Isolation of a *Pestalotiopsis Species* Degrading Mucilage from Fruit of *Opuntia ficus-indica* var. Saboten

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The high molecular-weight mucilage extracted and purified from cactus fruit of *Opuntia ficus-indica* var. Saboten was degraded by the cell-free culture filtrate of a fungus isolated from soil. TLC analysis of the polymeric mucilage after incubation with the fungal culture filtrate confirmed its degradation. When the degradation products were tested for their qualitative reactions with ninhydrin and phenol-sulfuric acid, only phenol-sulfuric acid gave positive development, and ninhydrin did not show any observable color reaction. This coloring reaction suggested the presence of a carbohydrate without an amino group within the mucilage. Analyses by HPLC and liquid gel permeation chromatography on sephadex G-100 also provided additional information on degradation of the mucilage by the fungal culture filtrate. The sequences of ITS-5.8S rDNA from the fungal isolate that was cultivated for the preparation of mucilage-degrading enzyme showed 99% similarity to those of *Pestalotiopsis aquatica*.

Key words: *internal transcribed spacer (ITS), mucilage degradation,* Opuntia ficus-indica, *Pestalotiopsis species*

Palm-shaped cactus *Opuntia ficus-indica* var. saboten cultivated in Jeju Island belongs to the Cactaceae family, and the appearance of its fruit is similar to the prickly pear [Hamdi, 1997]. The cactus has been used as a traditional folk remedy for such symptoms as abdominal pain, bronchial asthma, burns, diabetes, and indigestion.

Study on the composition of *O. ficus-indica* mucilage revealed that the mucilage was a high molecular weight polysaccharide behaving as a polyelectrolyte [McGarvie and Parolis, 1981a]. The mucilage was composed of 24.6-42, 21-40.1, 8-12.7, 7-13.1 and 22-22.2% of arabinose, galactose, galacturonic acid, rhamnose, and xylose, respectively [Trachtenberg and Mayer, 1981]. McGarvie and Parolis (1981b) discovered that the mucilage of nopals was composed of a family of highly branched polysaccharides.

The fruit of *O. ficus-indica* is rich with mucilages. However, because the mucilages show high viscosity due to their high molecular weights, they present limitation in their industrial applications. No report has yet been published on the microbial enzymatic degradation of the mucilage from the palm-shaped cactus cultivated in Jeju Island. The aim of this study was to isolate a fungus that produces the mucilage-degrading enzyme.

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Abbreviations: high performance liquid chromatography, HPLC; internal transcribed spacer, ITS; thin-layer chromatography, TLC

Materials and Methods

Extraction and purification. Fresh fruits of *O. ficus-indica* var. saboten were purchased from the cultivation field in Jeju Island. The juice was obtained by pressing the fruits with an oil pressure extractor. High molecular-weight polymers were precipitated by adding 95% ethanol to the juice at 3 : 1. The precipitate was separated by centrifugation at 7,000 × g for 20 min and dissolved in a small amount of distilled water. These steps were repeated several times until all colors were removed from the juice, and finally the mucilage polymers were lyophilized.

Chemical analysis. Carbohydrate contents were determined by the Molisch method [Foulger, 1931], and total sugar contents were determined by the phenol-sulfuric acid method [Bloor, 1956] using maltose as a standard. Protein contents were determined by the ninhydrin method [Moore and Stein, 1948].

Isolation of a mucilage-degrading fungus from soil. M9 medium [Sambrook and Russel, 2001] containing Congo red (0.01%) as an indicator was used to screen microorganisms from the soil. The medium contained the

mucilage of *O. ficus-indica* at a final concentration of 0.5% as the sole source of carbon. Soil samples were spread on the M9 agar medium, and the plates were incubated at 28°C for 3 days. Microorganisms that decolorized the regions around the colonies were screened and chosen for this study. Morphological characteristics of the fungus were examined through the optical microscope (Olympus CK2-TR3; Tokyo, Japan) using Image-Pro Plus.

Preparation of crude enzyme. A fungus isolated from soil was inoculated into the M9 broth medium, and incubated at 28°C for 4 days in a shaking incubator (Hanbaek Scientific Co., HB-201SLI; Bucheon, Korea) at 200 rpm. The supernatant was obtained by centrifugation at 7,000 × g for 20 min and freeze-dried. The lyophilizate was dissolved in distilled water and filtered with φ0.45 μm syringe filter prior for use as a crude enzyme. The crude enzyme was inactivated by heating in the water-bath at 100°C for 30 min when necessary.

Thin layer chromatography. Analyses of the mucilage and its degradation products were performed on an aluminum sheet silica gel 60 F_{254} (Merck, Darmstadt, Germany) by TLC with a mobile phase of buthanol, methanol, and water mixture (4 : 1 : 1, v/v/v) [Stahl, 1969]. The chromatograms were visualized using ninhydrin or phenol-sulfuric acid.

High performance liquid chromatography. Samples were prefiltered with φ0.45 μm syringe filter before injection into the HPLC column. HPLC analysis was carried out on a Waters 2690 Millenium[32] (Milford, Maryland, USA) apparatus equipped with a Waters 2414 RI detector employing a Phenomenex BIOSEP-SEC-S4000 column (300 × 7.0 mm). Elution was carried out with distilled H_2O. Flow rate was 1 mL/min, and detector temperature was 35°C. Molecular weight standards were dextrans (Sigma, St. Louis, Missouri, USA) produced from *Leuconostoc mesenteroides*.

Liquid gel permeation chromatography. Samples of the mucilage and its degradation products were loaded on to the Sephadex G-100 column (2.8 × 35 cm) and eluted with 0.1 M acetate buffer (pH 5.0) to obtain separate fractions. Three milliliters of each fraction was collected and examined for the presence of sugars and proteins.

Identification of fungus. Identification of the fungus degrading the mucilage of the fruit of *O. ficus-indica* was basically performed by Korean Culture Center of Microorganisms (KCCM). Genomic DNA from the isolated fungus was purified and used as template DNA for the amplification of the ITS regions of 5.8S rDNA. ITS and 5.8S rDNA sequences of the unknown isolate were determined and compared with those of other fungal strains. The identity of the fungus was derived from the comparison of the consensus DNA sequences using the National Center for Biotechnology Information (NCBI) Genbank (U.S. National Library of Medicine, Bethesda, Maryland).

Results and Discussion

Viscous mucilages of the *O. ficus-indica* fruits were extracted and purified. Carbohydrates were detected in the mucilage preparations using the Molisch method, and their total sugar concentration determined by the phenol-sulfuric acid method using maltose as a standard was 64.85 mg/mL in 0.5% (w/v) aqueous solution, which about 10 times higher than the expected value of 5 mg/

Fig. 1. Microscopic morphology of the fungal isolate Bx.

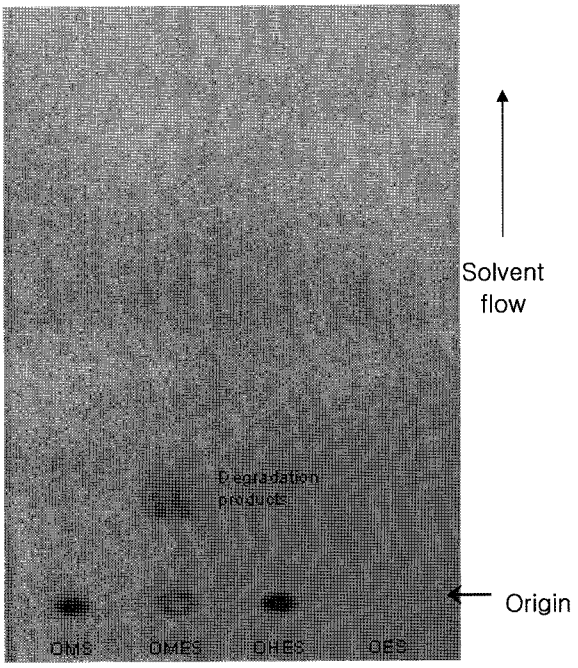

Fig. 2. Thin layer chromatogram of cactus fruit mucilage and its degradation products. OMS, untreated mucilage; OMES, mucilage after incubation with fungal crude enzyme; OHES, mucilage after incubation with heat-treated fungal crude enzyme; OES, fungal crude enzyme.

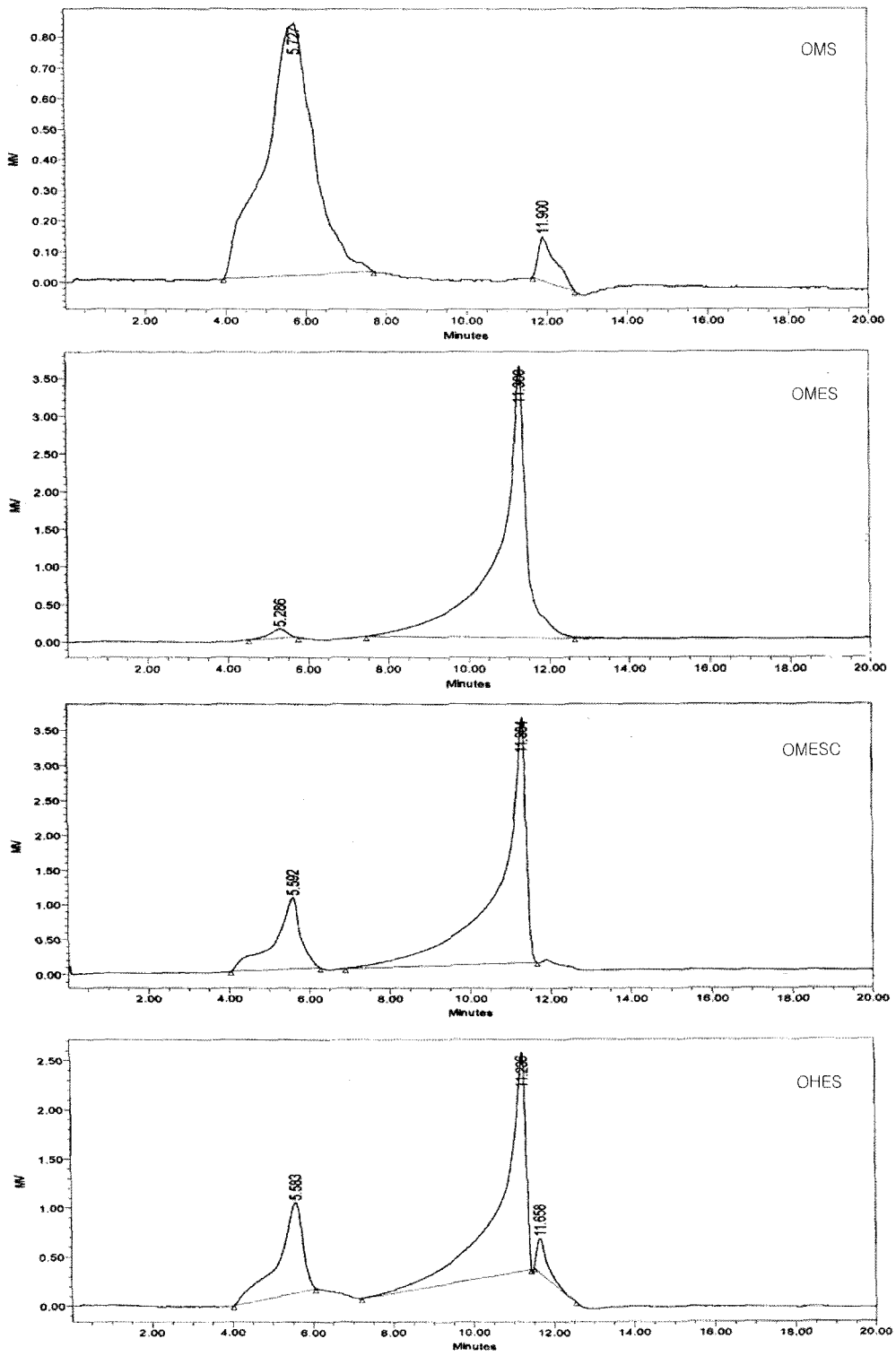

Fig. 3. Analysis of mucilage from cactus fruit by high performance liquid chromatography. OMS, untreated mucilage; OMES, mucilage after incubation with fungal crude enzyme; OMESC, mucilage immediately after mixing with fungal crude enzyme; OHES, mucilage after incubation with heat-treated fungal crude enzyme.

mL. Little ninhydrin-positive reaction was observed in the aqueous mucilage solution. Matsuhiro *et al.* [2006] reported that no protein was detected in the mucilage using the Bradford method. In contrast, the water-soluble polysaccharide of *O. ficus-indica* gave neutral fractions of glucans, glycoproteins, and acidic fractions that contain arabinose, galactose, rhamnose, xylose, and galacturonic acid [Paulsen and Lund, 1979]. Amin *et al.* [1970] found

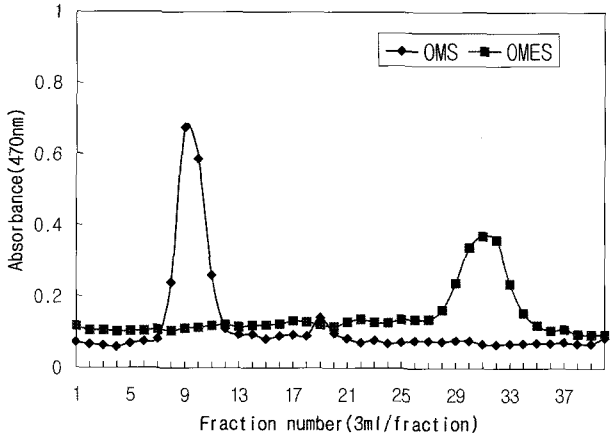

Fig. 4. Liquid gel permeation chromatograms on Sephadex G-100 of the mucilage before and after incubation with fungal crude enzyme. OMS, untreated mucilage; OMES, mucilage after incubation with fungal crude enzyme.

Fig. 5. The nucleotide sequences of internal transcribed spacers and 5.8S rRNA genes from the fungal isolate Bx and *Pestalotiopsis aquatica*. The nucleotide sequences of *Pestalotiopsis aquatica* (Pa) were obtained from NCBI Genbank (accession number DQ132823). (Note: the sequences 15~156, internal transcribed spacer 1; the sequences 157~314, 5.8S rRNA; the sequences 315~477, internal transcribed spacer 2).

that the mucilage was a neutral polysaccharide of approximately 55 sugar residues without uronic acid, composed of arabinose, rhamnose, galactose, and xylose. Our positive test results obtained using the ninhydrin method might have resulted from relatively small amount of proteins or peptides remaining in the mucilage as a result of the preparation process.

Many strains of fungi isolated from soil were grown on the M9 medium containing Congo red, using the mucilage as the sole carbon source. Congo red dye, which binds to the polysaccharide molecules having more than five contiguous β-linked D-glucopyranosyl units, has been widely used [Lee *et al.*, 1984]. The fungus with the best decolorizing ability of Congo red was selected. Its pure culture was obtained through repeated subculturing on M9 medium containing the mucilage as the sole carbon source. Symbol Bx was tentatively assigned to the purified fungal isolate. Crude enzyme was obtained from culture filtrate of the fungus Bx. The isolate Bx was filamentous and had cylindrical or fusiform spores (Fig. 1). The spores of Bx were scattered or gregarious, with two or more apical appendages and three layers of brown cells. *Pestalotiopsis* species has 3-septate conidia [Lee *et al.*, 2006].

The enzymatic degradation of mucilage by the isolate Bx was examined using TLC (Fig. 2). One spot was observed at R_f 0.19 after the incubation of the mucilage with fungal crude enzyme (OMES). Both the mucilage solution (OMS) and the fungal enzyme preparation (OES) showed no migration spot. Incubation of the mucilage with the heat-inactivated crude enzyme (OHES) also showed no degradation product. The spot at R_f 0.19 is a clear indication that the mucilage was degraded by the fungal enzyme. When the degradation products were tested for their qualitative reaction with ninhydrin or phenol-sulfuric acid, only phenol-sulfuric acid gave a brown color development, and ninhydrin did not show any observable color reaction. This coloring reaction suggested the presence of carbohydrates without amino group within the degradation products of the mucilage.

When the mucilage solution was analyzed by HPLC (Fig. 3), two major peaks could be observed, eluting at the retention times of 5.7 (high molecular-weight fractions) and 11.9 min (low molecular-weight fractions) (OMS). When the mucilage was incubated with the fungal crude enzyme for 2 h and subsequently injected into the same HPLC (OMES), two major peaks eluting at the retention times of 5.3 (molecular weight 2×10^6) and 11.3 min (less than molecular weight 2×10^4) could also be observed (molecular weight calibration data using dextran polymers as standards not shown here). However, significant molecular weight shifts were observed. The high molecular-weight fraction decreased, whereas the low molecular-weight fraction increased. The mixture of mucilage and enzyme immediately after mixing produced two major

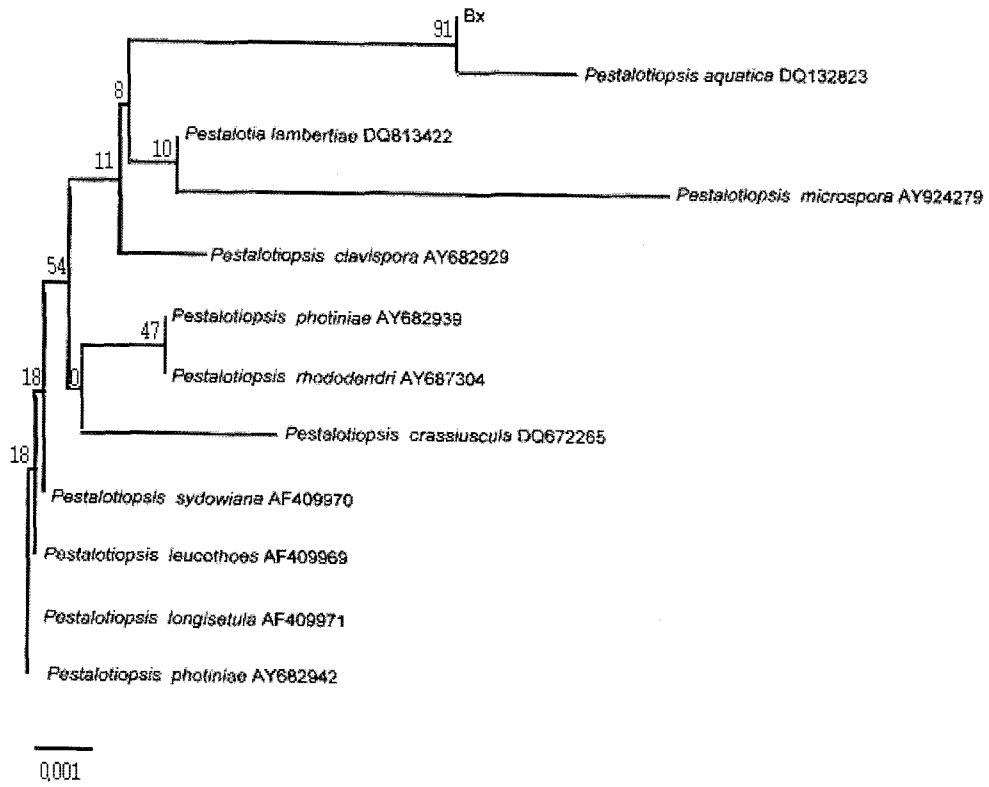

Fig. 6. Dendogram of the ITS-5.8S rDNA region sequences of 11 *Pestalotiopsis* strains showing the phylogenetic relationship of the fungus Bx with reference texa.

peaks eluting at the retention times of 5.5 and 11.3 min (OMESC), and the elution pattern was similar to the chromatogram OHES obtained after the incubation of the mucilage with the heat-treated crude enzyme. These HPLC chromatograms (Fig. 3) showed that most of the high molecular-weight mucilage fractions disappeared due to the degradation caused by the crude enzyme prepared from the fungus Bx.

Analysis by liquid gel permeation chromatography on Sephadex G-100 showed that total carbohydrates of OMS were detected in fractions 8-11, whereas the carbohydrates of OMES were detected in fractions 28-34 (Fig. 4). These results suggested that the fungal enzyme was responsible for the shift of mucilage size from high molecular weight to low molecular weight.

Comparison of DNA sequence similarity has been a powerful tool for the strain identification. rDNA-ITS sequences especially have been proved to be useful in the phylogenetic analysis of fungi [David and Leonard, 1997; Sousa *et al.*, 2004; Lee *et al.*, 2006]. Figure 5 shows the comparison of total 482 base pairs of nucleotide sequences of ITS and 5.8S rRNA gene from the fungal isolate Bx with those of *Pestalotiopsis aquatica* from NCBI data base (accession number DQ132823). The sequences from 15 to 156, from 157 to 314, and from 315 to 477 corresponded to ITS 1, 5.8S rRNA, and ITS 2, respectively. Only one base pair difference was observed between those Bx and *P. aquatica*. The ITS regions of 5.8S rDNA sequences from the isolate Bx showed similarity higher than 99% to that of *Pestalotiopsis aquatica*. Fungal morphology (Fig. 1), and the nucleotide sequences of ITS and 5.8S rRNA gene (Fig. 5) strongly suggested that the isolate Bx should be a *Pestalotiopsis species*. The distance tree gave the same topology (Fig. 6).

To the best of our knowledge, this is the first report on the isolation and identification of a fungus degrading mucilage from the cactus fruit of *O. ficus-indica*.

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