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Use of a recombinant protein for development of a DAS-ELISA serological kit for sensitive detection of witches' broom disease of lime

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Abstract

Witches' broom disease of lime (WBDL), associated with '*Candidatus* Phytoplasma aurantifolia' is the most devastating disease of acid lime in Southern Iran. Lack of an efficient approach for control of the disease has resulted in application of quarantine measures for protection of healthy plants and to limit the spread of disease to uninfected areas. Toward this aim, development of a rapid and efficient method for detection of infected plants is a major focus. The present study introduces application of recombinant DNA technology for development of a sensitive serological technique (DAS-ELISA) for detection of infected plants.

The immunodominant membrane protein (IMP), as a major protein present on the surface of phytoplasma cells, was selected as a target for generating specific antibody molecules. The gene encoding IMP of '*Ca*. P. aurantifolia' was obtained from infected plants. The region encoding the IMP fragment was isolated by PCR amplification followed by insertion into the pZ57R/T cloning vector. Intact clones containing the right sequences were selected and sub-cloned into the pET28a bacterial expression vector. Large scale expression of recombinant protein was performed in *E. coli* and purification was carried out through affinity chromatography in Ni-agarose columns.

To obtain specific polyclonal antibodies against WBDL, the purified recombinant IMP was used for rabbit immunization. The antisera titer was determined after each boosting via indirect ELISA. When the titer reached 1:100,000, the animal was sacrificed, blood was collected and serum was separated from blood cells. The IgG molecules were purified from serum content by affinity chromatography using protein A columns followed by conjugation to alkaline phosphatase (AP) enzyme. The purified specific antibodies and conjugate were used for detection of the corresponding antigen, IMP in infected plants in DAS-ELISA and dot-blot methods. The results confirmed the capability of this technique for efficient detection of infected plants, while no reaction was observed in negative controls. The detection limit of the DAS-ELISA method was determined at 70 µg IMP/ml leaf extract.

Key words: Witches' broom disease of lime, DAS-ELISA, IMP, phytoplasmas.

Introduction

The witches' broom disease of lime (WBDL) was observed in Iran in 1997 (Bové *et al*., 2000). The disease destroys thousands of trees yearly throughout these regions and it is associated with '*Candidatus* Phytoplasma aurantifolia' (Zreik *et al*., 1995).

The IMP proteins are predominant proteins located on the external surface of the cell membranes of phytoplasmas and could be used as a target for efficient detection of infected plants. They play important roles in the attachment of the bacteria to their host cell surface, and are involved in phytoplasma-host interactions (Kakizawa *et al*., 2010).

There are several diagnostic techniques to detect phytoplasmas. Molecular methods, especially the polymerase chain reaction (PCR), are specific and sensitive (Namba *et al*., 1993). Serological methods applying specific antibodies are convenient and economic in that many samples could be analyzed in a short time in a lab with minimal equipment.

This paper describes application of the recombinant IMP protein for production of polyclonal antibodies with high binding ability and development of a specific DAS-ELISA serological kit for efficient detection of WBDL.

Materials and methods

Total DNA was extracted from the midribs of healthy and infected lime plants as described by Zhang *et al.* (1998). The gene encoding the IMP protein of the phytoplasma was obtained by PCR amplification using specific primers and inserted into a pTZ57R/T vector.

The IMP coding region previously cloned in pTZ57R/T was digested by using *Sal*1/ *Not*1 restriction enzymes and sub-cloned into the pET-28a bacterial expression vector. The plasmids obtained from the recombinant clones were used to transform *E. coli* BL21(DE3) competent cells. The expression and purification of recombinant IMP from bacterial cultures was accomplished. Recombinant proteins were purified by a Ni-agarose column. Protein concentration was measured using comparative analysis with a known concentration of BSA protein.

Two white inbred rabbits were used for immunization. Five injections were given at intervals of 2 weeks. Each injection containing about 100 µg was carried out intramuscularly in the hind legs with an emulsion of equal volumes of IMP protein and Freund's complete or incomplete adjuvants. Animals were bled 4-5 times from the marginal ear vein at 14 day intervals for estimation of antibody titer by indirect ELISA.

Finally, blood was collected from rabbit hearts 14 days after the last immunization. The blood was clotted at room temperature and then the serum was separated by centrifugation.

Purification of polyclonal antibodies was performed by using protein A columns in an affinity chromatography method. Purity and concentration of polyclonal antibodies was measured by SDS-PAGE electrophoresis. Conjugation of purified immunoglobulin to alkaline phosphatase was accomplished using a conjugation kit (AbD serotec, UK).

The purified polyclonal antibodies and conjugate were used for detection of infected plants by DAS-ELISA, western blotting and Dot immunobinding assay (DIBA) methods.

In order to determine the detection limit of the constructed kit, a real time PCR analysis using specific primer pairs amplifying a 158-bp DNA fragment of the IMP gene was performed.

Results and discussion

The IMP proteins are predominant on outer membranes of phytoplasma cells and therefore are ideal targets for development of serological techniques for efficient detection of phytoplasma infected plants. The IMP gene of 540 bp encoding the immunogenic protein of the phytoplasma agent was amplified using specific primers. Database alignment of the sequences obtained using the universal M13 primer revealed a 100% homology with the accession number GU339497 in the NCBI database associated with '*Ca.* P. aurantifolia'. The digested IMP gene was then cloned into the pET-28a bacterial expression vector, transferred into the competent cells resulting in the successful expression and production of the recombinant IMP fused to a 6-His tag. The IMP produced by the clones was purified and its integrity was measured on an SDS PAGE gel at 35 kDa. The concentration of purified IMP was measured at around 6 mg/ml obtained from one liter culture medium. Western blot analysis using specific anti-His tag monoclonal antibodies confirmed the presence of recombinant fusion protein in the right position.

The specific polyclonal antibodies were obtained by intramuscular immunization of rabbits by the purified recombinant IMP protein. The antibody titer was determined after each immunization by indirect ELISA. When the antibody titer exceeded 1:100,000, the full serum including the whole antibodies was obtained from rabbits. Purification of IgG was performed by affinity chromatography using staphylococcus protein A columns. The purity and integrity of purified antibodies were evaluated by SDS-PAGE analysis. These results revealed the presence of two bands on the gel around 25 and 50 kDa belong to light and heavy chains of antibodies, respectively. The comparative analysis using BSA as standard protein showed the concentration of purified IgG to be about 0.5 mg/ ml.

To make conjugate, purified antibody was fused to the alkaline phosphatase enzyme. The conjugate and purified antibodies were used in DAS-ELISA, western blotting and DIBA serological analyses by using plant extracts and purified IMP as controls. These results confirmed the ability of the antibodies to efficiently detect infected plants. The sensitivity of the constructed kit was found to be as high as 100%. The optimum dilution of conjugate and purified antibodies were measured around 1:1,000. In order to quantify the detection limit of the constructed DAS-ELISA kit for the phytoplasma agents in the samples, real time PCR was used and it was determined around 70 µg IMP/ml leaf extract. To determine cross reactivity of the polyclonal antibody with other phytoplasma agents, DAS-ELISA and DIBA analyses were performed by using infected sesame, alfalfa and almond. These results showed no reaction when the leaf extracts obtained from the phytoplasma-infected sesame and almond samples were used. However, there was a positive reaction against the phytoplasma from alfalfa.

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