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In memorian Professor Luis Astudillo, Universidad de Talca, Chile

Structural studies of the exopolysaccharide produced by a submerged culture of entomopathogenic fungus *Metarhizium anisopliae*

[Estudio estructural del exopolisacárido producido por un cultivo sumergido del hongo entomopatogénico *Metarhizium anisopliae*]

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Abstract: The exopolysaccharide (EPS) separated from the entomopathogenic fungus *Metarhizium anisopliae* was determined by gel permeation chromatography to be homogeneous. The high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD) showed a content of monosaccharides D-galactosamine and D-fucose at a molar ratio of about 2:1. The results obtained from Fourier transform-infrared spectroscopy (FT-IR) and second derivative FT-IR spectrum confirmed the proposed structure.

Keywords: exopolysaccharide, entomopathogenic fungi, *Metarhizium anisopliae.*

Resumen: El exopolisacárido (EPS) separado desde el hongo entomopatogénico *Metarhizium anisopliae* determinado por cromatografía de exclusión en gel ser homogéneo. La cromatografía iónica de alto rendimiento con detección de pulso amperométrico (HPAE-PAD) mostró un contenido de monosacáridos D-galactosamina y D-fucosa en una relación molar de alrededor de 2:1. Los resultados obtenidos desde la espectroscopía infrarroja con transformada de Fourier (FT-IR) y la segunda derivada del espectro FT-IR confirmaron la estructura propuesta.

Palabras clave: exopolisacárido, hongo entomopatogénico, *Metarhizium anisopliae.*

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INTRODUCTION

The microbial exopolysaccharides (EPS) have a variety of beneficial functions such as gel formation, emulsifying, absorption, cohesion, and film formation. Various types of EPS have been examined for their use in the fields of medicines, foods cosmetics and other industries (Xu & Yun, 2004; Xu *et al.*, 2006). Most of the EPS produced by fungi are highly hygroscopic β-glucans, suggesting that its production could be related with the tolerance to desiccation; similarly to that observed and described in bacteria (Schnider-Keel *et al.*, 2001). The EPS consist of wide variety of glucans closely related in structure but possessing very different water solubilities. Purified β-glucans from Ganoderma lucidum demonstrated antitumor and bactericidal activities, and immunomodulating properties (Papinutti, 2010). Synthesis of exopolysaccharide by the fungus Aureobasidium pullulans and the many factors affecting its production have attracted considerable interest because of its potential applications in various industries (Orr *et al.*, 2009).

These microbial biopolymers are classified as homopolysaccharides and heteropolysaccharides depending upon their chemical structures. The homo- and heteropolysaccharides are synthesized by single and multi-enzyme systems on either single carbon or complex carbon sources (Kocharin *et al.*, 2010). Studies of the structure of the exopolysaccharide from fungus Aspergillus sp. Y16 showed that it consists of (1β2)-linked α-D-mannopyranose units, substituted at C-6 by the (1β6)-linked α-D-mannopyranose, (1β)-linked β-D-galactofuranose and (1β)-linked β-D-mannopyranose units (Chen *et al.*, 2011). The polysaccharide purified from Paecilomyces tenuis Samson was analysed using NMR and GC-MS data, this is composed of glucose with only an α-(1→6) linkage (Lu *et al.*, 2001). The difference in chemical composition, type of glycosidic likage, and branching degree of polysaccharides, influences the secondary and tertiary structures of the single chains and their macromolecular assembly, determining the physical properties of the polysaccharide which are related to their structural or physiological functions (Lim *et al.*, 2005).

Acidic exopolysaccharides represent the largest group of polysaccharides, which contain one or more carboxyl, sulphuric ester or phosphate groups in the saccharide repeating unit (Hwang *et al.*, 2003). Acidic polysaccharides are likely to be more bioactive than neutral polysaccharides, because of the acidic groups forming associations with the target biomolecules, such as proteins in the host, through electronic interactions (Xie *et al.*, 2007). An acidic polysaccharide was fractionated from the exopolysaccharide produced by a medicinal fungus Cordyceps sinensis in mycelia culture (Kim *et al.*, 2003; Zhong *et al.*, 2009). The molecular structure of polysaccharide was characterised and elucidated by spectral and chromatographic analyses, and its immunomodulatory effect was tested, in macrophage cell culture, on cytokine release (Wang *et al.*, 2011). Exopolysaccharides from Cordyceps taii has been shown to have a wide range of pharmacological effects including immunomodulatory, antitumor, antioxidant, hypoglycaemic and hypocholesterolemic activities (Xiao *et al.*, 2010).

On the other hand, entomogenous fungi of the genus Metarhizium have received considerable attention due to their great potential to be effective against several species of insects, including Lepidortera (Rao *et al.*, 2006). However, only the production of destruxins of the entomopathogenic Metarhizium anisopliae has been reported.

In present study, we report the extraction of exopolysaccharide (EPS) obtained from Metarhizium anisopliae. The monosaccharides constituents were determinate throughout high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD) and structural elucidation of EPS by Fourier transform-infrared spectroscopy (FT-IR) and second derivative FT-IR spectrum.

MATERIAL AND METHODS

General experimental procedures

FT-IR spectra were recorded in a FT-IR-4100 Jasco spectrophotometer. All spectra were recorded with an accumulation of 64 scans and resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}. For the IR spectra, the samples (2.0 mg), were dried 24 h at 60° C under reduced pressure and were mechanically mixed with 20 mg of KBr. Derivation, including Savitzky-Golay algorithm with 25 smoothing points was performed using the OPUS/I.R. version 1.4 software incorporated into the hardware of the instrument (Lillo *et al.*, 2011). The content of monosaccharides en el EPS was determined through of high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD) using a Dionex DX-500 BioLC system.

Organism collection

Metarhizium anisopliae were cultures in potato dextrose agar. Stock cultures were maintained on the same medium and transferred to fresh medium by four weeks interval. A voucher specimen of the fungus is deposited in the collection fungi of the Departamento de Ciencias Básicas, Universidad del Bío-Bío, Chillán, Chile.

Fungal strain and culture conditions

The native strain of *Metarhizium anisopliae* was grown in shaken-flask culture Hagen medium containing the following chemicals (per liter of distilled water): 0.05 g $CaCl_2$•$2H_2O$ (Merck), 0.025 g KH_2PO_4 (Merck), 0.25 g $(NH_4)2HPO_4$ (Merck), 0.15 g $MgSO_4$•$7H_2O$ (Merck), 1.3 ml $FeCl_3$ 1% (Merck), 3.0 g malt extract (Merck) and 10 g glucose (Merck). Each flask containing 100 ml of medium was inoculated with 2.0 ml suspension of the fungus obtained from the surface of stock slants (Lillo *et al.*, 2007).

In a 2,000 ml Erlenmeyer flask containing 500 ml of medium with aeration and agitation (150 rpm), the fermentation was performed. 125 ml of well grown culture (7 d) in the same medium were used as inoculum. The fermentation was stopped after 30 d. The pH value of the medium was adjusted to 6.5 with HCl (2 M) or KOH (2 M).

Purification of exopolysaccharide

The resulting culture filtrate was mixed with four volumes of absolute ethanol, stirred vigorously, and kept overnight al -10 ºC. The precipitated was centrifuged at 3,000 rpm for 15 min and the supernatant was discarded. After repeated precipitation steps, the resulting EPS was dialyzed at room temperature overnight in de-ionized water and lyophilized and the weight of EPS was estimated (Lillo *et al.*, 2007).

Gel permeation chromatography

An aqueous solution of polysaccharide (1mg/ml) was chromatographed on Fractogel TSK HW-55(S)-gel (Merck Co., Darmstadt) column (100 mm x 13mm) and eluted with 1 % v/v acetic acid (pH 5.3) (Lillo et al., 2008). The column was calibrated with 2 ml solution of Blue Dextran 2000 (4 mg/ml) and D-glucose (4 mg/ml). Elution was monitored spectrophotometrically with the phenol-sulfuric acid reagent for sugars (Matsuhiro *et al.*, 2006). The fractions collected were analyzed to 480 nm and the absorbance was graphed as function of the volume (each 5 mL) (Figure 1).

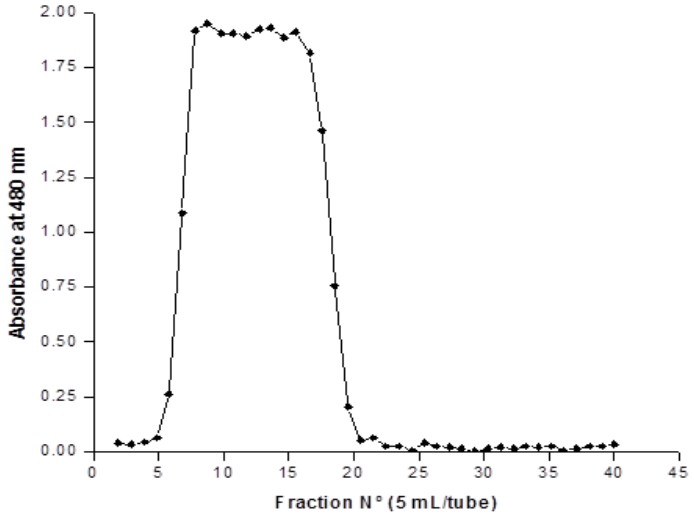

Figure 1
Elution profile of the gel permeation chromatography on Fractogel TSK HW-55 (S) of the EPS isolated from *Metarhizium anisopliae* (each point corresponds to collection of 5 mL).

RESULTS AND DISCUSSION

The development of entomopathogens fungal as biological control agents has been of considerable research in the last years. *Metarhizium anisopliae* is an entomopathogenic fungus and good alternative to chemical control of insects. From submerged culture of the fungus an exopolysaccharide by means of precipitation with cold ethanol was obtained. Fractogel TSK HW-55 (S) gel permeation chromatography of EPS (Figure 1) revealed the existence of homogeneous EPS. The molecular weight was estimated about 800 kDa. The constituent monosaccharides were determined by means of high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD). In the analysis of the EPS by HPAE-PAD were observed two peaks of in the chromatogram (Figure 2) assigned to D-galactosamine and D-fucose according to retention time of the monosaccharide standards. The 90% of the EPS corresponded to a mixture of these monosaccharides. The molar ratio of D-galactosamine and D-fucose was calculated to be about 2:1.

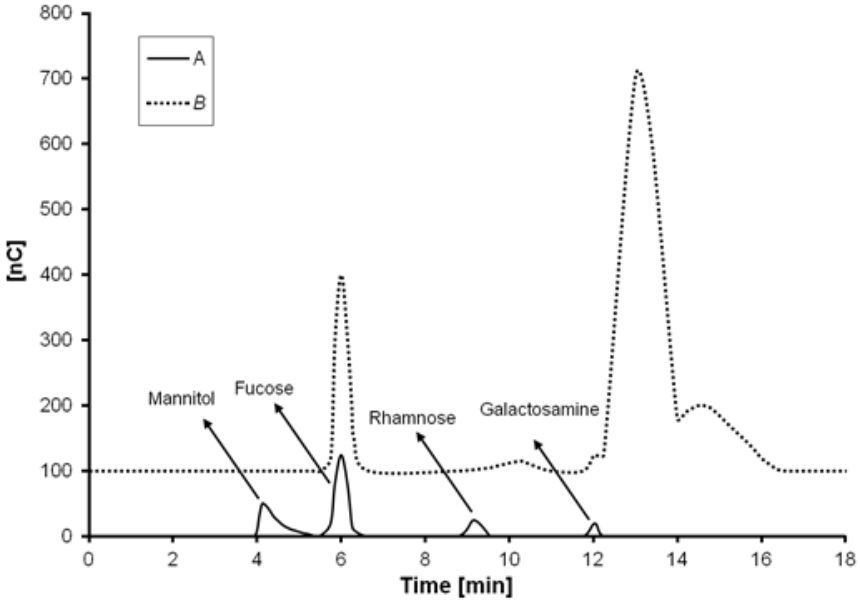

Figure 2
High-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD): (A) Reference sugars (D-mannitol, D-fucose, D-rhamnose and D-galactosamine) and (B) of the EPS isolated from *Metarhizium anisopliae*.

In order to investigate the functional groups of the EPS, the FT-IR spectrum (Figure 3A) presents characteristic absorption bands at 3402.2 cm^{-1} assigned to N-H and O-H stretching, at 2925.6 cm^{-1} assigned to C-H stretching, at 1423.3 cm^{-1} assigned to C-N stretching of a primary amine, at 1365.6 cm^{-1} due to the C-O deformation of a secondary alcoholic group and at 849.4 cm^{-1} characteristic of a C-H vibration of α-anomeric residues (Lu *et al.*, 2007). In comparison with the IR spectra of exopolysaccharides documented in literature, a characteristic absorption band appeared at 1669.8 cm-1 which is resolved into two bands, in the second-derivative mode (Figure 3B), one at 1652.9 cm^{-1} assigned to a C=O stretching vibration of the N-acetyl group and another at 1546.4 cm^{-1} assigned to the N-H deformation vibration of amine group (Leung *et al.*, 2009). Bands in the FT-IR spectrum of EPS between 1160 and 1027 cm^{-1} are assigned to stretching C-C and C-O modes, including a shoulder due to the asymmetric stretching C-O-C of glycosidic linkage (~ 1154 cm^{-1}) (Vijayabaskar *et al.*, 2011). These results suggest that the EPS are polysaccharides composed of galactosamine residues partially N-acetylated.

On the other hand, most studies with the production of EPS have focused on the water-soluble EPS. The major production of the EPS is obtained to the third-day culture (1.735 gL^{-1}). However, after the third-day the concentration of the EPS began to decrease, while there was an increase in the biomass of the fungus. This decreasing in the production of the EPS probably could be due to the exhaustive consumption of the carbon source in culture means. Some authors reported that is necessary an excess of carbohydrate in growing medium for stimulate biosynthesis of EPS (Mahapatra & Banerjee, 2013).

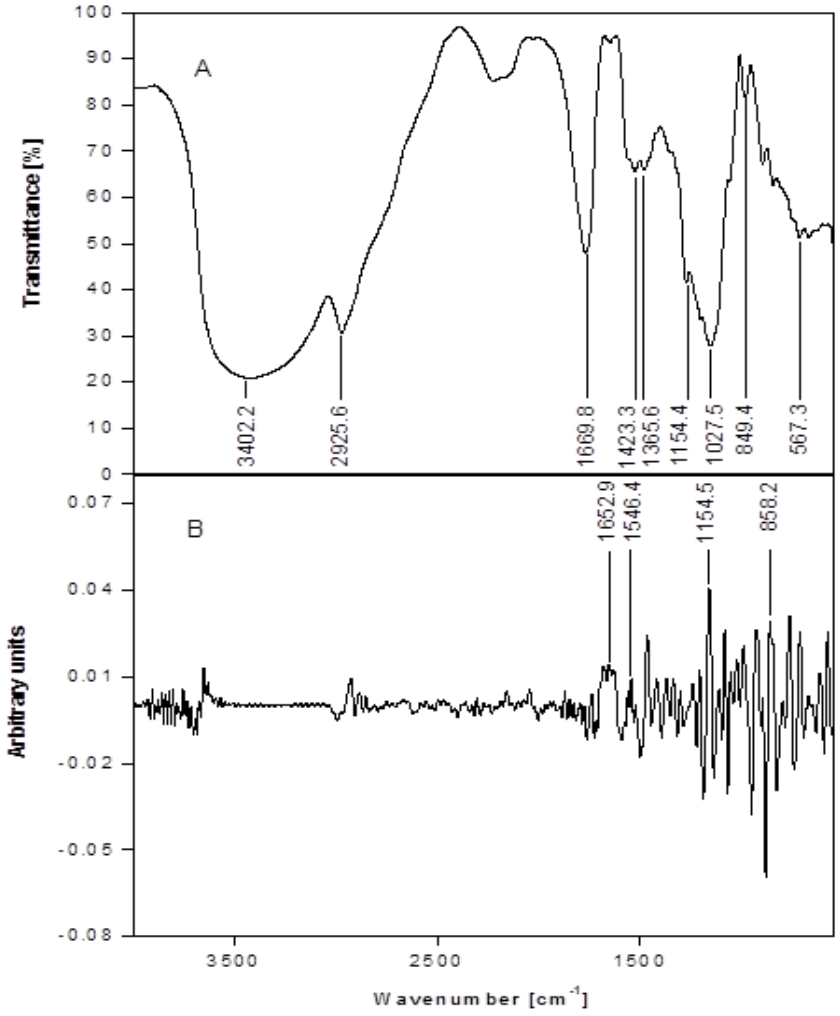

Figure 3
FT-IR spectra of the EPS isolated from *Metarhizium anisopliae*: (A) normal spectrum and (B) second-derivative FT-IR spectrum.

CONCLUSIONS

The Fourier transform-infrared spectroscopy (FT-IR) and high-performance anion-exchange chromate-graphy with pulsed-amperometric detection (HPAE-PAD) allowed to determine that the exopolysaccharide produced by the entomopatho-genic fungus Metarhizium anisopliae is a polysaccharide compose mostly of galactosamine residues partially N-acetylated. However, additional efforts are needed to investigate the relationship between the structure and its biological activity.

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