rabbit anti-GFLV and then its alkaline phosphatase conjugate (Loewe, Germany). The absorbance values of the treated wells were measured at 405 nm wavelength in STAT FAX 303+ ELISA plate reader. After serological diagnosis of symptomatic GFLV infections in Grapevine, ELISA assay was repeated on the same plants using supernatants of suitable clones as monoclonal antibody for antibody efficiency analyses.

Western blotting technique was used for confirming the result of ELISA and to see pattern of specificity of anti-GFLV monoclonal antibody. It was performed as above mentioned method and using supernatants of suitable clones as primary antibody and rabbit anti-mouse IgG conjugate (1/2000 dilution) as secondary one. The membrane was washed and detected by ECL (Amersham Phamacia Biotech Inc, USA) hyperfilm after exposure for 5 min.

Isotype Determination

The class and subclass of the 3B7 MAb were determined by ELISA with a mouse monoclonal sub-isotyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, following the procedure provided by the manufacturer (Thermo, USA).

RSSULTS

Chimeric Construct Synthesis and Expression

A synthetic DNA coding for potential epitopes of GFLV CP (GenBank KU640965) was chemically synthesized by ShineGene Molecular Biotech

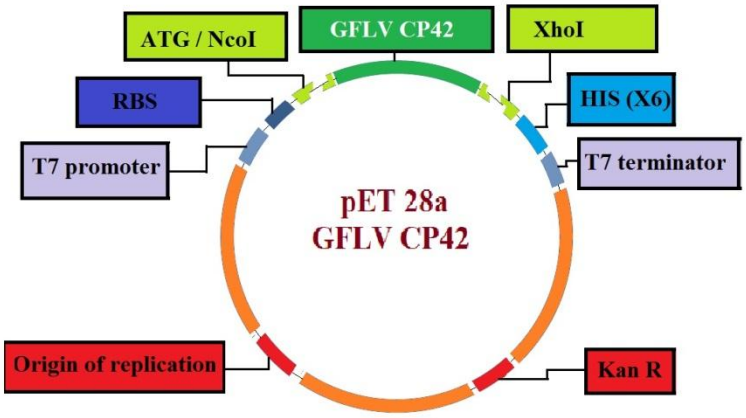


Figure 1. A diagram showing antigenic determinant in *Grapevine fanleaf* virus coat protein gene cloned into the expression vector. RBS: Ribosome binding site; Kan R: Kanamycin resistance gene; HIS: Histidine tag.

(Shanghai, China). The synthetic gene was sub-cloned into pET-28a expression vector (Novagen, USA) between NcoI and XhoI restriction sites (Figure 1), then the recombinant vector containing the antigenic determinants was transformed into *E. coli* strain DH5[32] and the transformants were selected on kanamycin.

Recombinant Protein Expression, Purification, and Characterization:

The synthetic gene was expressed in *E. coli* BL21 (DE3), with 6X-His-tag at 5′ ends. The optimum condition for expression was achieved after 7 h induction by IPTG (1 mM), at 37 ◦C and OD 600 of 0.9. Purification of the recombinant protein was carried out under denaturing conditions, and SDS-PAGE analysis revealed as a major band (~42 kDa) in all the eluted fractions (Figure 2). The approximate yield of purification was more than 15 mg/L culture. Western blotting analyzes using commercial anti-GFLV antibody (virus research center Turin, Italy) was confirmed CP42 as native virus coat protein (Figure 3).

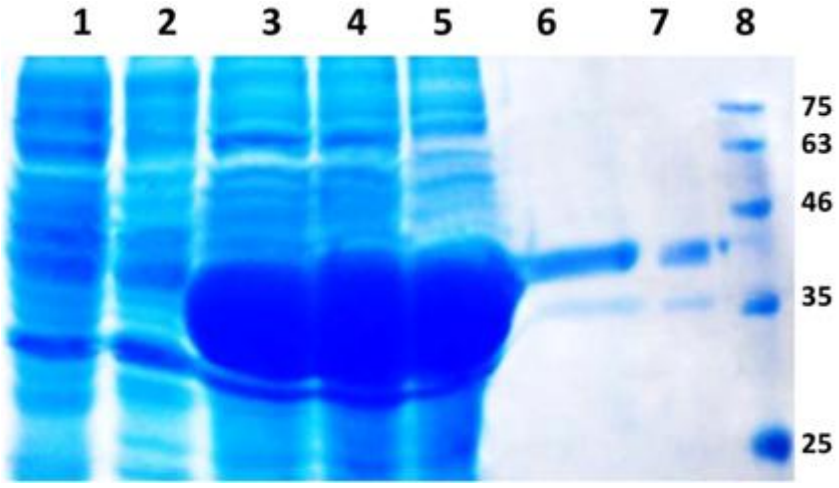


Figure 2. SDS-PAGE analysis of expression and refolding of CP42 recombinant protein. Lane 1, 2: un-induced bacterial extract; Lane 3, 4, 5: induced bacterial extract. Lane 6, 7: refolded protein; Lane 8: ladder.

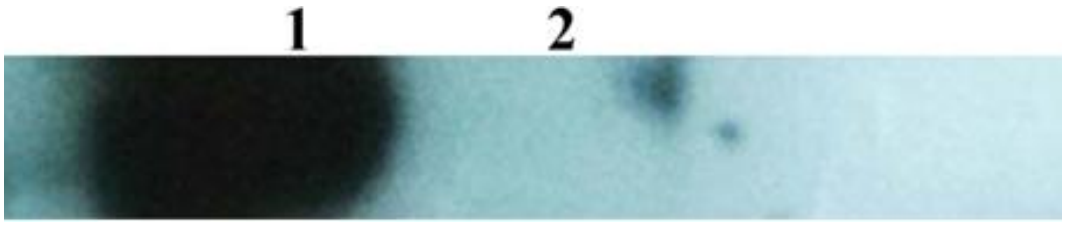


Figure 3. Western blot analysis of CP42 using commercial anti-GFLV antibody. Lane 1: induced Bacterial extract; Lane 2: un-induced Bacterial extract. GFLV: *Grapevine fanleaf* virus

Immunization Procedure and Limiting Dilution Assay

The titers of antibodies against determinants of GFLV coat protein (synthetic peptides), in the sera of immunized mice showed that all mice were immunized against the antigen, but in two mice increase of antibody titer was more than others and mice number 2 had higher anti-CP42 antibody (Figure 4). The serum of the immune mouse at 1:32000 dilution, indicated the highest absorbance in reaction with CP42 using ELISA method (Figure 5). So the immune mouse was selected for the fusion. The final result of the successful fusion of the immune mouse spleen cells with myeloma SP2/0 cells were about 250 wells, of which, 12 wells contained positive clones with high absorbance in reaction with synthetic peptides and 1 clone had anOD over than 3. Positive clone was named as 3b7 and was selected for cloning by LD method. The yield of LD was many clones with absorbance over than 3 and about 2 at 0.01 dilutions.

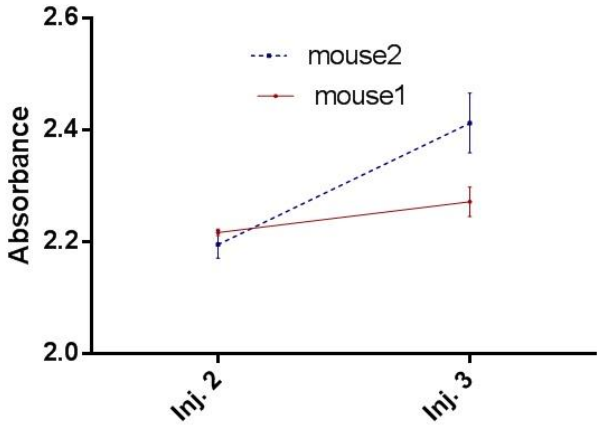


Figure 4. Enhancement of immune responses in mice by 2nd and 3rd injection (Inj.3 *p* value < 0.1) of CP42 in sera of two Balb/c mice evaluated by ELISA assay using 1:2000 dilution of immune mice serum.

Assessments of Monoclonal Supernatant for GFLV Detection

The specificity of the anti-GFLV was assessed by an ELISA and western blotting procedure. Grapevine samples were collected based on symptomatic signs such mosaic, open petiole leaf, vein banding, leaf deformation, mottling, and fanleaf.

For ELISA confirmation assay, an uninfected and four infected plants with typical symptoms were chosen. ELISA was done using standard polyclonal antibody in two repeats and one sample of purified CP42 was used as positive control. The rate of absorption in all infected samples and CP were greater than 2.5 times of healthy ones. The one way ANOVA test, also confirmed the result by a *p* value less than the significance level of 0.0001. Then, another ELISA assay was done using supernatant of monoclonal in two repeats. The results showed that monoclonal

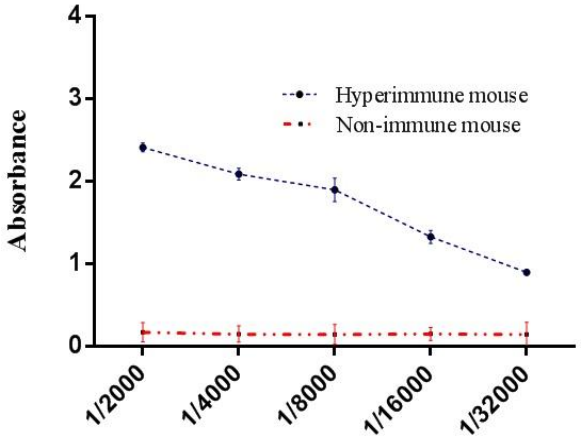


Figure 5. Titration of immunized mouse serum against chimeric CP42 antigen (Two way ANOVA, *p* value < 0.0001). A serial dilution of serum were added to CP42 pre-coated 96-well plates and titration of antibody was assayed by ELISA

antibody could isolate the contaminated and non-contaminated samples efficiently (Table 1). The one way ANOVA test, confirmed the result by a *p* value less than the significance level of 0.001. Result of western blotting shows that, harvested antibody against CP42 could acceptably recognize presence of whole GFLV in the infected samples (Figure 6).

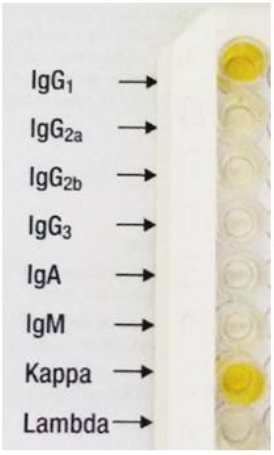
Isotype Determination

Isotype of this MAb was identified as IgG1 and Kappa light chain (Table 2); ince, their OD was more than twice the other chains. The ANOVA test, also confirmed the result by a *p* value less than the significance level of 0.0001.

Table 1. Mean absorbance of supernatant of the 3B7 Mab (*p* value <0.001) and commercial antibody (*p* value <0.0001) in two repeats. Optical density value of infected (8D, 13D, 14d, 20D) and non-infected (38D) Grapevine samples and recombinant CP42 as control (P), at 405 nm.

Sample	Commercial antibody		Supernatant of the 3B7	
	Rep.1	Rep.2	Rep.1	Rep.2
8D	2.965	2.999	2.986	2.861
13D	2.973	2.987	2.954	2.961
14D	2.031	2.093	2.781	2.983
20D	2.849	2.741	2.286	2.449
P	1.598	1.622	2.949	2.955
38D	0.182	0.171	1.101	1.202

Figure 6. Western blot determination of the 3B7 MAb. Lanes 1, 2, and 3 are related to the infected grapevine samples. Lanes 4 and 5 are the uninfected grapevine samples. Lanes 6 and 7 are CP42.



Class	OD
IgG_1	1.341
IgG_{2a}	0.149
IgG_{2b}	0.137
IgG_3	0.101
IgA	0.128
IgM	0.116
kappa	1.631
Lambda	0.105

Table 2. Determination of the isotype of MAb by ELISA. Classes and subclasses of 3B7 MAb. (*p* value < 0.0001)

DISCUSSION

This study has been conducted to evaluate diagnostic potential of anti-recombinant GFLV-CP, which is directed against a part of whole protein antigenic epitopes, for detection of intact virus particles. Sokhandan et al. (2011) reported cloning and sequencing of Iranian GFLV isolate′s coat protein gene.[11] Followed by their study, improvement of detection system and control strategies for GFLV infections in Iran, were the main aim of the current study. However, in this study, we produced a monoclonal antibody against antigenic determinant of GFLV coat protein instead of its full length. This antigen is a novel chimeric 377 amino acids containing two parts of antigenic regions of whole protein which are linked together by a linker peptide (GenBank KU640965). In this study, it was confirmed by ELISA that a high level of monoclonal antibody against chimeric CP42 was produced. The chimeric CP42 antigen had appropriate immunogenic properties to stimulate immune system for producing suitable level of antibody. Immunoblotting assay also confirmed these results and demonstrated that 3b7 MAb has been able to specifically recognize both CP42 and also native virus particle in the infected plant tissues.

In this study, mouse antiserum with titers of 1/32,000 was obtained, suggesting that CP42 is relatively a good immunogen. Related to this study, Etienne et al, obtained polyclonal antisera with titers of 1/20,000 using GFLV particles as antigen then they concluded that the virus particle is immunogenic.[33] In other experiment, Sokhandan et al, prepared polyclonal antibodies against GFLV coat protein expressed in *E. coli.* A 1/1,000 dilution of the IgG purified from the antiserum was efficient to detection of the virus in infected plant.[18] The same antibody at 1/500 dilution detected GFLV in infected grapevine leaf by immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR).[13] Therefore, CP42 is significantly more immunogenic than above mentioned proteins.

There are several ways to diagnose the GFLV disease. Symptoms and ELISA assay are two basic detection methods. Nevertheless, GFLV symptoms are affected by both environmental conditions such as symptoms attributed to herbicides and virus strain such as other grapevine-infecting viruses or Grapevine yellow speckle viroid (causing GFLV-like symptoms in ELISA-negative grapevines).[11] On the other hand, ELISA testing alone cannot be used to definitive diagnose of GFLV because other grapevine-infecting viruses such as ArMV that serologically cross reacts with anti-GFLV polyclonal IgG can cause false-positive result, with the ELISA screen.[34] Therefore, the combination of both methods was used to detect viral infection in the present study. In order to analyse3b7 MAb efficiency, firstly some of the infected and the non-infected grapevine were confirmed based on morphological symptoms and ELISA of commercial anti-GFLV antibody. Verified samples were used to evaluate the performance of 3B7 MAb.

In the present study, supernatant of 3b7 MAb efficiently detect CP42 and infected grapevine in ELISA and western blot., There are some similarities and differences between our experiment and other studies, who immunized mice with whole virus particle for production of hybridoma.[35,36] Huss et al., in 1987 reported the production and characterization of first monoclonal antibody to GFLV and use of them for diagnostic tests.[37] These antibodies were also used in attempts to differentiate between serologically closely related GFLV isolates that differ in their geographical origin and virulence.[36] Relative to above study, Nolke et al. using purified GFLV particles, produced GFLV-specific monoclonal antibodies and used its ScFv sequences for conferring GFLV and *Arabis mosaic virus* (ArMV) resistance in *Nicotiana benthamiana*[35], however, some scientists suggest the possibility of using recombinant virus coat protein as antigen, instead of whole virus. Kusano et al. produced mouse monoclonal antibodies against recombinant viral coat protein. They used two lines of antibody from different paratopes as immunochromatic strip test for direct detection of *Apple stem grooving virus* in citrus samples.[38] Fasihi-Ramandi et al., were other scientists who produced monoclonal antibody against recombinant protein and used it for ELISA and agglutination test.[39] In the present study, CP42 has been used for both immunization of mice and screening of hybridoma cells in order to production of monoclonal antibodies. Our work is similar to those of other groups of scientists who produced monoclonal antibody against chimeric antigen that was designed using selected part of protein instead of its full length. Based on the high affinity and appropriate reactivity of

produced MAb with the native antigen, they concluded that their MAb could be a beneficial candidate for further application in genetically engineered monoclonal antibody.[40]

Results of ELISA and western blot demonstrated the binding activity of 3b7 to both native and denatured form of antigen and this is probably due to the specificity of produced MAb for linear epitopes instead of conformational. These results also indicate the specificity of 3b7 MAb that binds to all infected samples, but not to uninfected grapevine plant.

A non-specific background reaction was encountered in the ELISA with 3B7 mab. ELISA kits involve several steps and reagents and in some cases significant background noise can result from the interaction of these reagents. Furthermore, depending on reagent, purity of chemicals, type of microtiter plate, handling (especially washing), and incubation conditions, background values may vary not only from reagent to reagent but also from plate to plate even within a series of plates testing the same pathogen. However, it may be managed by optimizing the procedure and calculation of the cut-off value.[41] In this experiment, optical absorption, due to a delay in the appearance of signals, was measured after 24 hours of adding the substrate. For this reason it is important to ensure that, results are statistically significant when compared to the negative control. On the other hand, a sample is considers as positive if its absorbance is at least twice as much as the absorbance of the negative.[42-45] The late appearance of the signals should not challenge efficiency of the Mab because western blotting confirmed the efficiency.

Ability of the MAb, which was produced against CP42 to detect the related viral antigens, can be explained by several hypothesis. First, the icosahedral GFLV capsid forms by 60 copies of the CP arranged according to a pseudo T=3 symmetry.[46] Only external regions of icosahedral complex are considered as antigenic epitope for the whole virus. As ELISA kit detects the whole virus, only paratope of antibodies against outside region of virus will be useful. Expression of recombinant subunit does not necessarily lead to formation of virus like particle. Gottschamel et al. induced expression of CP in transgenic plant and observed only a few viruses like particles and most of subunits remain disassociated from other.[47] Then, deletion of some regions of protein affects the antibody′s breadth in the immunized mouse. Previous research has shown that any change of protein structure lead to a change in the number of targeting related B-cell.[48] It seems that, deletion of non-effective regions of CP probably has caused increased production of specific antibodies. As for second hypothesis, some experimental studies showed recombinant capsid protein self-assemble into a hollow virus particle which retains native virus epitopes. They suggested its potential value as a vaccine[49]; however, assemble of virus particle needs different factors such as: hydrophobic attraction, electrostatic repulsion and specific contacts between certain pairs of amino acids[50]. Moreover, environmental factor such as ionic strength, pH, and temperature can affect them[51] Conformation of recombinant gene expressed in various variant hosts may be different from native and actually may not be predictable. So any variation in protein shapes, lead to change of conformational epitopes and related B-cell. Approximately, 90% of B-cell epitopes from native proteins are conformational.[52] It was not considered as an important problem because despite the change of protein tertiary structure, protein secondary structure remained intact and guarantied preservation of some of conformational epitopes. In addition to them, linear epitopes are only related to amino acid sequence of protein and position of epitope on the surface of protein according to a large number of amino acids related to native protein are retained in the CP42, it retain a large number of linear epitopes.

Newly synthesized recombinant protein is prone to misfolding. In this experiment, protein was synthesized in the form of inclusion body and then refolding process was performed during the purification using chromatography process. However, possibility of misfolding was an important concern. In order to overcome this issue, many hybridoma cells have been analyzed (instead of specific B-cell selection) against bot recombinant protein and crude sap prepared from GFLV infected young grapevine shoot as the antigen. One hybridoma was positive with high affinity for recombinant protein and enough affinity for crude sap. Kusano et al. in 2014 analyzed as high as 8000 hybridomas 9 of which, were positive for crude sap preparation from ASGV-infected young citrus shoots and in order to increase the sensitivity of the kit, a combination of two colonies was used for test line and for colloidal gold conjugation.[38]

In general, although the use of recombinant proteins

as antigens is an efficient method in production of monoclonal antibody, but it is not cost-effective due to the necessity of simultaneous analyze for a large number of colonies. Consequently obtaining a fast result is not guaranteed. The limitation of our study could be the lack of cross reactivity, specificity, affinity, and avidity data. However, in this study, a new anti-GFLV monoclonal antibody, 3B7, was developed and characterized based on novel 42 KDa chimeric coat protein antigen for diagnostic purposes.

Our results indicate that anti-CP42 antibodies showed reactivity against whole GFLV particle in ELISA and western blot performed in this study. Utilization of recombinant proteins as antigen is a helpful method to produce monoclonal antibody and production of diagnostic kits. Mass usage of it, will also be affordable. Based on the high affinity of 3b7 MAb and its efficacy in recognition of GFLV particle together with its specific binding to CP42 antigen, it could be concluded that 3b7 MAb is a suitable choice for production of ELISA kits or applying in other methods.

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