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Research Article

Preparation of polyclonal antibody against recombinant coat protein of *Cucumber mosaic virus* isolate B13

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Abstract: *Cucumber mosaic virus* (CMV) is one of widely-spread viruses of plants with the broadest host range encompassing over 1200 species. One major limiting factor for detection of the virus is unavailability of the virus-specific antibodies especially in developing countries. Recombinant DNA technology facilitates antibody preparation without requiring special equipment. In this study, coat protein (CP) gene cDNA of CMV was subcloned from pTZ57CMVCP into pET21a expression vector and transformed into *Escherichia coli* strain Rosetta. Expression of CMV CP was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and its identity was confirmed by western blotting, dot blot immunobinding assay (DIBA) and enzyme- linked immunosorbent assay (ELISA) using anti- CMV antibody. The expressed protein was purified using T7•Tag affinity purification kit and used as antigen for raising polyclonal antibodies in two mice. The purified anti-CMV CP IgG and the conjugated IgG performed favourably in terms of specificity and sensitivity to detect both expressed CP (antigen) and CMV isolates in infected cucurbit plants using plate trapped antigen (PTA)- ELISA, double-antibody sandwich (DAS)-ELISA and western blotting. The prepared antibodies can be applied in serological and sero-molecular tests in studies on the virus and in screening of plants for the infection. This is the first report of preparation of antibodies against CP of an indigenous isolate of CMV.

Keywords: Antibody, Coat Protein, *Cucumber mosaic virus, E. coli*, Expression, western blot

Introduction

Cucumber mosaic virus is the type member of the genus *Cucumovirus* in the family *Bromoviridae*. CMV is composed of three particles, which are identical in shape but differ in their densities (King *et al.*, 2011; Palukaitis and Garcia-Arenal, 2003). CMV has a broad host range, infecting about 1200 species in 85 plant families including cereals, fruits, vegetables and ornamentals (Roossinck, 2001). It is endemic in most temperate regions of the world causing important epidemics in a variety of crops.

Serological assays such as ELISA are commonly used for simultaneous screening of a large number of samples due to its robustness, accuracy, and reasonable cost (Lima *et al.,* 2012). Conventionally, purified virioins from the infected plant material are used to prepare the polyclonal antibodies. Quality of such polyclonal antibodies however depends on the purity of virus purification (Lima *et al.,* 2012; 2005). It also requires maintenance of virus culture on live

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plants as antigen material, generation of large quantity of plant tissue through mass propagation of the virus isolate and accurately removal of plant proteins during the purification process. Expression of viral genes is an important strategy to generate the recombinant proteins that may be used in antibody production (Barbieri *et al.*, 2004). Based on immunogenicity of CP it is a good candidate for use as the recombinant antigen for antibody production.

Generally, recombinant proteins are purified as specific antigens for immunizing an animal for obtaining high-quality virus-specific antibodies (Nickel *et al.*, 2004). Expression of foreign genes in *E. coli* is relatively simple, fast and inexpensive for producing high quantities of proteins with minimal post-translational modifications (Hartley, 2006).

Proteins from several plant viruses have been produced in *E. coli* and used for raising virus-specific antibodies for immunodiagnostics (Cerovska *et al.*, 2012; Jain *et al.*, 2005; Lee and Chang, 2008; Plchova *et al.*, 2011). Such antibodies are widely used in serological methods for detection of viruses in different studies. ELISA is a sensitive, specific, inexpensive, robust and suitable assay for testing many samples simultaneously (Lima *et al.*, 2005; Lima *et al.*, 2012). Additionally, antibodies to recombinant viral proteins are applied in extremely sensitive techniques such as immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) and western blotting (Koolivand *et al.,* 2014; Komorowska *et al.*, 2010; Wetzel *et al.*, 1992).

The objectives of this study were to express CMV CP in *E. coli* to prepare antigen and production of CMV-specific polyclonal antibody for serological and sero-molecular tests such as ELISA, western blotting and IC-RT-PCR.

Materials and Methods

CMV isolate

CMV isolate B13 CP (AY871070) from Basmenj district (East Azarbaijan, Iran) which was isolated from cucumber and belonged to subgroup I was previously cloned into pTZ57R/T and sequenced (Sokhandan-Bashir *et al.*, 2006; Rostami *et al.,* 2014). The CP gene was released from pTZ57CMVCP by digestion with *Bam*HI and *Sac*I and ligated into the bacterial expression vector, pET21a (Novagen, USA) digested by *Bam*HI and *Sac*I. The new construct (pET21CMVCP) was transformed into *E. coli* strain Rosetta by heat-shock procedure and the desired construct was selected based on sequencing by T7-promotor and T7-terminator primers (Macrogen, Seoul, South Korea).

Expression and Purification of CMV CP in *E. coli*

As a start culture, *E. coli* strain Rosetta containing pET21CMVCP was grown overnight in LB medium containing 100 µg/ml ampicillin. Next, an overnight culture was diluted 50 times in 10 ml of LB medium and grown (37 ^{o}C, 200 rpm) until the optical density (OD_{600}) was ~0.6. Then, 1.5 ml of cell suspension was taken as non-induced control before isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture at 1 mM to induce the T7 promoter. Both induced and non-induced cell suspensions were grown in incubator under the same conditions as mentioned earlier. Samples from the incubated culture were analyzed after 4 hours of the induction by taking 1.5 ml of culture from the flask. Cell suspension samples were centrifuged at 10,000 rpm for 7 to 10 minutes in a Heraeus Megafuge 1.0R rotor 3041 (Germany).

Analysis of the expressed protein was done by SDS-PAGE (Laemmli, 1970) and the expression of CMV CP was approved by western blotting, DIBA (Surendran *et al.,* 2015) and PTA-ELISA (Dijkstra and de Jager, 1998) using anti-CMV antibody (Agdia, USA) in 1: 1000 dilution.

To achieve CMV-CP purification, *E. coli* Rosetta containing pET21CMVCP was expressed in 100 ml LB medium containing 100 µg/ml ampicillin as described above. The cells were harvested by centrifugation (Heraeus Megafuge 1.0R, Germany) at 6,000 *g* for 15 min and the expressed protein was purified using T7•Tag Affinity Purification Kit (Novagen, USA) as per manufacturer's instruction. The soluble extract was loaded directly onto T7•Tag

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Affinity columns for purification. Concentration of the purified recombinant protein was determined by Bradford's method (Bradford, 1976).

Confirmation of purified recombinant CMVCP

10µl aliquot of the purified protein was re-suspended in 90 µl of Laemmli buffer, boiled for 5 minutes and 30 µl aliquots were loaded onto 12% polyacrylamide gel containing SDS (Laemmli, 1970). Commercial anti-CMV antibodies (Agdia, USA) were applied to verify the purity and antigenicity of the purified CMV CP by subjecting to PTA- ELISA (Dijkstra and de Jager, 1998), DIBA (Surendran *et al.,* 2015) and western blot.

Raising antiserum and purifying antibody

The immunization program consisted of five injections. Once a week, 10 µg of purified CMV CP was injected into two mice. After collecting the pre-immune blood, the first dose was prepared using a 1:1 ratio (v/v) of CP in complete Freund's adjuvant (Sigma-Aldrich, USA) and injected subcutaneously. Subsequently, booster injections were administered subcutaneously with 5 µg of CMV CP emulsified in incomplete Freund's adjuvant at 1:1 ratio (v/v).

The mice were bled one week after the last injection and the collected blood was subjected to centrifugation at 3,000 g for 10 min to separate serum fractions and stored at -20 °C until required. Titration of the antiserums was done by PTA-ELISA in the dilution range of 1:512 to 1:8192. The immunoglobulin G (IgG) was obtained from the antiserums using Protein-A IgG Purification Kit (Thermo Scientific, Waltham, MA, USA) as per manufacturer's instructions and stored at -20 °C before conjugating with alkaline phosphatase using EasyLink Alkaline Phosphatase Conjugation Kit (Abcam, Cambridge, UK) according to a protocol provided by the manufacturer.

Calibration of prepared antibody

The polyclonal antibodies (IgG and IgG-conjugate) developed against recombinant CMV CP were evaluated for sensitivity and specificity by Western blotting (1:500), DAS-ELISA and PTA-ELISA (1:500, 1:1000 and 1:2000 dilutions). Crude leaf extracts from healthy and CMV-infected tissues (both 1:10 dilution) and also purified CP protein (10 µg/ml) from *E. coli* were used as test materials. Furthermore, some leaf samples infected by potyvirus (*Bean common mosaic virus*, BCMV and *Zucchini yellow mosaic virus*, ZYMV) were used for checking the cross reactivity. The result was evaluated after adding universal AP-conjugated antibody (Agdia USA) by measuring light absorbance at 405 nm wavelength.

The samples were positively identified if the mean DAS-ELISA (A405 nm) value of samples exceeded at least twice the mean of the healthy control (s).

Crude protein extract (30µl) and the purified CMV CP (10µg/ml) were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Hybound, UK) by a wet electroblotter transfer system (Bio-Rad, USA). The membrane was blocked with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 X $2H_2O$, 1.5 mM KH_2PO4; pH 7.5) containing 2% (w/v) skim milk powder, and the blotted proteins were probed with a prepared anti-CMVCP IgG dilution of 1:500. The universal alkaline phosphatase-conjugated IgG (Rabbit anti mouse) (Agdia USA) as the secondary antibody was used at a dilution of 1: 500. The target proteins were finally revealed by adding to substrate 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT).

Result

Expression of CMV CP

Sequencing data confirmed in-phase insertion of the full-length CMV CP gene into the expression vector pET21a. Expression of the CP gene in *E. coli* was proved on 12% SDS-PAGE by an intense band with a size of about 31 kDa, which roughly corresponded to the predicted size of the complete CMV CP plus the fused amino acid tags (Fig. 1A).

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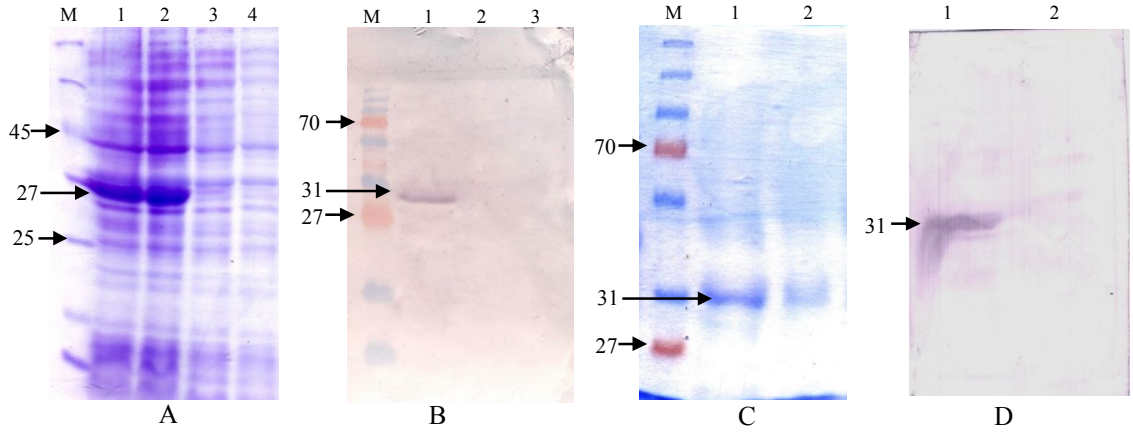

Figure 1 SDS-PAGE (A and C) and western blotting (B and D) of the expressed CMV-B13 CP in *E. coli* (A) Lane M: Prestained Protein Marker; lane 1 and 2: total protein from induced cells at 4, 6 hours of inductions by 1mM IPTG, respectively; lane 3: protein sample from non-induced cells; Lane 4: total protein from *E. coli* carrying pET21a(+) lacking the CP gene (B) Western Blot of CMV CP on nitrocellulose membrane. Lane M: Prestained Protein Marker, Lane 1: expressed CMV CP; Lane 2: protein sample from non-induced cells; Lane 3: protein sample from *E. coli* pET21a (+) lacking CP gene. C) Electrophoretic patterns of purified CMV CP protein on 12% SDS-PAGE. Lane M) Prestained Protein Marker, Lanes 1 and 2) CMV CP purified protein. D) Western Blot of purified CMV CP on nitrocellulose membrane. Lane 1) Purified CMV CP protein, Lane 2) protein sample from non-induced cells.

Antibody preparation and analysis

Western blotting and DIBA with a rabbit commercial anti-CMV polyclonal antibody confirmed the recombinant protein as CMV CP. Accordingly, a protein band with the expected size (based on comparison with the protein size marker) was observed in the western blotting (Fig. 1B). CMV-infected samples produced a signal after 15 to 30 min of incubation with the substrate (BCIP/NBT) in the DIBA assay. No reaction occurred with the extract from the bacterial carrying pET21a without insert, or non- induced (negative controls).

The recombinant CMV CP was purified from 100 ml culture by T7.tag purification kit under denaturing condition (Fig. 1C). SDS-PAGE analysis of the purified protein showed a distinct expected band of about 31 kDa corresponding to CMV CP plus the fused tags (Fig. 1C). Concentration of the purified CP protein was estimated as~500 µg/ml by spectrophotometer.

The result of western blotting revealed that the expressed CMV CP could react strongly with the commercial anti-CMV antibody when the protein bands were transferred onto nitrocellulose membrane (Fig. 1D).

Titer of the anti-CMVCP antiserum was determined to be about 1:4096 using PTA-ELISA (Fig. 2). Titration of the anti-CMVCP IgG purified from the antiserum showed that the IgG at 1:500 and 1:1000 dilution still reacted with the CMV-infected leaves and the purified expressed CP (Fig. 3). Further, results from DAS-ELISA which was performed to evaluate efficiency of the prepared anti-CMVCP IgG (1:500 and 1:1000 dilution) and the conjugated IgG (1:500 dilution) showed that both IgG and the conjugate reacted efficiently with the purified expressed protein and CMV-infected leaf tissue (Fig. 4). Furthermore, the anti-CMVCP antibody reacted in the western blotting with the expressed protein at 1:500 antibody dilution. However, no signal was

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observed with the crude extracts from healthy plants (Fig. 5).

Discussion

Advances in virus research have made it possible to use recombinant proteins as antigens to raise antibodies for diagnosis of plant viruses. The ability to produce recombinant CMV CP in the bacterium has several implications. Preparation of the antigenic recombinant CP brings about the possibility to produce antibody against the virus, which could be used in serological tests. This is especially helpful in developing countries where reagents such as antibodies are imported and costly and the process of importation is time consuming.

The expressed CMV CP when analysed by SDS-PAGE, presence of a band with a molecular mass of approximately 31kDa corresponding to that of CMV CP was observed (Fig. 1A). An about 4 kDa difference with the CMV-CP mass was due to the fused tags. Identity of the expressed protein was also confirmed by western blotting (Fig. 1B). As in the case of similar studies, no significant differences have been reported in the immunogenicity of recombinant antigens compared to conventional antigens (Kumari *et al.*, 2001; Mutasa-Gottgena *et al.*, 2000).

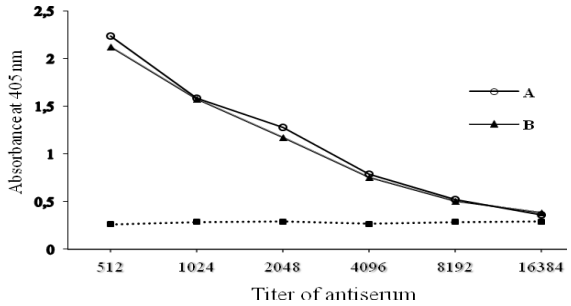

Figure 2 Titration of antiserum prepared against recombinant coat protein of cucumber mosaic virus isolate B13 by the use of indirect ELISA. The graph is based on absorbance values of the microtiter plate wells treated with different dilutions of the antiserum. A and B represent antisera of two mice as replicates. The purified protein was used as antigen.

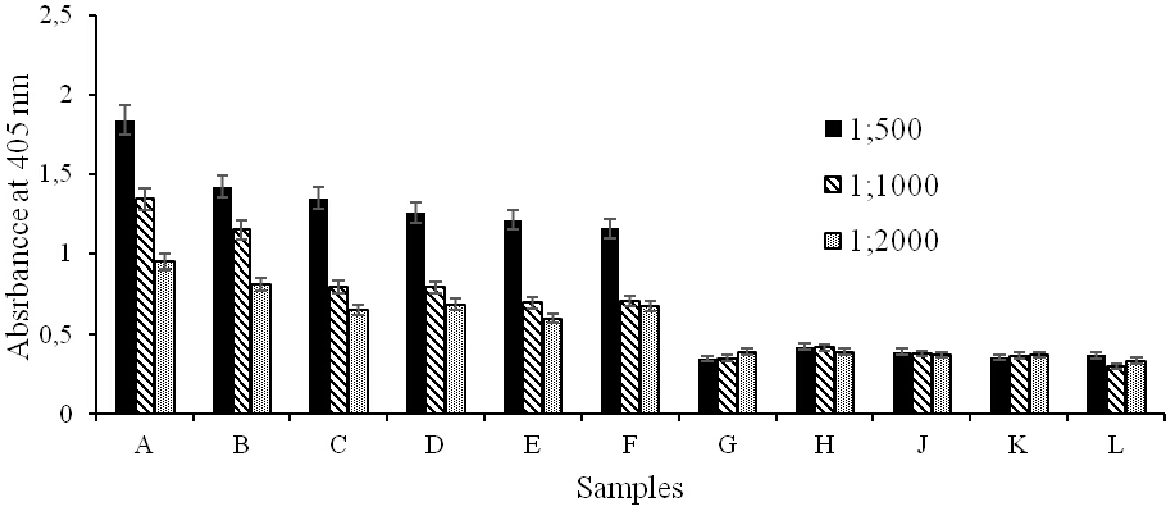

Figure 3 The result of PTA-ELISA by the use of anti- CMV-B13 CP IgG based on absorbance values at 405 nm. A: injected CP protein purified by affinity chromatography, B: expressed protein in *E. coli*, C, D, E and F: CMV infected plant, G: healthy plant, H: protein extracted from non-induced colony, J: protein extracted from a colony containing pET21a (+), K: plant extraction buffer, L: substrate.

[DOR: 20.1001.1.22519041.2017.6.1.8.4]

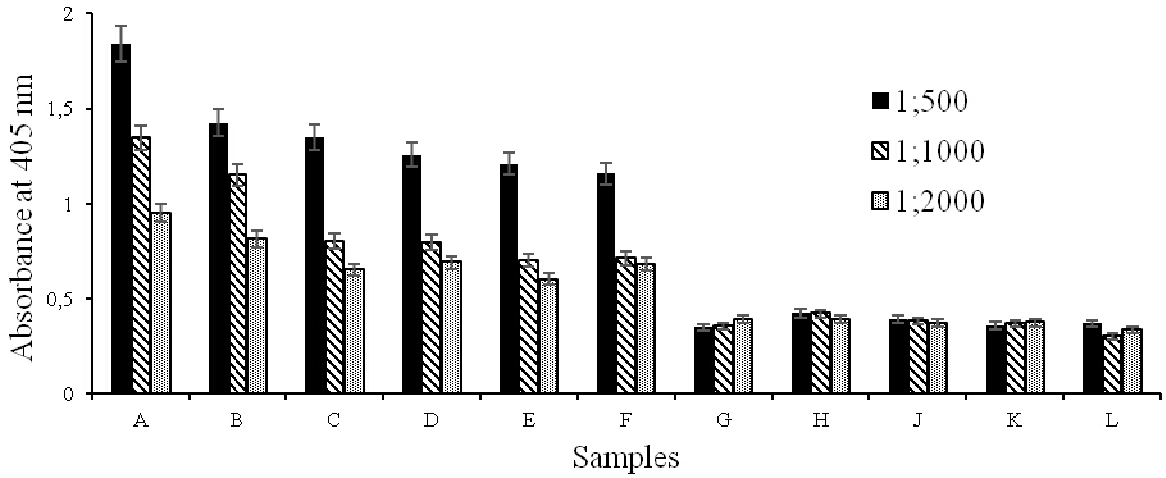

Figure 4 The result of DAS-ELISA by the use of anti- CMV-B13 CP IgG and conjugated IgG (both 1:500 dilution) based on absorbance values at 405 nm. A: injected purified CP protein by affinity chromatography, B: expressed protein in *E. coli*, C, D, E and F: CMV infected plant, G: healthy plant, H: protein extracted from non-induced colony, J: protein extracted from a colony containing pET21a (+), K: plant extraction buffer, L: substrate.

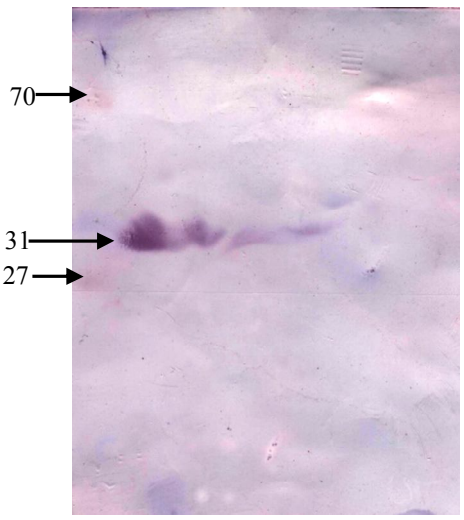

Figure 5 Western blotting by the use of anti-CMV-B13 CP IgG (diluted 1:500). M: Prestained Protein Marker, 1) injected purified CMV CP protein, 2) protein sample from a colony containing pET21aCMVCP after induction by IPTG, 3) protein sample from non-induced cells.

Because the fusion CMV CP possessed T7.tag at the NH2- terminus, T7•Tag affinity chromatography kit was used for the protein purification (Fig. 1C, D) and the purified protein was confirmed by SDS-PAGE, western blotting, DIBA and ELISA. Similarly, *Potato virus Y* (PVY) CP expressed in *E. coli* has been reported to be detectable in DIBA (Amer *et al.*, 2004). However, ELISA is preferable due to its more sensitivity. The use of purified expressed fusion protein as the antigen gives consistent results in terms of quantity and quality of the antigen compared to conventional methods (Raikhy *et al.*, 2007; Koolivand *et al.,* 2016). Thus, the recombinant viral CPs expressed in bacterial cells have great potentials as alternative sources of antigens for raising specific antibodies to plant viruses (Lima *et al.,* 2012). They can be produced in large quantities and manipulated or modified as needed for specific uses. In contrast to the conventional method of antigen preparation, which is associated with very poor yields for CMV, the purification procedure described above can be completed in three days. Different dilutions of the polyclonal antibodies were tested to find out an optimal concentration for the detection with anti- CMV CP antibodies. The results indicated that the CP can be detected in CMV-infected plant and in the purified preparation from *E. coli* by PTA-ELISA (Fig. 3). The optimal

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conditions for the diagnosis were 1:10 dilution of the leaf extracts, 1:1000 dilution of anti-CMV CP IgG in PTA-ELISA (Fig. 3).

Moreover, anti-CMV CP IgG and the conjugated IgG efficiently detected the CP and the virus in the infected plant tissue in DAS-ELISA (both diluted 1:500) (Fig. 4). However, there are reports of antibodies prepared against recombinant viral proteins, which do not detect the related virus in DAS-ELISA (Cerovska *et al.*, 2006; Korimbocus *et al.*, 2002) due to inability of the coated antibodies to react with native viral epitopes even though it was not the case in this study which it could be due to high yield the expressed protein and refolding the injected protein.

It is noteworthy that as reported previously, non-specific background reactions are not unusual in the western blotting when polyclonal antibodies prepared against recombinant proteins are used (Abou-Jawdah *et al.*, 2004; Cerovska *et al.*, 2012; Gulati-Sakhuja *et al.*, 2009; Xu *et al.,* 2006; Kumari *et al.*, 2001).

This study demonstrated expression of recombinant CMV CP from isolate B13 in *E. coli* and its application as an efficient immunogen in preparation of anti-CMV CP antibodies, which reacted efficiently with the CP as well as virus particles. This success has implications as to the virus detection which is critical in management of viral diseases.

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چکیده: ویروس موزاییک خیار یکی از ویروس‌های گیاهی شایع با دامنه میزبانی وسیع بیش از ۱۲۰۰ گونه گیاهی است. یکی از مؤلفه‌های محدودکننده ردیابی این ویروس در دسترس نبودن آنتی بادی اختصاصی ویروس علی‌الخصوص در کشورهای در حال توسعه است. تکنولوژی دی‌ان‌ای نوترکیب امکان سهل‌تر تولید آنتی بادی را بدون نیاز به تجهیزات پیشرفته فراهم کرده است. در تحقیق حاضر ژن پروتئین پوششی ویروس موزاییک خیار که در سازه pTZ57CMVCP همسانه‌سازی شد درون ناقل بیانی pET21a همسانه‌سازی شد و درون استرین Rosetta باکتری *Escherichia coli* منتقل شد. بیان CMV CP توسط الکتروفورز در ژل پلی‌آکریل آمید (SDS-PAGE) بررسی و با استفاده از وسترن بلات، سنجش لکه‌گذاری نقطه‌ای و الیزا توسط آنتی بادی ویروس موزاییک خیار تأیید شد. پروتئین بیان شده با استفاده از کیت خالص‌سازی T7.Tag تخلیص و به‌منظور تهیه آنتی بادی در بدن دو عدد موش استفاده شد. IgG تخلیص و IgG کانژوگه شده علیه پروتئین پوششی ویروس موزاییک خیار دارای اختصاصیت و حساسیت مناسب برای ردیابی پروتیئن پوششی بیان شده و هم‌چنین گیاهان کدو آلوده به CMV در الایزای غیرمستقیم، الایزای دوطرفه و وسترن بلات بودند. آنتی بادی‌های تهیه شده می‌توانند در تست‌های سرولوژیکی و سرومولکولی در مطالعه ویروس و غربال گیاهان آلوده استفاده شود. این اولین گزارش از تهیه آنتی بادی در برابر پروتیئن پوششی یک جدایه بومی ویروس موزاییک خیار می‌باشد.

واژگان کلیدی: آنتی بادی، پوشش پروتئینی، ویروس موزاییک خیار، *E.coli*، بیان، وسترن بلات

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