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Purification and Amplification of Garlic Latent Virus

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마늘 잠복 바이러스의 순수분리 및 증식

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ABSTRACT

To understand the molecular structure and pathogenesis mechanism of garlic latent virus (GLV), it was purified by serial infection in *Vicia faba* which shows local necrotic spot by inoculation. For the preparation of GLV in large quantities, it was amplified in leek which was considered as a systemic host for GLV. GLV particles showed 690 nm long in average and particle shape was slightly curved filamentous rod. Particle length of garlic viruses from mosaic diseased garlic ranged from 200 nm to 2000 nm, but most of the particle was in the range of 600-900 nm. The structural protein of garlic viruses isolated from mixed-infected garlic leaves distributed between the molecular weight range of 24,500-38,000 Da but the molecular weight of GLV coat protein was 34,000 Da.

INTRODUCTION

Garlic (*Allium sativum* L.) is an important vegetable crop for the Korean people and has long been cultivated extensively. Garlic mosaic disease is the most prevalent in the field throughout Korea and is considered to be the most important disease of garlic. Two sap-transmissible elongated viruses from garlic plants were described and named garlic latent virus (GLV) and garlic mosaic virus (GMV) based on the symptoms produced in infected garlic plants and the observation by electron microscope (Lee *et al.*, 1979; La and Choi, 1987; Chang *et al.*, 1988). GLV was assigned to be a carlavirus of around 650-700 nm long flexuous rod shape and GMV to potyvirus of about 750-800 nm long on electron micrograph. Mixed infection of GLV and GMV was found in most of the garlic plants showing mosaic symptoms collected from various parts of Japan and Korea (Lee *et al.*, 1979; La and Choi, 1987; Chang *et al.*, 1988).

Garlic plants inoculated with GLV produced visible symptoms at early stage of infection but less prominent at later stage, whereas infected broad beans (*Vicia faba*) produced systemic necrotic spots. *Chenopodium amaranticolor, C. quinoa* and *Tetragonia expansa* infected with GLV also produced local necrotic and chlorotic lesions, differently from with GMV (La, 1972; Lee *et al.*, 1979; La and Choi, 1987). Leek (*Allium porrum* L.) was found to be a useful systemic assay host for garlic viruses (La and Choi, 1987).

To study molecular structure of GLV, purification of GLV from mixed infected garlic plants was undertaken by serial infection on local lesion host, broad bean. Identification of GLV amplified in systemic host, leek, is described in this report.

MATERIALS AND METHODS

Host plants. Garlic (*Allium sativum* L.) samples used in this experiment were grown at the experimental farm of Seoul National University. To purify GLV, broad bean (*Vicia faba*) was employed as a local lesion host and leek (*Allium porrum* L.) as a systemic host (La, 1972; La and Choi, 1987).

Virus inoculation. For virus inoculation test plants were treated with carborundum (500 mesh) and inoculated with expressed sap from excised lesions macerated with 5 volumes of 0.05 M phosphate buffer (pH 7.3) containing 0.01% 2-mercaptoethanol and 0.1 M sodium diethyldithiocarbamate (1 : 5, w/v) (La and Choi, 1987). Necrotic spots on broad bean developed after 2 weeks of inoculation. But leek plants were examined for the presence of virus particles after 2 months of inoculation.

Virus preparation. GLV particles were isolated from the inoculated leaves of leek by the procedure described by Langenberg (1973). After 2 months of inoculation leaves of leek were ground in cold TAC extraction buffer (2.5 ml/g leaf tissue) with sea sand in pre-chilled mortar. TAC extraction buffer consisted of 0.1 M Tris, pH 7.2, 0.05 M citric acid, 0.8% polyvinyl pyrrolidone and 0.2% 2-mercaptoethanol. Extract was transferred and 1 m*l* of 2.0 M $CaCl_2$ and 2 m*l* of 2.0 M K_2HPO_4 were added for each 50 m*l* extract with stirring. It was centrifuged for 10 min at 10,000 rpm with JA-20 rotor at 4°C. To the supernatant recovered, Triton X-100 and polyethylene glycol MW 6,000 (PEG 6,000) were added to make final concentrations of 0.5% and 6%, respectively. The solution was stirred for 1 h at 4°C and stood for at least 1 h at 4°C. The precipitate was centrifuged for 20 min at 10,000 rpm with JA-20 rotor at 4°C. Pellets were resuspended in cold TACm buffer (0.01 M Tris, pH 7.2; 0.005 M citric acid; 0.01 M 2-mercaptoethanol; 0.1% Triton X-100) to give a final volume one-twentieth of the extract. The resulting suspension was centrifuged for 10 min at 10,000 rpm with JA-20 rotor to remove insoluble matter. The supernatant was overlaid on 30% sucrose dissolved in TACm buffer and centrifuged for 1 h at 68,000×g with fixed angle rotor at 4°C. The pellet was dissolved in TACm buffer and transferred into an Eppendorf tube. Insoluble matter was removed by centrifugation for 1 min at 10,000 rpm. The supernatant was overlaid on linear 10~40% sucrose density gradient and centrifuged for 2 h at 55,000×g with aluminium swing-out rotor at 4°C. Centrifuged fractions were scanned photometrically at 260 nm and analyzed by SDS-PAGE.

Electron microscopy of virus particles. Inoculated plants were examined for the identification of virus particles by leaf-dip preparations (Hitchborn and Hills, 1965) using transmission electron microscope (TEM) (Hitachi-800). Sample grid was coated with formvar, carbon and then charge-treated. The virus was fixed with equal volume of 2.5% glutaraldehyde solution, negatively stained with 1% uranyl acetate or 2% phosphotungstic acid for 10 sec, and then observed with TEM.

SDS-polyacrylamide gel elecrtrophoresis. Protein samples were electrophoresed on an SDS-containing discontinuous polyacrylamide gel electrophoresis system (Choi and Dreyfuss, 1984). The separating gel was prepared from a stock of 33.5% acrylamide and 0.3% N,N'-bisacrylamide to a final concentration of 12.5% acrylamide. The separating gel buffer contained 0.38 M Tris-HCl, pH 9.1. The stacking gel was prepared from a stock of 30% acrylamide and 0.44% N,N'-bisacrylamide to a final acrylamide concentration of 4% in 0.125 M Tris-HCl, pH 6.8. Both gel contained 0.1% SDS and were polymerized with ammonium persulfate and N,N,N',N'-tetra-methylenediamine. The electro tank buffer was 25 mM Tris and 192 mM glycine containing 0.1% SDS. Samples were prepared by boiling for 3 min in a 0.125 M Tris-HCl, pH 6.8, buffer containing 5% 2-mercaptoethanol, 1% SDS, 10% glycerol and bromophenol blue.

RESULTS AND DISCUSSION

Purification of garlic latent virus from mixed-infected garlic. To purify GLV from mixed-infected garlic, *Vicia faba* was inoculated with expressed sap from mosaic diseased garlic leaves. The necrotic lesions appeared on the inoculated leaves after two weeks and expressed sap from the lesion was applied on the leaves of another broad bean plant again (Fig. 1). These processes were repeated three times and the necrotic spots excised were used as a stock for pure GLV. Leek which was considered as a systemic host for GLV was inoculated with expressed sap from excised lesions of broad bean serially infected. Even though there was no visible symptom for viral infection, after two months of inoculation leek plants were examined for the presence of virus particles by electron microscopy.

Electron microscopy of virus particles. Inoculated leek plants were examined for the identification of virus particles by leaf-dip preparations (Hitchborn and Hill, 1965) using transmission electron microscope. Fig. 2a showed electron micrograph of GLV particles of 690 nm

Fig. 1. Chlorotic spots appeared on the leaves of *Vicia faba* infected with garlic latent virus. Arrows indicate typical necrotic lesions.

long in average. Their particle shape was slightly curved filamentous rod, which is consistent with the previous observation (Lee *et al.*, 1979). It has been known that carlavirus group is slightly flexuous filamentous rod, normally 610-700 nm long and 12-15 nm in diameter, often appearing curved to one side (Koenig, 1982). In contrast, Fig. 2b showed electron micrograph of garlic virus particles isolated from mixed-infected garlic leaves. Particle length of viruses ranged from 200 nm to 2000 nm, but most of the particle was in the range of 600-900 nm. There were mostly two types of virus particles, flexuous filamentous rod and slightly curved filamentous rod.

GLV coat protein. To determine the molecular weight of purified GLV coat protein, SDS-PAGE analysis was carried out. GLV isolated from inoculated leek and garlic viruses isolated from mixed-infected garlic leaves were loaded on 12% SDS-polyacrylamide gel (Fig. 3). The structural protein of garlic virus isolated from mixed-infected garlic leaves distributed between the molecular weight range of 24,500-38,000 Da. Electrophoresis of dissociated polypeptides from virus preparation purified by serial infection on broad bean and amplified in leek shows two strong bands (MW 28,000 and 34,000 Da) and a single weak band (MW 52,000 Da). The weak bands

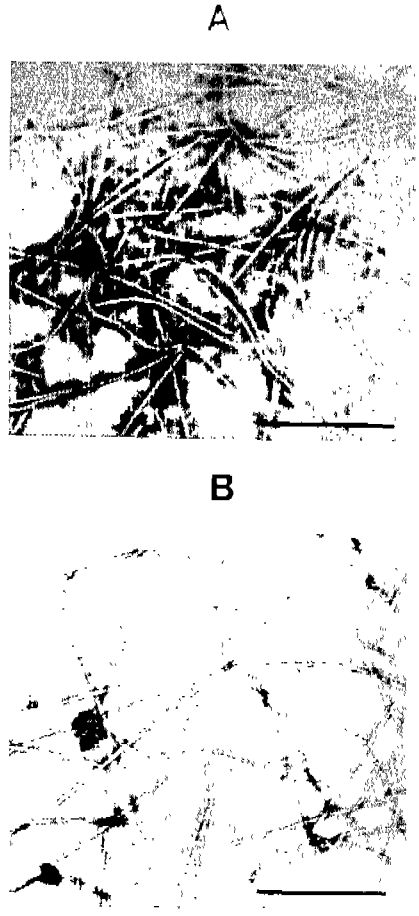

Fig. 2. Electron micrographs of virus particles in leaf-dip preparations of infected plants. A. leek plant inoculated with pure GLV strain obtained by serial infection in *V. faba*. B. Garlic plant showing mosaic symptom. The scale bar indicates 500 nm.

of MW 52,000 Da and 28,000 Da are also present in extracts from control leek plants indicating that these could be contaminants of host leek proteins. The polypeptide of MW 34,000 Da is specific for virus-inoculated leek plant and is probably structural protein of GLV. It has been known that carlavirus particles are composed of a single protein species of MW 31,000-34,000 Da (Koenig, 1982). The MW 28,000 Da polypeptide, however, could be a degradation product of the MW 34,000 Da polypeptide. The relative intensity of 28,000 Da polypeptide was quite variable to that of 34,000 Da polypeptide depending on preparation to preparation. Huttinga and Mosch (1974) found a similar degradation of the coat protein of other potyviruses and concluded that the smaller protein was

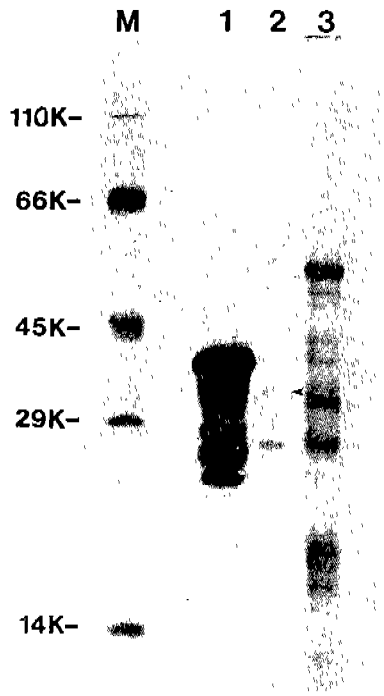

Fig. 3. SDS-PAGE analysis of garlic latent virus prepared from virus-inoculated leek. Protein samples were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. Arrowhead indicates 34,000 Da coat protein of GLV. Lane 1: Garlic virus particles from the leaves of garlic with mosaic symptom. Lane 2: Garlic latent virus particles prepared from the leaves of GLV inoculated leek. Lane 3: Leaf extract of virus-free leek.

a breakdown product of the larger. In case of garlic yellow streak virus, the lower MW protein was always present in higher concentration than the higher MW protein. This was perhaps a result of the lengthy purification procedure which would allow extensive degradation of the coat protein by proteolytic enzymes and proteolytic enzyme might be present in small amount in virus particle. It would be a virus gene product, which is the case in potyvirus.

To study molecular structure of GLV, it was purified by serial infection on broad bean and SDS-PAGE analysis of structural protein for GLV was carried out in this study. Serial infection on local lesion host plants and amplification in systemic host could be a way to prepare pure virus from mixed infected plant. SDS-PAGE analysis for structural protein could be another way of studying identity and purity of virus preparation. Combination with serological identification such as Western blot analysis could be proven to be a sure way. Nucleic acid analysis should be followed for further identification and understanding its molecular structure.

적 요

한국 마늘에 감염되어 있는 주된 바이러스 중의 하나로 알려진 마늘 잠복 바이러스 (GLV)의 분자 구조와 병 발생 메카니즘을 이해하기 위하여, 국부 감염 숙주 식물인 *Vicia faba*에 연속적으로 감염시킴으로써 마늘 잠복 바이러스를 정제하였고, GLV에 대한 전신 감염성 숙주 식물로 생각되는 leek에서 GLV를 대량으로 증식시켰다. 투과전자현미경을 이용하여 바이러스 입자를 관찰한 결과, 마늘 바이러스들의 입자의 길이는 200-2000 nm의 분포를 보였으나 입자의 대부분은 600-900 nm의 범위 안에 존재하였다. 반면 순수 분리된 GLV 입자는 평균 690 nm의 길이를 보여주었고, 유연한 실 모양이었다. SDS-PAGE 분석으로 혼합 감염된 마늘 잎으로부터 분리된 마늘 바이러스의 구조 단백질은 분자량 24,500-38,000 Da의 분포를 나타내었으나 GLV 껍질 단백질의 분자량은 34,000 Da으로 나타났다.

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