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AN IDEAL METHOD FOR SEPARATION OF PROTEIN FROM PURIFIED COCONUT ROOT (WILT) PHYTOPLASMA

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ABSTRACT

Separation of protein component from phytoplasma is important for its characterization and use as antigen for production of phytoplasma-specific antiserum, since proteins make the best antigens. Protein was isolated from purified phytoplasma of coconut root (wilt) disease by three methods and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Comparative analysis of different methods showed that the method in which water-saturated phenol with 8-hydroxyquinoline and 0.1% sodium dodecyl sulphate were used, yielded more undegraded and readily soluble proteins that were distinctly separated in SDS-PAGE. The other two methods wherein water-saturated phenol with 8-hydroxyquinoline and liquid phenol alone was used, gave much lower protein yield that were insoluble and degraded in electrophoresis. SDS-PAGE analysis of the proteins fractionated by the three methods showed that 2 bands resolving at 28 and 29 kDa were observed only in the case of phytoplasma proteins isolated by water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS method, which were observed as a single band in the other two methods. These results thus indicate that the water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS method is most suitable for separation of protein from purified phytoplasma.

INTRODUCTION

Detection of phytoplasma in yellows diseased plants is possible using serological methods utilizing monoclonal and polyclonal antisera and these antisera were made with partially purified phytoplasma preparation from host plants (Lin and Chen, 1986 and Gomez *et al.*, 1996). After the implication of phytoplasma as etiological agent of root (wilt) disease of coconut (Solomon *et al.*, 1983a), one of the objectives has been to produce pathogen - specific antiserum, which can be used in a serological test. Solomon *et al.*, (1983b) have developed a serodiagnostic test for identification of root (wilt) disease of coconut, which is based on a polyclonal antiserum raised to partially purified disease - associated antigen. However, the specificity of this test relies on elimination of common antibodies in the antiserum owing to plant materials in the antigen preparation.

Antiserum for culturable mollicutes like *Spiroplasma citri* was made against *in vitro* cultured organisms (Clark *et al.*, 1983). Such an approach is not possible for non-culturable phytoplasmas like root (wilt) phytoplasma of coconut. Recently, root (wilt) phytoplasma has been purified from coconut (Mayilvaganan *et al.*, 2000). In the production of antiserum, though phytoplasmas as such can be used as antigen, protein component of phytoplasma is preferable because proteins are highly immunogenic that would induce better immune response. This paper describes various methods tried for separation of protein from purified coconut root (wilt) phytoplasms. Methods for isolation of protein are also of interest for biochemical characterization of phytoplasma protein.

MATERIALS AND METHODS

Source of Phytoplasma: Phytoplasma was originally isolated and purified from coconut with root (wilt) disease by Percoll-gradient centrifugation method (Jiang and Chen, 1987 and Mayilvaganan *et al.*, 2000). Several purification schedules were carried out to collect sufficient phytoplasma fraction. The final phytoplasma preparation was collected in

phosphate buffer saline (PBS), pH 7.0 and dialyzed in PBS.

Protein Isolation: Proteins were separated from purified phytoplasma preparation by three methods, which were originally used for separation of protein component from isolated purified viruses (Ralph and Bergquist, 1967) and followed with operational modifications for separation of protein from phytoplasma. The methods were compared for their suitability for separation of protein by quantifying the proteins by Lowry procedure (Lowry *et al.*, 1951) and by analyzing the separated proteins by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS - PAGE).

Method-1: About 10 ml of phytoplasma preparation was mixed with an equal volume of water-saturated phenol (100 g phenol with 40ml distilled water and 0.14g 8-hydroxyquinoline) and 10% sodium dodecyl sulphate was added to adjust the mixture a final concentration of 0.1% SDS. The mixture was gently rotated for 10 min in a semiclear centrifuge tube and centrifuged at 12,000 g for 10 min to separate the emulsion into lower phenolic phase containing protein component of phytoplasma, upper aqueous phase containing nucleic acid of phytoplasma and a thin interface of gelled material. The lower phenolic phase was removed carefully with a micropipette. The aqueous phase was re-extracted twice with 5 ml of water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS mixture. To the pooled total phenolic phase, 30 ml of methanol and several small crystals of sodium acetate were added and stored at 4°C overnight. The protein precipitate was pelleted by centrifuging at 12,000g for 10 min. The protein was washed thrice with methanol and once with peroxide-free ether, and dried in air to powder. The protein was dissolved in sterile double distilled water in the concentration of 0.4 µg/ml by warming at 60°C.

Method-2: Ten ml of phytoplasma preparation in PBS was mixed with 10 ml of water - saturated phenol and 8-hydroxyquinoline mixture. The mixture was gently shaken for 5-10 min and allowed to stand for another 10 min which was centrifuged at 12,000 g for 10 min to obtain a clear lower phenolic phase containing protein component of phytoplasma. After collecting the phenolic phase, the upper aqueous phase was re-extracted with 5 ml of extraction mixture (water-saturated phenol and 8-hydroxyquinoline). The phenol collections were pooled together and the total phenolic phase was mixed with 30 ml of methanol and small crystals of sodium acetate, which was kept at 4°C for precipitation of protein. The protein was collected by centrifuging at 12,000 g for 10 min and the protein pellet was washed three times with methanol and several times with ether, and dried in air. The protein powder was dissolved in sterile water at a concentration of 0.4 µg/ml.

Method-3: Ten ml of phytoplasma preparation was mixed with 6 ml of liquid phenol, which was gently shaken for 5-10 min and allowed to stand for 10 min. The mixture was centrifuged at 12,000 g for 10 min to obtain clear phenolic phase containing protein component of phytoplasma. The phenolic phase was collected and the aqueous phase was twice re-extracted with 2 ml of phenol. The total phenolic collections were mixed with 50 ml of methanol and a pinch of

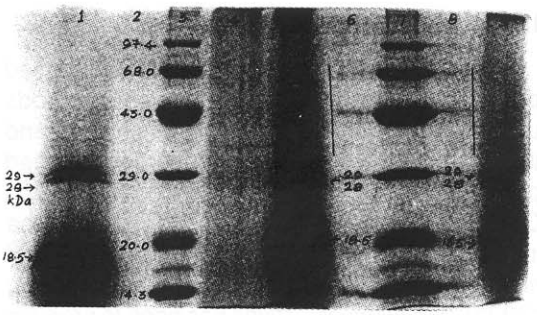

Fig. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis. (SDS - PAGE) pattern of proteins (samples containing 0.2 µg of protein) separated from purified coconut root (wilt) phytoplasma preparation; lane 1, method-1 (water - saturated phenol, 8-hydroxyquinoline and 0.1% SDS); lane 3 and 7, relative molecular masses of standard proteins; lane 4, corresponding preparation from healthy coconut; lane 5, method-2 (water-saturated phenol and 8 - hydroxyquinoline); lane 9, method-3 (liquid phenol).

sodium acetate crystals, which was kept at 4°C for precipitation of protein. The protein was collected by centrifuging at 12,000 g for 10 min and washed with methanol and peroxide-free ether several times. The protein was dried, and dissolved in sterile double distilled water in a 0.4 µg/ml concentration after estimation by Lowry method.

SDS - PAGE of Protein: Proteins separated by three methods were processed for electrophoresis according to Laemmli (1970). Before electrophoresis, each protein sample in a concentration of 0.4 µ/ml was mixed with solublisation sample buffer (0.5 Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 2% β - mercaptoethanol and 1% bromophenol blue) 1:1 v/v and heated at 98°C for 4 min. Similarly standard marker proteins in the concentration of 3 µg/ml were processed. Protein samples were resolved on a discontinuous gradient gel with 4% acrylamide in stacking gel and 12% in the resolving gel in a Bio-Rad Protien dual slab cell (Richmond, CA) and run at 40 mA till the tracking dye reached the separating gel, after which the gel was run at 60 mA for 120 min. The electrophoresed gels were stained in 0.1% Coomassie Brilliant Blue R - 250 overnight and destained.

RESULTS AND DISCUSSIONS

Separation of Protein: Initial attempts to isolate protein from two or three ml of phytoplasma preparation resulted in little protein yield, which was observed as faint bands on electrophoresis indicating low titre of phytoplasma cells in the purified preparation. This could be due to low concentration of phytoplasma in coconut as generally woody plants contain less concentration of phytoplasma (Thomas, 1979; Solomon, 1997). Hence, phytoplasma preparation used for protein separation was increased to 10 ml to obtain more protein.

The quantity of protein obtained and pattern of resolution of proteins in SDS - PAGE widely varied depending upon the separation method. Among the methods adopted for separation of protein from phytoplasma, the method-1, wherein water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS used was most consistent in terms of protein yield, solubility in water and distinct separation pattern of proteins in SDS- PAGE. The method-1 generally yielded about 0.5 mg protein, which corroborates low titre of phytoplasma in the purified preparation. Seddas *et al.*, (1995) had isolated 1.8 mg of protein from five ml fraction of flavescence doree phytoplasma obtained from 12 g of fresh tissue of broadbean. The methods-2 and -3, in which water-saturated phenol with 8-hydroxyquinoline and liquid phenol alone used respectively gave much lower protein yield of around 0.3 mg protein. In method-3, mechanical treatment of vigorous shaking of the mixture (phytoplasma preparation and phenol) apparently did not improve protein yield. The use of 0.1% SDS has helped in lysing the phytoplasma cells and releasing proteins from membranes and also acted as protein solubilizer (in Method -1).

When the color and solubility of proteins obtained by various methods compared, the protein obtained by water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS was light brown in color and readily soluble in sterile water when the protein was warmed to 60°C which remained in solution on cooling. In contrast, the protein obtained from phytoplasma by water-saturated phenol and 8-hydroxyquinoline was light brown in color, however, the protein was only partially soluble in water even after heating to 60°C. The phenol - prepared protein (Method -3) was dark phenolic color and partially soluble. The color and insolubility of proteins obtained by methods -2 and -3 could be due to aggregation of proteins by phenol - protein complex formation. Despite several washings of protein pellets with peroxide-free ether, the phenol residues from proteins (from Methods -2 and -3) could not be removed. The function of 8-hydroxyquinoline is to act as chelating agent in the phenolic phase, denaturing the metal-bound degradative proteins (Ralph and Bergquist, 1967). The ideal proportion of methanol to phenolic phase was found to be 5:1 for better protein yield. The quantity of sodium acetate did not have any effect the precipitation of protein in phenolic phase.

Protein Separation Pattern in SDS-PAGE: When the various methods were compared in terms of separation of protein pattern in

electrophoresis(Fig), the method-1 (water-saturated phenol, 8-hydroxyquinoline and 0.1% SDS) provided best result of distinct separation of protein bands. As shown in the Figure, only three proteins of 29, 28 and 18.5 kDa (lane 1) were seen without any minor peptides from phytoplasma proteins isolated by method-1. The methods-2 (water- saturated phenol and 8-hydroxyqinoline) and -3 (liquid phenol) revealed that coinciding with reduction in 18.5 kDa protein, many new peptides of smaller molecular weight have appeared (lanes 5 and 9) but not in the protein obtained by method-1. The detection of many minor peptides in electrophoresis from proteins obtained by methods 2 and 3 could be due to degradation of 18.5 kDa protein into smaller fragments during separation of protein from purified phytoplasma preparation. Moreover, the two bands resolving at 28 and 29 kDa could be observed only in the phytoplasma protein isolated by water -saturated phenol, 8-hydroxyquinoline and 0.1% SDS method. The same proteins had appeared as single protein entity (Figure, lanes 5 & 9) in the phytoplasma proteins obtained by methods 2 and 3. In addition, the appearance of 18.5 kDa protein was not distinct by the methods 2 and 3 owing to association of phenol with protein. These results indicate that the water-saturated phenol with 8-hydroxyquinoline and use of 0.1% SDS is suitable for separation of protein component from purified phytoplasma.

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