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Original Article

Applying of Recombinant Major Coat Protein for Production of Specific Antibody and Efficient Detection of *Citrus Tristeza* Virus (CTV)

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Abstract

Background and Aims: *Citrus tristeza* virus (CTV) is the most economically important pathogen of *citrus* throughout the world. To avoid the ominous effect of disease in *citrus* growing area, producing of virus free plants and elimination of infected plants are imperative. For this aim obtaining simple and sensitive diagnosis tools is crucial. The main objective of present study is applying of recombinant protein technology for production of specific antibody and developing of serological *assays* for efficient detection of CTV within infected plants.

Materials and Methods: The major coat protein (p25) of CTV was selected as a target for preparation of polyclonal antibody. The gene encoding p25 was recombinantly expressed in bacterial host and the protein was purified through affinity chromatography approach. The purified recombinant coat protein was used for immunization of rabbit. Specificity of the prepared serum against CP was confirmed through serological assay. The immunoglobulin molecules were purified from serum through staphylococcus protein A followed by conjugation to alkaline phosphatase (AP) and horse radish peroxidase (HRP) enzymes. The prepared antibodies and conjugates were used for detection of infected plants by double antibody sandwich- enzyme linked immunosorbent assay (DAS-ELISA) and dot-blot immune binding assay (DIBA).

Results: The p25 protein was expressed in bacterial host. The SDS-PAGE results confirmed high purity and integrity of CTV major coat protein with the expected size of about 29 kDa. The indirect ELISA results revealed that the antibody titer was around 1:65000. The IgG molecules purified through protein A column and SDS-PAGE results confirmed purity of the prepared antibody. The concentration of IgG was quantified by comparison to standard protein, BSA, which was estimated around 1 mg/ml. The results obtained from DAS-ELISA and DIBA assays proved that prepared antibodies could be effectively applied for detection of CTV infected plants.

Conclusions: The prepared antibody and conjugates were powerful tools for detection of infected plants. To the best of our knowledge this is the first work for applying of peroxidase enzyme in developing of ELISA assay against CTV.

Keywords: *Citrus tristeza* virus, Polyclonal antibody, Recombinant protein, Serological assay.

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Introduction

Citrus tristeza virus (CTV) is distributed worldwide and causes one of the most economically important virus diseases of *citrus* (1). The virus is transmitted by aphids in a semi-persistent manner (2) CTV has long, flexible and filamentous virions and a positive sense single strand RNA genome consisting around of 19296 nucleotides organized in 12 open reading frames and encoding at least 19 proteins (3, 4).

The RNA genome is encapsidated by two capsid proteins, a 25 kDa and 27 kDa protein identified as the major (CP) and minor (p27) capsid components, respectively (4, 5). CTV exists as a large number of distinct strains including widely differing symptoms in various species and cultivars of *citrus* (2, 3). The *tristeza* disease is thought to have been introduced to Iran on satsuma mandarin trees (*Citrus* unshiu) imported from Japan and planted in Mahdasht orchards in Mazandaran province, northern Iran between 1968 and 1970 (6). The use of serological methods, mostly the various forms of enzyme- linked immuno-sorbent assay (ELISA) (7-10) has become an indispensable tool for large-scale diagnosis of CTV worldwide, for both research and virus control purposes (2, 9, 11-13).

Antisera production for diagnosis of CTV, either as polyclonal antibodies (8, 14, 15) or as monoclonal antibodies (16), was commonly accomplished in the past by using purified virus preparations as immunogenes.

Raising polyclonal antibodies requires large amounts of purified virions to be used in immunization procedures. In addition, the antigen may not maintain its characteristics along successive purifications. The use of CTV coat protein (CP) expressed in and purified from an Escherichia coli system to raise polyclonal antibodies overcomes these limitat-ions, and avoids the production of antibodies against host proteins that may be present in the viral preparations (17). Production of antisera against bacterially expressed CTV coat protein (rCP-CTV) has been previously described (18, 19). However, with the sequencing of the cp gene from CTV isolates of the north, of Iran (20), CTV specific antisera have been raised against the recombinant coat protein expressed in bacteria for CTV diagnosis (19, 21, 22). This approach has been applied for developing of specific antibodies against several plant viruses (23-27). In the present paper, we describe the development of a specific serological kit for diagnosis of CTV in infected plants.

Methods

Expression and purification of CTV-CP. The bacterial expression vector harboring the gene encoding of coat protein, pET28a-CP (kindly provided by G. Hosseini Salekdeh, ABRII, Karaj, Iran), was used for production of recombinant CP. Transferring of the cons-truct to BL21 strain of E. coli, led to produc-tion of recombinant CP having a N-terminal 6x His tag. To produce recombinant CP, a bacteri-al colony consisting abovemen-tioned cons-truct, was grown on LB agar plate containing 50 µg. mL-1 Kanamycin, induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by affinity purification under native conditions through immobilized metal ion affinity chromatography (IMAC) in columns contain-ing Ni-NTA agarose beads (Qiagen, UK).

Protein concentration was measured in comparison with serial dilutions of bovine serum albumin as a standard protein using SDS-PAGE gels as described by Laemmli (leammli et al., 1970).

Integrity of purified protein was further evaluated by applying of Western Blot analysis. Therefore, purified CP protein separa-ted by SDS-PAGE. Protein was transferred to Milipore polyvinylidine difluoride (PVDF) membrane, blocked with Phosphate Buffer Saline (PBS) (137 mm NaCl, 2.7 mm KCl, 10 mm Na2HPO4, 2mM KH2Po4 pH7.4) containing 2% (W/V) skim milk powder and probed with a primary antibody (anti- His tag)

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at a dilution of 1/1000. The alkaline phosphatase conjugated secondary antibody was used at a dilution of 1/3000. The target proteins were finally revealed by adding substrate 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and Nitro blue tetrazolium (NBT) (Sigma, Diesen hofen, Germany).

Antibody preparation. Two white New Zealand rabbits were used for immunization. Five intramuscular injections in the hind legs were performed at intervals of two weeks. Each injection contained about 100 µg of recombinant CP (20 µL) protein and 500 µL of Freund's complete adjuvant, mixed with 480 µL PBS 1x for the first injection and incomplete Freund's adjuvant for the subsequent injections.

Indirect ElISA was performed to determine titer of the polyclonal antibodies. A micro titer plate (MaxisorpTM, Nunc, Roskilde, Denmark) was initially coated by 10 µg mL-1 of purified recombinant CP protein using a carbonate coating buffer (15 mM Na2Co3, 35 mM NaHco3, pH 9.6). Then the plate was blocked with 2% (W/V) skim milk in 1x PBS. serial dilutions of serum (1/512-1/262144) in 1x PBS was added to the coated plate and incubated at 37°C. Finally, the P-nitrophenyl phosphate (pNPP) as substrate (sigma) for AP conjugated antibody and ABTS subestrate for HRP conjugated antibody (Roche, Almere, Netherlands) was added and incubated at room temperature for 6-20 min followed by measurement of the OD at 405 nm using a ELISA reader (Teca, Austria). When antibody titer reached to preferred amount, blood was collected from rabbit hearts, 14 days after the last boosting. The serum fraction was collected and stored at -20°C.

Antibody purification from serum was performed using protein A spin column according to the manufacturer's manual (AbD serotec, UK). Concentration of purified antibody was estimated on SDS-PAGE.

Conjugation with enzymes. In order to apply the prepared antibody in ELISA, the purified antibody was subjected to conjugation with AP and HRP enzymes. The purified antibody was conjugated to HRP by applying periodate conjugation method (28) with slight modifications. For this aim, 10 mg of HRP was added to one mL double distilled water. 200 µL of freshly prepared 0.1 M Sodium periodate was added to the HRP solution and was stirred for 20 min at room temperature. After stirring, the solution was dialyzed against acetate buffer (1 mM, pH 4.4) for overnight at 4°C. After dialysis, the pH of the activated enzyme solution was adjusted to 9.0 by addition of 20 µL of 0.2 M carbonate-bicarbonate buffer (pH 9.5). After that, 200 µL of antibody solution (1 mg/ml) and 40 µL of carbonate-bicarbonate buffer (pH 9.5) were added to the solution immediately. It was then stirred at room temperature for 2h. After stirring, to separate the unreacted enzyme from the mixture by salt precipitation, an equal volume of saturated ammonium sulfate was added to the solution and was incubated at 4°C for 30 min.

Then it was centrifuged for 20 min at 4000 xg and the supernatant was discarded. The precipitate was dissolved in 1 mL saline and dialyzed extensively against several changes of PBS at 4°C. It was next ultra-filtered using an ultrafiltration membrane with an MWCO of 10 kDa for separating the conjugate from free unreacted enzyme.

The conjugate thus obtained was then preserved at -20°C. The AP was conjugated to antibody using LYNX rapid conjugation kit based on the manufacturer's manual (AbD serotec, UK).

Immunoassay analysis. In order to evaluate specificity of prepared antibody, infected plant samples, was tested using immunoassays analysis including, dot-immunoblotting and ELISA (29). 51 plant samples (shoots, fully expanded leaves and peduncles) from *citrus* yards of north and southern part of Iran (e.g., Saari from Mazandaran and Jiroft from Kerman provinces, respectively). The samples include orange, sweet orange, mexican lime trees showing chlorosis, vine clearing, and dieback. Presence of CTV in plant samples was evaluated by DAS-ELISA using prepared antibody and conjugate.

The wells were coated with purified anti-CP polyclonal antibody diluted to 1/1000 in PBS and incubated at 37°C for 2 hours. Extraction of infected and healthy plant was performed

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with mortar and pestle. Plant materials were extracted 1:10 (W/V) in extraction buffer (Tris buffer pH 7.4 containing 137 mM NaCl, 3 mM KCl, 2% PVP 24 kDa, 0.05% Tween 20 and 0.02 % NaN3). The plant extracts and purified protein control (CP positive control) were added to the plate and incubated overnight at 4°C. Next, the AP/HRP conjugated anti-CP polyclonal antibodies at dilution 1:1000 were added and incubated at 37°C for30 min. Absorbance values were read at 405 nm. The sample was positively identified if the mean DAS-ELISA (A405nm) value of samples exceeded at least twice the mean of the healthy control. The accuracy of results was re-assessed by applying trademarked ELISA kit (Bioreba, Switzerland).

Further serological analysis was performed using a Dot Immune Binding Assay (DIBA). Healthy and infected plants were extracted in extraction buffer. Then 4µL of each was disposed on nitrocellulose membrane.

Saturation of the free binding sites was performed with 2 % (W/V) skim milk in 1x PBS. The target protein was detected by 1:500 diluted AP labeled anti CP IgG. The bound antibody was revealed by addition of substrate NBT/BCIP.

Results

Production of recombinant CP protein. To produce sufficient protein for immunization of rabbit and further analysis, the major component of CP of CTV was selected for bacterial expression. For this aim, the construct pET28a-CP, harboring the coat protein of CTV was inserted and expressed in BL21 (DE3) strain of E. coli. The purification of recombinant CP protein was performed under native condition by using Ni-NTA columns in affinity chromatography method.

Samples from different steps of expression and purification were prepared and followed by SDS-PAGE analysis. This result revealed the high purity and integrity of CP and showed a protein with the expected size of about 29 kDa (Figure 1). Generally, the total yield of purified protein in the culture medium varied from 8 to 20 mg. mL-1.

Western blot analysis using anti 6x His tag monoclonal antibodies proved successful expression of CP in E. coli cell (Figure 2).

Antibody production and characterization. The purified CP with the optimized density of 100 µg. mL-1 was injected intramuscularly into rabbits. The antibody titer determined after each boosting. After 6 weeks, when the antibody titer exceeded 1:65000, blooding was performed and whole serum was isolated from other blood cells (Figure 3). IgG molecules were affinity purified by using staphylococcus protein A. The IgG monitored for purity by SDS-PAGE which appeared as approximately 25kDa and 50kDa bands. Also the concentration of IgG was quantified by comparison to standard protein, BSA, which was calculated at

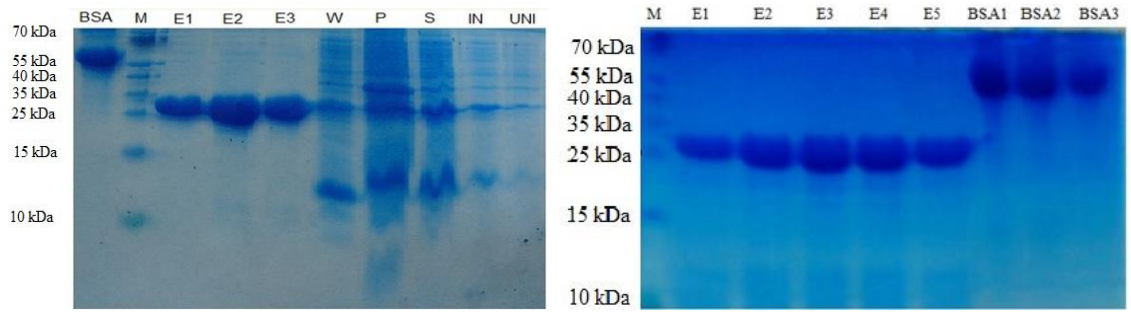

Fig. 1. Expression of recombinant CP in E. coli BL21 (DE3). Proteins were analyzed by SDS-PAGE, 12% Polyacryl amide gels were stained with Coomassie brilliant blue. A: analysis of total protein of E. coli. UNI: uninduced cells, IN: IPTG-induced cells, S: Supernatant, P: Pellet, W: after washing, E: elution steps, M: prestained protein marker (Fermentas SM0671). B: analysis of IMAC-purified recombinant CP., M: prestained protein marker (Fermentas SM0671), E1-E5: different elution steps of recombinant CP, BSA1-3: different concentration of BSA: 850, 1700 and 3500 µg mL^{-1}.

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Fig. 2. Western blot analysis of purified CP with Alkaline Phosphatase -conjugated polyclonal antibody at 1:500 dilution. M: prestained protein marker (Fementas SM0671), CP: Coat protein.

about 1mg mL-1 (Figure 4). The purified antibody was applied to preparing conjugation with AP and HRP.

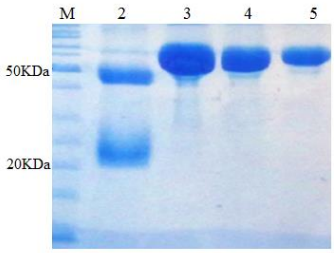

Fig. 4. IgG purified by using staphylococcus protein A. (M): Protein marker (Fermentas SM0661), (2): IgG purified, 3, 4 and 5: BSA different concentrations (3.5, 1.75 and 1 mg.mL-1, respectively).

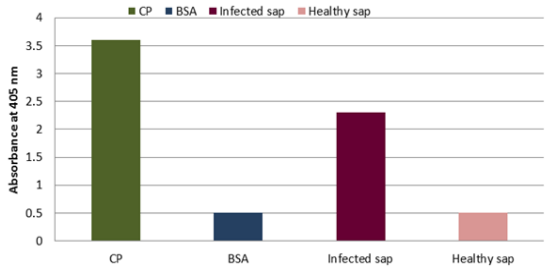

Fig. 5. Detection of infected plant samples using DAS-ELISA with polyclonal antibody prepared against recombinant CP protein, at a dilution 1:1500 (AP conjugated antibody were used).

Fig. 6. DIBA test for confirming the ability of purified antibody for distinguish infected and healthy sample. 1: recombinant CP protein, 2-4: infected samples, 5: healthy sample.

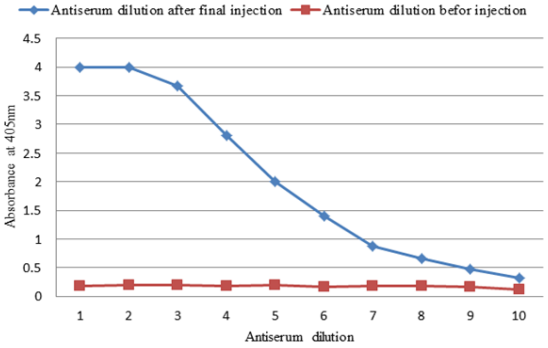

Fig. 3. Comparison of the optical absorption coefficient at 405 nm for different dilutions of antiserum. (1:1/512, 2:1/1024, 3:1/2048, 4:1/4096, 5:1/8192, 6:1/16384, 7:1/32768, 8:1/65536, 9:1/131072, 10:1/262144).

Immunoassay analysis. Ability of prepared antibody and conjugates for detection of infected plants was evaluated by DAS-ELISA and DIBA analysis. Applying of DAS-ELISA with AP conjugate led to successful detection and differentiation of infected samples from the healthy ones at a dilution of 1:1500 (Figure 5).

Complementary DIBA analysis was performed for evaluation the specificity of prepared conjugate antibody against recombinant and native CP protein. The results proved the binding ability of antibody against *Citrus tristeza* virus present in infected plant as well as against recombinant CP (Figure 6).

Applying of HRP for conjugation to immuno-globulin led to successful detection of infected plant (Figure7). The major advantage of this conjugate was the low background staining in negative control leading to higher accuracy in detection of infected samples. Beyond this,

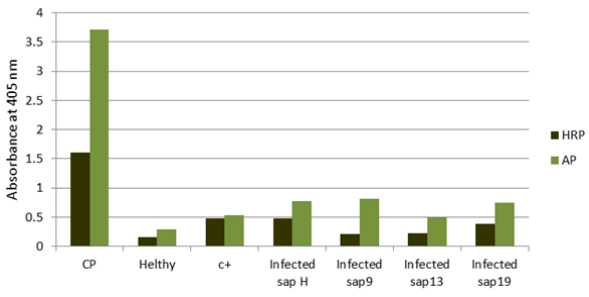

Fig. 7. Comparison of AP antibody conjugate, and HRP antibody conjugate after 15 minutes.

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higher concentration of conjugate, 1:200, needed for efficient detection of samples.

The prepared antibodies were applied for detection of infected plants collected from *citrus* growing area from north, Sari, and southern, Jiroft, part of Iran. The samples obtained from *citrus* plants showing decline in foliage with no fruit and new shoots. The midrib and bark of plant samples were used in DAS-ELISA by using prepared antibodies. The results revealed that 17 samples of *Citrus* plants collected from northern part are infected with CTV while no positive results received from Jiroft in southern area (Figure 8).

Generation of specific antibodies against plant viruses is greatly complicated due to the problems associated with obtaining pure material for immunization. It is confirmed that recombinant viral coat protein can be satisfactory applied as antigen for developing of specific antibodies against plant viruses (30).

In present study the CTV recombinant coat protein was used for immunization of rabbit and preparation of a polyclonal antibody against the *tristeza* disease. The alkaline-phosphatase (AP) and horseradish peroxidase (HRP) labeled the pathogen and exhibited a high specificity. Both polyclonal and

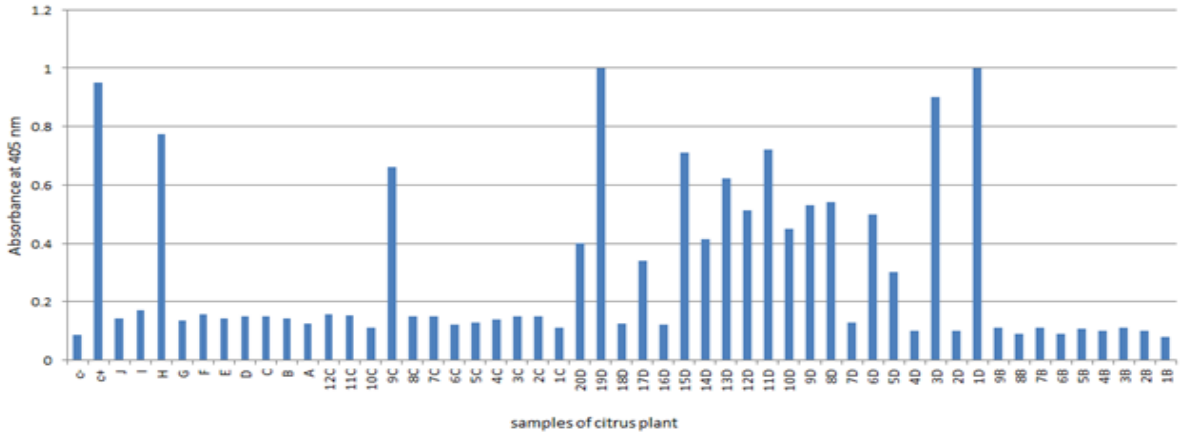

Fig. 8. Plant samples suspected disease compared with DAS-ELISA, by AP conjugated polyclonal antibody prepared against recombinant CP protein, at a dilution 1:1000, Healthy sweet orange was used as negative control.

Discussion

The *tristeza* is the major disease of *citrus* throughout the word. The most important strategies against the disease are use of virus free plants and prevent dispersion of disease in regions by applying of quarantine strategies and eradication of infected plant. For this aim, accessibility to fast and rapid diagnostic methods is a major issue. Present article describes producing of specific antibodies and developing of serological assays for efficient detection of infected CTV plants. The samples gathered from symptomatic plants in *citrus* gardens both northern and southern area of Iran, which it is commonly believed they are different in pathogenicity behaviors.

monoclonal antibodies have generated against CTV for their detection and differentiation (11, 31). The antibody generated against CP can be used for detection of procedures and also for other purpose like the characterization of secretion pathway or the study of host-pathogen interaction.

Antibodies against CP of CTV have been proven to be very powerful tools to detect the pathogen. Applying of produced antibody for detection of infected plants collected from different area of Iran revealed that it could be effectively used for different isolates of CTV disseminated in north area of country.

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