Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 60 (2005) 199–203
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Structural elucidation of bioactive fungi-derived polymers

Pawadee Methacanon[a,*], Siribhorn Madla[b], Kanyawim Kirtikara[b], Malinee Prasitsil[a]

[a]National Metal and Materials Technology Center, 114 Thailand Science Park, Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand
[b]National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

Received 22 November 2004; accepted 22 December 2004
Available online 3 February 2005

Abstract

Chemical composition and molecular structure of exopolysaccharides (EPS) from three strains of fungi, *Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744, which can promote the production of IL-8 (a cytokine enhancing wound healing), were elucidated. The results from HPLC after acid hydrolysis revealed that the EPS were mainly composed of glucose indicating the presence of glucan. Galactose, mannose and arabinose were also found as minor monosaccharides. In addition, the protein content in the EPS was determined to be approximately 6–7% with the exception of *Phytocordyceps* sp. BCC2744 (21%). To identify the linkages between the monosaccharides and the molecular structure of the EPS, methylation followed by reductive cleavage and ^{13}C-NMR analyses were performed. They were shown to be composed of a (1→3)-β-D-glucan backbone substituted at *O*-6 with side chains of (1→6)-β-D-glucopyranosyl units. The highest branching structure was shown in the EPS from *A. pistillariiformis* BCC2694, followed by *C. dipterigena* BCC2073 and *Phytocordyceps* sp. BCC2744, respectively. Apart from the highly branched at *O*-6 of (1→3)-β-D-glucan, (1→2) mannan and (1→3) galactan were also found in *C. dipterigena* BCC2073.

Keywords: Monosaccharide composition; Methylation analysis; Fungal polysaccharides

1. Introduction

Recently, microbial polysaccharides have received considerable attention due to their potential use in a wide variety of industries including cosmetics, pharmaceutical, and food industries, etc. (Selbmann, Onofri, Fenice, Federici, & Petruccioli, 2002). Several groups of polysaccharides from fungi have potent biological and pharmacological activities, including immunostimulating and anti-tumour activities (Ooi & Liu, 2000). Furthermore, several species of fungi are used as traditional medicines in treatment of different human diseases such as hepatitis, hypertension, hypercholesterolaemia, and gastric cancer (Park, Kim, Hwang, & Yun, 2001).

From our observations, entomopathogenic fungi in particular produce viscous fermentation broths due to the presence of exopolysaccharide (EPS) products. In our previous study, 16 strains have been selected as a representative of 15 different genera collected from various natural places in Thailand. The polymer production and some biological properties (anti-viral and anti-fungal activities and cytotoxicity) of the polymers have been previously described (Madla, Methacanon, & Kirtikara, 2005). Among all these polymers, the EPS from three strains (*Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744) have potential as a wound dressing material due to their biological and physiological properties. They are biocompatible and strong inducers of IL-8, a cytokine responsible for enhancing wound healing process. In this report, we provide further information on the composition and molecular structure of these EPS.

* Corresponding author. Tel.: +66 2564 6500x4422; fax: +66 2564 6445.
E-mail address: pawadeem@mtec.or.th (P. Methacanon).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2004.12.006

2. Experimental

2.1. Materials

2.1.1. Microorganisms and culture condition

Three strains of fungi, *Akanthomyces pistillariiformis* BCC2694, *Cordyceps dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744, belonging to a group of entomopathogenic fungi were obtained from the BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The medium and microorganism culture conditions were as previously described (Madla et al., 2005).

2.1.2. Isolation of extracellular materials

Extracellular materials were prepared as reported in Madla et al. (2005). Briefly, after fermentation, distilled water was added to the crude broth and any insoluble materials were removed by centrifugation. Then, polymer was separated by precipitation of supernatant in ethanol, dialysis against distilled water for 24 h and lyophilization.

2.2. Chemical analyses

2.2.1. Identification of monosaccharides

Sample (~25 mg) was soaked in trifluoroacetic acid (conc. TFA, 2 mL) for overnight at ambient temperature. Then, TFA was diluted to 3 M and the sample was hydrolysed at 120 °C for 5 h. The TFA was removed using a rotary vacuum evaporator. Distilled water was subsequently added to the solid to wash the sample and this was followed by re-evaporation; the procedure was repeated until the obtained hydrolysate was neutral. Finally, the dry hydrolysate solid was dissolved in water (5 mL) and used for monosaccharide analysis by HPLC.

Twenty microliters of hydrolysate was applied to a CarboSep CHO-682 Lead column (300 mm; Transgenomic Inc, USA). Products eluted in water at a flow rate of 0.4 mL/min were monitored by RI (refractive index) detector. Five monosaccharides (arabinose, xylose, mannose, galactose and glucose) were used as standards.

2.2.2. Linkage analysis

Samples (100 mg) were methylated three times using the procedure described by Tischer, Gorin, de Souza, and Barreto-Bergter (2002). After leaving overnight, the solution was neutralised with conc. HCl and dialysed against water. Then the reductive cleavage of the freeze-dried methylated product was performed as described by Jun and Gray (1987).

The resulting mixture of anhydroalditols was examined by gas chromatography/mass spectrometry (GC-MS) coupled with FID (flame ionization) detector at 300 °C. A capillary column of BPX-5 (30 m × 0.25 mm i.d., 0.25 μm film thickness, SEC Co. Ltd.) was used and temperature was programmed from 70 °C and held for 3 min, then heated to 120 °C at 20 °C/min and held for 5 min, and heated again to 300 °C at 5 °C/min. Helium was used as a carrier gas with a flow rate of 1.5 mL/min. Mass spectra were obtained with electron impact ionization at 70 eV. The integrated peak area was corrected according to the effective-carbon-response (e.c.r.) method (Bowie, Trescony, & Gray, 1984; Sweet, Shapiro, & Albersheim, 1975).

Table 1
Monosaccharide composition of the EPS

EPS from	Monosaccharide composition (%w/w)			
	Ara	Man	Gal	Glc
A. pistillariiformis BCC2694	–	4.20	1.42	71.60
C. dipterigena BCC2073	1.86	29.08	25.86	43.05
Phytocordyceps sp. BCC2744	2.84	8.02	6.23	57.94

Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

2.2.3. ^{13}C-NMR spectroscopy

Spectra of polymers (solid state) were recorded on a Bruker DPX-300 Nuclear Magnetic Resonance Spectrometer at 125 mHz (30 °C). Chemical shifts were referred to adamantane.

2.2.4. Amino acid analysis

Samples (100 mg) were hydrolysed with HCl (6 M, 2 mL) at 112 °C for 22 h. The composition of amino acids was derivatised with AccQ®Fluor reagent before analysis by HPLC (Waters Alliance 2695) with AccQ-Tag column (150 x 3.9 mm i.d., 4 μm film thickness) and a Fluorescence detector (EX: 250, EM: 395). A gradient system, phosphate buffer pH 5, acetonitrile and water, with a flow rate 1 mL/min was used.

2.2.5. Molecular weight

The molecular weight of samples was estimated by Gel Permeation Chromatography (GPC) using pullulan (MW 5900–788,000) as a standard. Analysis was performed on Ultrahydrogel linear column (7.8 i.d. × 300 mm, Waters, USA) connected in line with a Waters GPC apparatus. Samples (2 mg/mL) were eluted with 0.1 M NaOH with a flow rate of 0.6 mL/min at 25 °C and monitored by RI and photodiode array detectors.

3. Results and discussion

Monosaccharide compositions of the EPS from the three fungi are shown in Table 1. Glucose was the predominant

Table 2
Molecular weight of the EPS

EPS from	Molecular weight (kDa)
A. pistillariiformis BCC2694	8.3
C. dipterigena BCC2073	20.1
Phytocordyceps sp. BCC2744	9162, 9.04

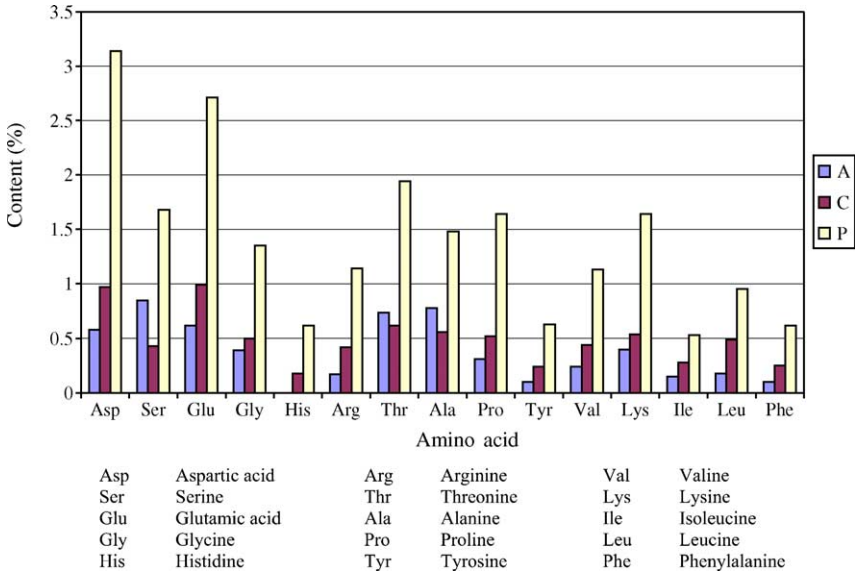


Fig. 1. Amino acid content found in the EPS from *A. pistillariiformis* BCC2694 (A), *C. dipterigena* BCC2073 (C) and *Phytocordyceps* sp. BCC2744 (P).

Table 3
Linkage analysis data of the EPS

	Linkage type	Relative mole percentage		
		A. pistillariiformis BCC2694	*C. dipterigena* BCC2073	*Phytocordyceps* sp. BCC2744
1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol	→3)-Glc*p*-(1→	23.36	24.43	60.23
1,5-anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol	→6)-Glc*p*-(1→	11.73	4.96	5.03
1,5-anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol	→3,6)-Glc*p*-(1→	64.91	47.87	30.03
1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol	Terminal Glc*p*	–	–	4.71
1,5-anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→2)-Man*p*-(1→	–	20.89	–
1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galacitol	→3)-Gal*p*-(1→	–	1.85	–

sugar found in all samples, particularly from *A. pistillariiformis* BCC2694 and *Phytocordyceps* sp. BCC2744, comprising 72 and 58%, respectively. The results strongly indicated that the EPS were mainly composed of glucan. It was in agreement with many literatures reporting that glucan has many biological properties such as anti-tumor, anti-microbial, anti-viral, antioxidant, free radical scavenging (Ooi & Liu, 2000) and anti-inflammatory activities (da Silva & Parente, 2003). In addition to glucose, arabinose, mannose and galactose were present at significant levels especially in the EPS from *C. dipterigena* BCC2073. This observation suggested that the samples might be composed of other polysaccharides apart from glucan.

The molecular weights of the EPS from the three strains of fungi were completely different as shown in Table 2. In addition, the GPC chromatogram of the EPS from *Phytocordyceps* sp. BCC2744 shows the presence of protein with molecular weight of approximately 9 kDa.

Protein contents of the samples from *A. pistillariiformis* BCC2694, *C. dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744, determined as total free amino acids after hydrochloric acid hydrolysis, were 5.61, 7.43, and 21%, respectively. The principal amino acids found in the samples from *A. pistillariiformis* BCC2694, *C. dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744 were serine, glutamic acid, and aspartic acid, respectively (Fig. 1). Many bioactive polysaccharides previously isolated from fungi belong to homoglycans or heteroglycans while others mostly bind to protein residues as polysaccharide–protein complexes (Ooi & Liu, 2000; Peng, Zhang, Zeng, & Xu, 2003).

Table 4
^{13}C-NMR results

EPS from	Chemical shift (ppm)	Assignment
A. pistillariiformis BCC2694	62.29, 72.84, and 104.09	C6, C2 and C1
C. dipterigena BCC2073	25.27, 30.69, and 174.71 63.51, 74.17, 104.50	Protein C6, C2 and C1
Phytocordyceps sp. BCC2744	19.10, 173.71 62.74, 69.35, 74.76, 86.97, 104.12	Protein C6, C4, C2, C3 and C1

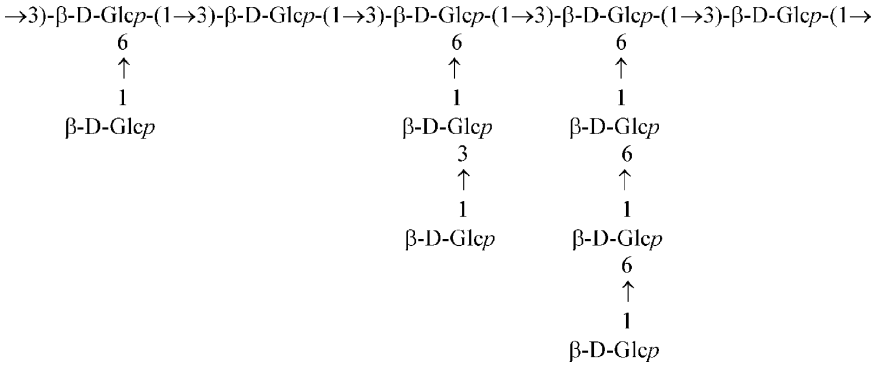
Determination of linkage position of the monosaccharides in the EPS was carried out by GC/MS after methylation and reductive cleavage. Methylation analysis is a widely used method for determining polysaccharide structure. However, reductive cleavage depolymerisation has several advantages compared to standard methylation analysis. It is an effective method for structural characterisation of complex carbohydrates which have different acid-labile groups or sugar residues. In addition, time consuming during hydrolysis step is avoided (Kiwitt-Haschemie, Renger, & Steinhart, 1996). The main reaction derivatives found in all samples were 1,5-anhydro-3-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5-anhydro-6-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol and 1,5-anhydro-3,6-di-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol (Table 3), revealing the presence of (1→3)-linked glucose (main chain), (1→6)-linked glucose (side chain) and (1→3; 1→6)-linked glucose (branch point), respectively. Apart from the EPS from *Phytocordyceps* sp. BCC2744, terminal glucose was not detected in the samples. The EPS from *A. pistillariiformis* BCC2694 possessed the highest degree of branching, followed by that of *C. dipterigena* BCC2073 and *Phytocordyceps* sp. BCC2744, respectively. It was confirmed that a highly branched structure of EPS is generally considered a positive factor in stimulating the immune system (Selbmann et al., 2002).

For *C. dipterigena* BCC2073, in addition to a highly branching glucan, (1→2) mannan and (1→3) galactan were also detected. This corresponded to the result from the GC (monosaccharide composition) of which mannose and galactose were found in a higher concentration (ca. 26–30%) compared with those in *A. pistillariiformis* BCC2694 and *Phytocordyceps* sp. BCC2744 (ca. 6–8%).

The ^{13}C-NMR chemical shifts (Table 4) were in accordance with values reported in the literatures (Barbosa, Steluti, Dekker, Cardoso, & da Silva, 2003; Gorin, 1981). The chemical shift of anomeric carbons occurring at approximately 104 ppm indicated a β-configuration. Furthermore, evidence supporting β-anomeric carbons in the samples was confirmed by FTIR spectroscopy (data not presented), with bands at 890 cm^{-1} being typical of (1→3)-β-glucan, and that at 1370 cm^{-1} being characteristic of β-glucan (Barbosa et al., 2003). Signals at 20–30 ppm and 174 ppm were attributed to alkyl and carbonyl group of protein, respectively.

4. Conclusion

The structure of fungal polysaccharides isolated from *A. pistillariiformis* BCC2694, *C. dipterigena* BCC2073, and *Phytocordyceps* sp. BCC2744 were investigated by means of chemical analyses and NMR spectroscopy. The polysaccharides were mainly composed of glucan possessing an irregular structure demonstrated as follows:



Acknowledgements

This work was supported by the Biodiversity Research and Training Program (BRT), Thailand (Grant BRT R_645001).

References

Barbosa, A. M., Steluti, R. M., Dekker, R. F. H., Cardoso, M. S., & da Silva, M. L. C. (2003). Structural characterization of Botryosphaeran: A (1→3; 1→6)-β-D-glucan produced by the ascomyceteous fungus, *Botryosphaeria* sp.. *Carbohydrate Research*, *338*, 1691–1698.

Bowie, J. U., Trescony, P. V., & Gray, G. R. (1984). Analysis of linkage positions in *Saccharomyces cerevisiae* D-mannans by the reductive-cleavage method. *Carbohydrate Research*, *125*, 301–307.

da Silva, B. P., & Parente, J. P. (2003). Bioactive polysaccharides from *Costus spicatus*. *Carbohydrate Polymers*, *51*, 239–242.

Gorin, P. A. J. (1981). Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *38*, 13–104.

Jun, J.-G., & Gray, G. (1987). A new catalyst for reductive cleavage of methylated glycans. *Carbohydrate Research*, *163*, 247–261.

Kiwitt-Haschemie, K., Renger, A., & Steinhart, H. (1996). A comparison between reductive-cleavage and standard methylation analysis for determining structured features of galactomannans. *Carbohydrate Polymers*, *30*(1), 31–35.

Madla, S., Methacanon, P., Kirtikara, K. (in press). Characterization of fungi-derived polymers that are biocompatible and inducers IL-8 production, *Carbohydrate Polymers*.

Ooi, V. E. C., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medical Chemistry*, *7*, 715–729.

Park, J. P., Kim, S. W., Hwang, H. J., & Yun, J. W. (2001). Optimization of submerged culture conditions for the mycelial growth and exo-biopolymer production by *Cordyceps militaris*. *Letters in Applied Microbiology*, *33*, 76–81.

Peng, Y., Zhang, L., Zeng, F., & Xu, Y. (2003). Structure and antitumor activity of extracellular polysaccharides from mycelium. *Carbohydrate Polymers*, *54*, 297–303.

Selbmann, L., Onofri, S., Fenice, M., Federici, F., & Petruccioli, M. (2002). Production and structural characterization of the exopolysaccharide of the Antarctic fungus *Phoma herbarum* CCFEE 5080. *Research in Microbiology*, *153*, 585–592.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various GLC response factor theories for partially methylated and partially ethylated alditol acetate. *Carbohydrate Research*, *40*, 217–225.

Tischer, C. A., Gorin, P. A. J., de Souza, M. B., & Barreto-Bergter, E. (2002). Structures of phosphonogalactomannans isolated from mycelia of *Aspergillus versicolor*. *Carbohydrate Polymers*, *49*, 225–230.