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Research Article

Production of polyclonal antiserum against beet western yellows virus coat protein expressed in *Escherichia coli*

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Abstract: Serological methods are commonly used methods for detection of viruses. Preparation of pure viral antigens is a crucial step in production of antibodies required for serological studies. In this research the gene encoding coat protein of a *Beet western yellows virus* (BWYV) isolate from Iran was amplified by PCR and was ligated into a bacterial expression vector (pET26b) to obtain pET-BWYV-CP clone. *Escherichia coli* BL21 was transformed with pET-BWYV-CP and expression of the recombinant coat protein was induced by IPTG. The expressed recombinant coat proteins were purified and used as an antigen for rabbit immunization. The antiserum was able to detect recombinant coat protein in total protein extracts of induced *E. coli* BL21 cells in western blot analysis.

Keywords: *Polerovirus*, Expression, Western blot, BWYV

Introduction

Beet western yellows virus (BWYV) is a species of the genus *Polerovirus* in the family *Luteoviridae* (Mayo and D'Arcy, 1999). This virus has a wide host range and infects almost 150 dicotyledonous plants including canola, wild radish, wild turnip, shepherd's purse, field pea, soybean, faba bean, chickpea, and subterranean clover (Hertel *et al.*, 2004). Virions of BWYV are icosahedral in shape, 25 nm in diameter and harbor a single-strand positive sense RNA of 5.6 kb in length which encodes for six proteins. BWYV is transmitted in a persistent (circulative, non-propagative) manner by several different aphid species among which *Myzus persicae* is the most important known vector. In sugar beets, BWYV causes foliar chlorotic spots that expand to the whole leaf and change its color to yellow. Older leaves tend to thicken and become brittle (Stevens *et al.*, 2005). In canola, the virus has the potential to cause stunting and stiffening of leaves (Hertel *et al.*, 2004).

Since there is no effective way to control plant viruses, timely detection and prevention of its distribution is very important. Enzyme-linked immunosorbent assay (ELISA) is a common, reliable and inexpensive method to test large number of samples in a short time. A BWYV ELISA system was optimized for the detection of less than 2 ng/ml of virus (Hewings and D'Arcy, 1984). This method requires a large amount of antibodies which their production depends on the availability of adequate viral antigen for animal immunization. In view of the fact that BWYV is a phloem-limited virus with very low concentration in infected plants, obtaining an adequately pure viral antigen is difficult. On the other hand, the production of antibodies based on purified virus preparations from propagative hosts would result in backgrounds in diagnostic methods due to contamination of the preparation with host proteins. To overcome these problems,

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recombinant structural- (Shams-Bakhsh and Symons, 2004; Cerovska *et al.,* 2010) or nonstructural- (Osman and Buck, 1991) viral proteins have been used for the production of diagnostic antibodies.

In this study, we produced a polyclonal antiserum against a recombinant BWYV coat protein expressed in *Escherichia coli* and investigated its potential for BWYV detection.

Materials and Methods

Plasmid DNA and bacteria

The coat protein gene of an Iranian isolate of BWYV was amplified from pTZ57R/T-BWYV-IR10 (Accession No. JX501663) (Zahedi Tabarestani *et al.,* 2012). Plasmid pET26b (+) (Novagen, USA) was used for recombinant protein expression in *Escherichia coli* BL21 (DE3).

Cloning

The BWYV coat protein gene was amplified by PCR using two specific primers BWYV-CP-F (5′ CGGAATTCGATGAATACGGTCGTGGGTAG 3′) and BWYV-CP-R (5′CTCAAGCTTTTT GG GGTTGTGGAATTGGC 3′) (the underlined nucleotides represent *Eco*RI and *Hind* III restriction enzymes sites, respectively) and the PCR products were purified by agarose gel electrophoresis using Gel Extraction Kit (Qiagen, Valencia, USA), were double digested with *Eco*RI and *Hind*III and were ligated into corresponding restriction sites of pET26b. The recombinant plasmid (pET-BWYV-CP) was transformed into *E. coli* DH5α by heat shock method (Sambrook and Russell, 2001). To verify the integrity of the plasmid, colony PCR with specific primers (BWYV-CP-F, BWYV-CP-R) and restriction digestion were performed to analyse the recombinant pET-BWYV-CP plasmids.

Expression of recombinant BWYV coat protein

Recombinant plasmid pET-BWYV-CP was transformed into *E. coli* strain BL21 (DE3) by the heat shock method. One transformed colony was cultured overnight at 37 °C in 5 mL Luria-Bertani (LB) medium containing kanamycin (0.05mg/ml). Then 100 µl of it was inoculated in fresh 5 mL LB supplemented with kanamycin until an OD_{600nm} of 0.7. At this stage, expression of the coat protein gene was induced by addition of isopropyl-D-thiogalactoside (IPTG) to a final concentration of 0.8 mM. To find best set up, cells were collected at 2, 4, 6, and 8 hours after induction at 37 °C by centrifugation at 13000 rpm for 1 min and pellets stored at-20 °C till the last sample was collected. Then pelleted cells were resuspended in loading buffer (25 mM Tris-HCl, pH 6.8, 192 mM glycine, 0.1% SDS, 0.5% bromophenol blue, 5% mercaptoethanol), boiled for 5 minutes at 96 °C, and total proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 100 V. Finally, protein bands were stained with Coomassie brilliant blue R250 for 2 h and destained overnight.

To test the solubility of the recombinant coat protein, pelleted cells were resuspended in lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8) and sonicated as described by Noueiry *et al.,* (1994) and centrifuged at 10000 rpm for 20 min; then, supernatant and bacterial pellet were separated by 12% SDS-PAGE.

Western blot analysis

Proteins from SDS-PAGE were electroblotted onto a nitrocellulose membrane for 1 hour at 100 V using a wet transfer cell (Bio-Rad, USA). Then the membrane was blocked overnight with 3% BSA, 0.1% Tween 20 in TBS buffer (TBS-T) at room temperature and proteins were incubated with 1:1000 diluted commercial monoclonal antibody prepared against purified virus (DMSZ, Germany) in blocking buffer for 1 h. After three washes of 5 min each in TBS-T (TBS + %0.1 Tween 20), proteins were incubated in blocking buffer containing anti-rabbit IgG-AP conjugate (1: 2500) for 1 h at room temperature. After three washes of 5 min each, proteins were incubated in 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma) in water until the desired band was visible and then the reaction was stopped with tap water.

Protein purification

To purify the recombinant BWYV coat protein the corresponding band was excised from 12%

SDS-PAGE gel and electro eluted in dialysis tubing filled with protein running buffer at 100 V for 6 h. Gel slices were then discarded and the purified protein was dialysed against water for 24 h at room temperature to remove free SDS and then concentrated by freeze-drying.

Immunizations

The freeze-dried BWYV coat protein was resuspended in sterile water, emulsified with an equal volume of complete Freund's adjuvant (Sigma, USA), and injected subcutaneously into a white, New Zealand rabbit. The first injection was followed by three booster injections at two-week intervals by mixing recombinant coat protein with an equal volume of incomplete Freund's adjuvant (Sigma, USA). Ten days after the last injection, blood samples were collected and allowed to coagulate for 1 hour at 37 ˚C, then stored overnight at 4 ˚C, and finally centrifuged at 5,000 rpm for 5 min. The antiserum was collected, aliquoted, and stored at-70 ˚C.

The working dilution of this antiserum was determined by western blot analysis using BL21 *E. coli* cells transformed with pET-BWYV-CP and different titrations from 1:50 to 1:45000

The specificity and sensitivity of the antiserum was evaluated by western blot analysis. Leaves from healthy and BWYV-infected canola plants (0.1 g) were ground in 2.5 volumes (w/v) of extraction buffer (0.5 M Tris-HCl pH 8.8, 2% SDS, 40% sucrose, 4% 2-mercaptoethanol), boiled for 3 min at 96 ˚C, and centrifuged at 4000 rpm for 3 min. Also, aliquots (18 µl) of the supernatant were mixed with 1 µg of the recombinant purified coat protein and analyzed by electrophoresis. Western blot analysis was carried out as described above, except that the proteins were probed with a 1:45000 dilution of antiserum against the recombinant coat protein. Plant extracts without recombinant coat protein, i.e. uninduced samples, and transformed *E. coli* BL21 cells with pET26b plasmid were used as negative controls.

Plate trapped antibody (PTA)-ELISA

Leaf samples collected from healthy and BWYV-infected canola plants were ground at a dilution of 1:10 (w:v) in extraction buffer (0.05 M Tris-HCl buffer, pH 8, containing 0.005 M EDTA, 20 g/l PVP, and 0.5 ml Tween 20). One hundred microliters of plant extracts were added to the microtiter plate and incubated at 4 ˚C overnight. Then the plates were washed three times with PBS-T and plates were blocked with 3% bovine serum albumin (BSA) in PBS-T at room temperature for 1 h. After three washes in PBS-T, antiserum diluted to 1:45000 with conjugate buffer (50mMTris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) was added to the plates and incubated at 37 ˚C for 1 h, followed by three washes in PBS-T. Anti-rabbit IgG-AP conjugate (diluted 1:2500 with conjugate buffer) was added and incubated at 37 ˚C for 1 h and, finally after three washes, substrate (1mg/mL ρ-nitrophenyl phosphate dissolved in 9.7% diethanolamine buffer, pH 9.6) was added and absorbance was measured at 405 nm.

Results

PCR amplification of the BWYV coat protein gene from a recombinant plasmid (pTZ57R/T-BWYV-IR 10) by the use of specific primers BWYV-CP-F and BWYV-CP-R produced a 624 bp fragment (Fig. 1). Colony PCR of transformed bacteria with recombinant plasmid pET-BWYV-CP yielded a same size bands as expected. After mini-preparation and double digestion of recombinant plasmids with *Eco*RI and *Hind*III, a 613 bp fragment was released, confirming the successful cloning (data not shown).

SDS-PAGE analysis of total protein extracts of induced *E. coli* BL21 cells transformed by pET-BWYV-CP showed the presence of a distinct band of about 25.9 kDa, this band was absent in negative control which was non-induced *E. coli* BL21 cells transformed with pET26b. The highest expression level of BWYV coat protein was obtained at 8 h after induction.

Based on the sequence of BWYV CP gene and flanged tags, the molecular weight of the recombinant protein was 25.9 kDa and consistent with that of the BWYV coat protein (22.98kDa) plus 11 and 7 amino acids (2.08 kDa) fused to the N-and C-terminals of the expressed protein, respectively, and 6 histidine residues (0.84 kDa).

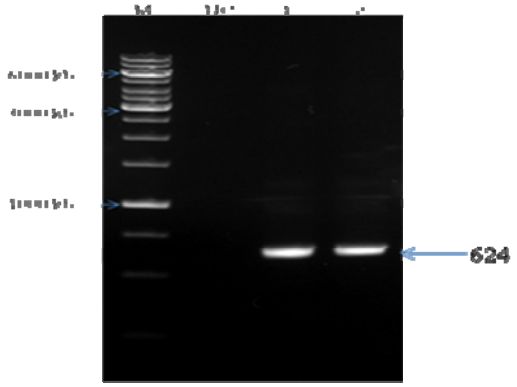

Figure 1 Agarose 1% gel stained with ethidium bromide showing PCR amplification of coat protein gene of BWYV using recombinant plasmid pTZ57R/T-BWYV-CL10 as template. Coat protein specific primers BWYV-CP-F and BWYV-CP-R (lanes 1 and 2) and NC represent negative control (PCR mixture with no template). M: standard DNA marker (SM 0313, Fermentas).

After sonication of transformed bacterial cells, pellets and supernatants of induced bacterial cultures were analyzed by SDS-PAGE. The highest amount of recombinant protein was insoluble and found in sonicated bacterial pellets, while little recombinant protein was observed in the supernatant.

The volume of transformed bacteria used for protein production had no effect on the level of coat protein expression (data not shown). Western blot analysis with a monoclonal antibody raised against purified BWYV could detect a band corresponding to the BWYV coat protein in total protein extracts of transformed *E. coli* BL21 cells. No reaction was observed in non-induced *E. coli* BL21 cells transformed with pET26b (Fig. 2).

Electroelution of the purified BWYV recombinant coat protein revealed its purity for immunization.

The working dilution of antiserum was determined at 1:45000 by western blot analysis. The lower titrations showed cross reaction with bacterial protein and higher titration showed weak signal with recombinant coat protein.

The antiserum prepared against the recombinant BWYV coat protein was able to react with the corresponding protein expressed in induced *E. coli* BL21 cells as well as mixture of purified recombinant coat protein and total protein extract of healthy canola plants in western blot. No cross-reaction was observed with plant or bacterial proteins. This antiserum was not able to detect BWYV in infected plants (Fig. 3)

No significant difference was observed between absorbance of healthy and BWYV-infected plants at 405 nm in PTA-ELISA using the prepared antiserum (data not shown).

Discussion

Recombinant plant viral coat proteins produced in bacterial cells are suitable alternatives of purified viral particles in production of antiserum (Dea *et al.,* 2000; Vaira *et al.,* 1996). In this work, the coat protein gene of BWYV was cloned and expressed in *E. coli* BL21 cells with no toxic effect. Toxicogenic effects of viral coat proteins in bacterial cells have been reported for a number of plant viruses (Jakab *et al.,* 1997; Shams-Bakhsh and Symons, 2004). However the expression vector pET26b contains the signal peptide sequence *pel* B to facilitate protein solubility, the expressed recombinant BWYV coat protein was insoluble. This result was in agreement with previous findings (Howard, 2005). Aggregation of recombinant proteins overexpressed in bacterial cells could result either from accumulation of high concentrations of folding intermediates or from inefficient processing by molecular chaperones. To obtain soluble expression, some strategies such as protein expression at reduced temperatures, using other *E. coli* strains [C41(DE3) and C43(DE3)], and modification of cultivation strategies have been proposed (Sørensen and Mortensen, 2005). To overcome this problem, duration of the induction period with IPTG was increased and the recombinant BWYV coat protein was successfully purified by electroelution of the corresponding band from SDS-PAGE via dialysis tubing (Sa´–Pereira *et al.,* 2000).

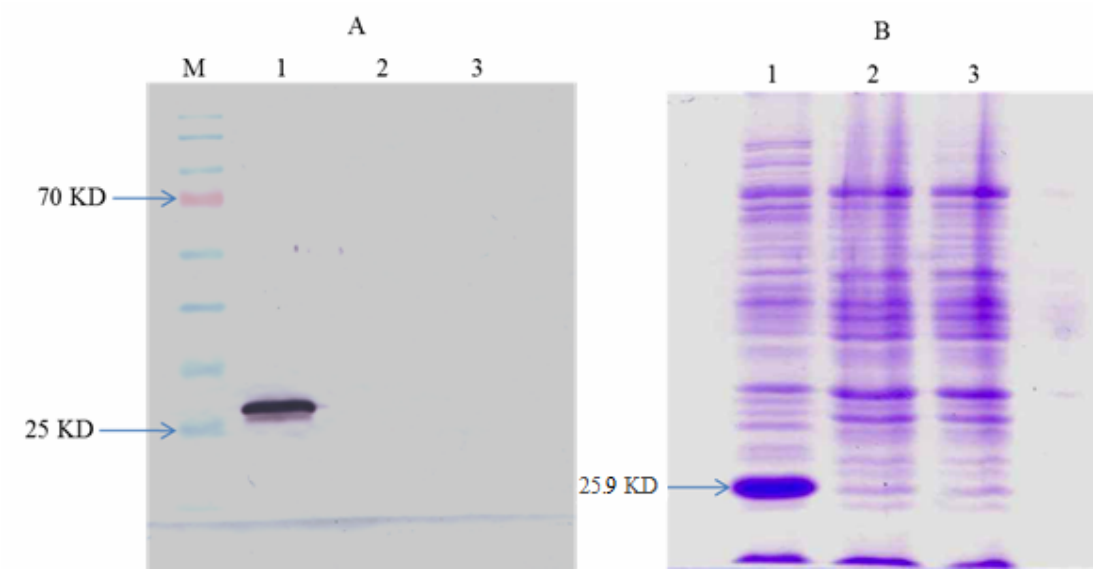

Figure 2 Western blot analysis of recombinant BWYV coat protein (A) followed by SDS-PAGE (12%) (B). The blot on nitrocellulose membrane was probed using commercial MAbs raised against purified virus. Lane 1: total protein extracts from induced cells. Lane 2: total protein extracts from uninduced cells. Lane 3: total protein extract from *E. coli* BL21 cells transformed with pET26b. M: pre-stained protein ladder SM0671 (Fermentas, Lithuania).

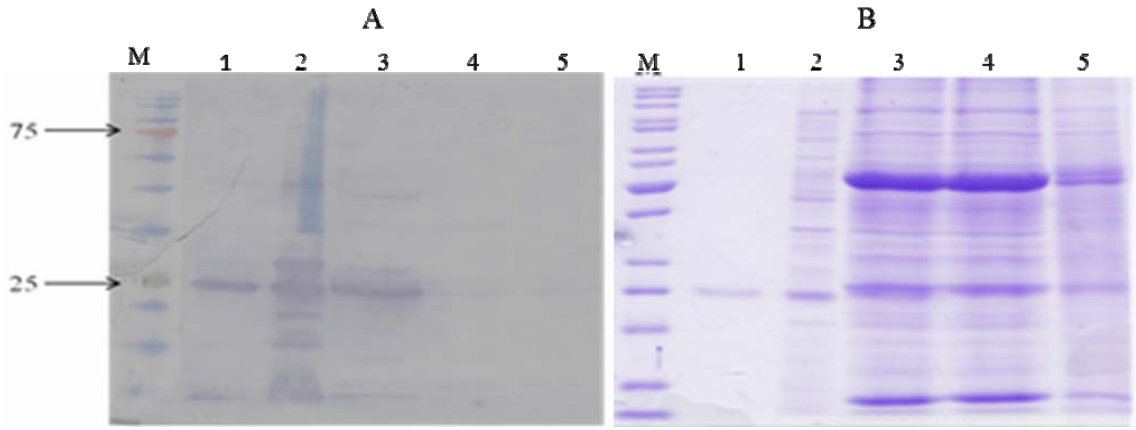

Figure 3 Western blot analysis with prepared antiserum against BWYV recombinant coat protein expressed in *E.coli* for BWYV detection followed by SDS PAGE (12%). Lane 1: purified recombinant coat protein. Lane 2: total protein extract from induced cells. Lane 3: mixture of the purified recombinant coat protein and the total protein extract of healthy canola plant. Lane 4: total protein extract from BWYV- infected canola plant. Lane 5: total protein extract from healthy canola plant. M: pre-stained protein ladder SM0671 (Fermentas, Lithuania).

Western blot analysis of expressed recombinant coat protein using a commercial antibody against native BWYV gave a positive signal, confirming the accuracy of the expressed recombinant coat protein. The polyclonal antiserum raised against the recombinant BWYV coat protein gave a strong reaction with the homologous protein from induced *E. coli* BL21 cells. However, the BWYV-CP antiserum did not detect BWYV in infected canola plant extracts in western blot and PTA-ELISA. Komorowska and Malinowski (2009) reported

similar results for the coat protein of *Apple stem pitting virus*. These authors suggested that removing of His-tag before immunization may improve the serological properties of the fusion protein. Moreover some modifications of immunization protocols seems to be beneficial.

On the other hand, this may result due to low concentration of luteoviruses in infected plants, because Shams-Bakhsh and Symons (2004) successfully applied the barley yellow dwarf virus-CP (a luteovirus) polyclonal antibody in western blot analysis with partially purified virus while, in immunogold localization in electron microscopy studies needed higher concentration of the virus particles.

In addition to our result, production of polyclonal antiserum against viral coat protein expressed in *Escherichia coli* has been reported for a number of plant viruses and these antisera have been used successfully for plant virus detections (Nikolaeva *et al*., 1995; Jelkmann and Keim-Konrad, 1997; Iracheta-Cardenas *et al*., 2008).

On the whole we believe that the advantages of viral coat proteins expressed in *E. coli*, for large scale production and avoidance of drawbacks of viral purification and contamination with plant proteins, make it an efficient strategy for antiserum preparation.

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تولید آنتی‌سرم چند همسانه‌ای علیه پروتئین پوششی ویروس زردی غربی چغندر بیان شده در *Escherichia coli*

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چکیده: روش‌های سرولوژیکی از متداول‌ترین روش‌ها برای تشخیص ویروس‌ها می‌باشند. برای انجام این روش‌ها آماده‌سازی آنتی‌ژن‌های ویروسی خالص مرحله‌ای مهم در تولید آنتی‌بادی‌ها است. در تحقیق حاضر ژن رمزکننده پروتئین پوششی جدایه ایرانی BWYV در طی واکنش زنجیره‌ای پلیمراز (PCR) تکثیر و در ناقل بیانی pET26b همسانه‌سازی گردید و pET-BWYV-CP به‌دست آمد. سپس سویه (BL21) باکتری *Escherichia coli* با pET-BWYV-CP ترانسفورم شد و بیان پروتئین پوششی نوترکیب با IPTG القاء گردید. پروتئین پوششی نوترکیب بیان شده خالص‌سازی و به‌عنوان آنتی‌ژن برای ایمنی‌زایی خرگوش استفاده شد. آنتی‌سرم تولید شده توانست پروتئین پوششی نوترکیب را در عصاره کامل پروتئین سلول‌های BL21 القاء شده توسط وسترن بلات تشخیص دهد.

واژگان کلیدی: پولرووویروس، بیان، وسترن بلات، BWYV