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Structure and antioxidant activity of a novel poly-N-acetylhexosamine produced by a medicinal fungus

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ABSTRACT

A novel poly-N-acetylhexosamine (polyhexNAc) about 6 kDa average molecular weight (MW) was isolated from the low-MW fraction of exopolysaccharide produced by liquid fermentation of a medicinal fungus *Cordyceps sinensis* Cs-HK1. The composition and linkage of sugar residues were determined by mass spectrometry and methylation analysis, and the anomeric configuration and chain linkage were confirmed by NMR. From the analytical results, the molecular structure was elucidated as a [-4-β-D-ManNAc-(1→3)-β-D-GalNAc-(1→] disaccharide repeating unit in the main chain with a Gal branch occurring randomly at the 3-position of ManNAc. This polyhexNAc showed notable antioxidant activities with a Trolox equivalent antioxidant capacity of 330 μmol Trolox/g, a ferric reducing ability of plasma of 45.7 μmol Fe(II)/g, and significant cytoprotective effect against H_2O_2-induced PC12 cell injury. This is the first report on the structure and bioactivity of an extracellular amino-polysaccharide from the *Cordyceps* species.

1. Introduction

There has been an increasing research and commercial interest in recent years in the structures and functions of natural polysaccharides from various sources because of their important roles in cell signaling and great potential for nutraceutical and pharmaceutical uses (Mourao & Pereira, 1999). Edible and medicinal fungi (mushrooms) have been widely applied as a source of nutritive and medicinal products. Polysaccharides including the protein-bound polysaccharides or polysaccharides peptide (PSP) complexes are major bioactive constituents of medicinal fungi with a wide range of health protecting and promoting effects, such as antitumor, immunomodulatory and antioxidant (Li & Tsim, 2004; Wasser, 2002). The most common polysaccharides isolated from edible and medicinal fungi are β-glucans and PSP complexes, some of which have been widely applied to immunotherapy and anticancer products.

Cordyceps sinensis (Berk.) Sacc. generally known as the Chinese caterpillar fungus or DongChongXiaCao in Chinese, is a highly valued medicinal fungus in traditional Chinese medicine with a broad spectrum of health promoting effects on the kidney, lung, liver and immune functions (Li & Tsim, 2004; Zhu, Halpern, & Jones, 1998). Since natural *Cordyceps* is very rare and not sufficient to meet the increasing demand, mycelial fermentation has become a major and more economical source of Cordyceps materials such as mycelial biomass and polysaccharides. Cs-HK1 is a fungus species isolated from the fruiting body of a natural Cordyceps organism in our lab and Cs-HK1 mycelial culture has been established (Leung, Zhang, & Wu, 2006). The Cs-HK1 mycelial culture in a liquid medium was able to produce high yields of exopolysaccharides (EPS) with a complex composition of carbohydrates and proteins in a wide molecular weight (MW) range. We have isolated a few polysaccharides from the crude EPS via column chromatography and characterized their structures, which were heteroglycans composed mainly of α-D-glucose residues with an average molecular weight about 40–50 kDa (Wang, Cheung, Leung, & Wu, 2010; Yan, Li, Wang, & Wu, 2010).

Poly-N-acetylhexosamines (polyhexNAc) are carbohydrate polymers composed of N-acetylhexosamine residues, which have been isolated from the secondary walls of *Bacillus* species (Amano, Hazama, Araki, & Ito, 1977; Ekwunife, Singh, Taylor, & Doyle, 1991; Fox, Black, Fox, & Rostovtseva, 1993). The polysaccharide reported by Ekwunife et al. (1991) was composed of Gal, GlcNAc and ManNAc in an approximate ratio of 3:2:1, extracted from the cell wall of *Bacillus anthracis* with hydrogen fluoride (HF). To the best of our knowledge, however, there is no literature report on a polyHexNAc

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isolated from *C. sinensis* or other medicinal fungi. The polysaccharide structures documented in the literature from *C. sinensis* and other *Cordyceps* species are mostly neutral or acidic glycans (Methacanon, Madla, Kirtikara, & Prasitsil, 2005; Wang et al., 2010; Yan et al., 2010; Yu et al., 2009; Zhong et al., 2009).

In our previous studies, the crude EPS was precipitated as one mixture with ~80% (v/v) ethanol in the liquid medium of Cs-HK1 mycelial culture (Leung, Zhao, Ho, & Wu, 2009). In this study, we applied a two-step precipitation protocol, using a low ethanol concentration (30%, v/v) to exclude the high-MW EPS in the first step, and a high ethanol concentration (70%, v/v) in the next step to obtain the low-MW EPS. The low-MW EPS was fractionated and further purified via a series of steps and one fraction was identified as a polyhexNAc. The molecular composition and structure of polyhexNAc was analyzed by a number of methods such as IR, ES-MS–MS, methylation analysis and NMR. Its antioxidant capacity was evaluated by radical scavenging and reducing ability assays, and by cell culture test against H_2O_2-induced cell injury.

2. Materials and methods

2.1. Materials

The Cs-HK1 fungus used in this study was previously isolated in our lab from the fruiting body of a wild Cordyceps organism and identified as a *Tolypocladium* sp. fungus, and an anamorph of *C. sinensis* both morphologically and genetically as reported in details previously (Leung et al., 2006) which is preserved at China General Microbiological Culture Collection Center (Reg. No. 6004). Ultrahydrogel 250 columns were acquired from Waters Corp. (Milford, MA), DEAE-cellulose anion-exchange resin from Whatman (Brentford, England), papain and cysteine from Fluka (Seelze, Germany). The dextran molecular weight standards (6 ranging from 1 to 1500 kDa) and the carbohydrate standards, D-mannose, ManNAc, GlcNAc, GalNAc, L-fucose, L-arabinose, D-galacturonic acid and lactose were from Sigma (St. Louis, MO), the derivatization reagent, 1-phenyl-3-methyl-5-pyrazolone (PMP), from Sinopharm Chemical Reagent Co., Ltd. (Shanghai), and the D_2O (99.8%) for NMR from Cambridge Isotope Laboratories Inc. (Andover, MA). Trolox ([(S)-(2)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid]) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) for the antioxidant assays were purchased from Calbiochem/EMD (Gibbstown, NJ). The PC12 cell line for the cell culture tests was obtained from ATCC (American Type Culture Collection), the RPMI 1640 medium, and fetal bovine serum from Gibco-BRL (Grand Island, NY), H_2O_2 and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Sigma.

2.2. Fermentation production of EPS and isolation by two-step ethanol precipitation

For the production of EPS by liquid fermentation, the Cs-HK1 fungus was cultivated in 250 mL Erlenmeyer flasks each containing 50 mL of a liquid medium, shaken constantly at 150 rpm and 20 °C for 7 days, as reported previously (Leung et al., 2006). The mycelial broth was then centrifuged and the supernatant liquid medium was collected for the recovery of EPS.

Considering the wide MW range of EPS produced by the Cs-HK1 fungus in liquid fermentation, we applied a two-step ethanol precipitation to separate the EPS into a high-MW and a low-MW fraction. In the first step, 2 volumes of ethanol (96% grade) were added to each volume of the liquid medium to precipitate the high MW EPS. After removal of the precipitate, the remaining supernatant was treated with another step of precipitation with 3 volumes of ethanol to precipitate the low MW EPS. In both steps, the precipitation proceeded at 4 °C overnight, followed by centrifugation (6000 rpm, 15 min). The remaining supernatant layer was removed and the precipitate settled to the bottom of the centrifugal tube was collected as the EPS crop. The EPS precipitate was washed twice with acetone and then dissolved in distilled water, lyophilized, and collected for further fractionation. From 1 L of the fermentation medium, about 2.3 g (0.23%, w/v) was attained in the first step and 0.3 g (0.03%, w/v) in the second step. The low MW EPS fraction from the second step, designated EPS-2, was further fractionated in the following experiments.

2.3. Fractionation of EPS-2 and purification of polyhexNAc

EPS-2 (~0.3 g) was dissolved in 2 mL distilled water and loaded into a Superdex 75 column (2.6 cm × 60 cm), eluted by 0.3 M NH_4HCO_3 at a flow rate of 0.3 mL/min. The eluate was monitored with RI, and one of the fractions showing absorption on the RI but no absorption on the UV was collected as the oligosaccharide fraction OF-III, with a yield of 20% from the original ESP-2. OF-III (~50 mg) was fractionated by anion-exchange chromatography on a DEAE-cellulose column (2.6 cm × 40 cm), eluted with NaCl on a linear gradient from 0 to 0.6 M (in 0.1 M sodium acetate at pH 5.0) at 1.0 mL/min (~500 mL total elution volume). The amino sugar in the eluate was detected by Elson method (Reissig, Strominger, & Leloir, 1955). The fraction collected was desalted on a Bio-Gel P2 column, yielding the polyhexNAc for further structure analysis and bioactivity assays.

2.4. Analysis of polyhexNAc molecular properties

2.4.1. Molecular weight by GPC

High pressure gel permeation chromatography (HPGPC) was performed on a Waters instrument consisting of a Waters 1515 isocratic pump and a Waters 2414 refractive index detector. The column was a Waters Ultrahydrogel 250 and maintained at 40 °C, and eluted with 0.2 M NaCl solution at a flow rate of 0.5 mL/min. The polysaccharide sample was dissolved in distilled water at 1 mg/mL, injecting 20 μL into the system. The chromatograms were recorded on a computer with LC solution Version 1.25 software, and analyzed with a GPC Postrun function. The average molecular weight at a given elution time was calculated by the calibration equation generated with dextran MW standards.

2.4.2. Chemical composition by HPLC

Monosaccharide composition was determined by the PMP-HPLC method (Strydom, 1994). In brief, the polysaccharide sample (~1 mg) was hydrolyzed with 2 M TFA at 110 °C in nitrogen atmosphere for 8 h, and lactose was added after acid hydrolysis as an internal standard. The hydrolysate was dried under vacuum, and then derivatized by adding 450 μL 1-phenyl-3-methyl-5-pyrazolone (PMP) solution (0.5 M, in methanol) and 450 μL of 0.3 M NaOH, and incubation at 70 °C for 30 min. The reaction was stopped by neutralization with 450 μL of 0.3 M HCl, followed with chloroform extraction (1 mL, three times). The extract solution was analyzed by HPLC on a Waters 2870 instrument with an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm) at 25 °C with UV detection at 250 nm. The mobile phase was composed of 0.05 M KH_2PO_4 (pH 6.9) with 15% acetonitrile (solvent A) and 40% acetonitrile (solvent B) in water on a gradient from 8% to 19% B in 25 min.

Sulfate content was analyzed by ion chromatography (Ohira & Toda, 2006) and the results showed no sulfate content in polyhexNAc.

2.4.3. Mass spectrometry

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) was carried out on a TOF Spec-2E instrument (Waters, Manchester, UK). The polyhexNAc sample was dissolved in methanol at 5 μg/mL, and 1 μL was deposited on the sample plate together with a matrix of 2-(4-hydroxyphenylazo) benzoic acid. The instrument was operated on the positive-ion mode with laser energy 20% (coarse) and 60% (fine), and resolution 1000.

Positive-ion electro-spray and tandem mass spectrometry with collision-induced dissociation (ES-MS and CID MS/MS) were performed on a Waters Ultima mass spectrometer with a Q-TOF configuration. Nitrogen was used as the desolvation and the nebulizer gas at flow rates 250 L/h and 150 L/h, respectively. The source temperature was 80 °C and the desolvation temperature 150 °C. A cone voltage 30 V was used for positive-ion detection and the capillary voltage was maintained at 4.5 kV. Product-ion spectra were obtained from CID using argon as the collision gas at 0.17 MPa pressure and with the collision energy between 45 and 60 V for optimal fragmentation. A scan rate of 1.0 s/scan was used for both ES-MS and CID MS/MS experiments, and the acquired spectra were summed for presentation. The polysaccharide sample was dissolved in acetonitrile/water (1:1, v/v) with 0.5% of formic acid, typically at 20 μg/mL, and 5 μL being injected. Solvent (acetonitrile/1 mM ammonium bicarbonate at 1:1, v/v) was delivered at 5 μL/min with a Harvard syringe pump (Harvard Apparatus, Holliston, MA).

2.4.4. Methylation analysis

The linkage analysis was performed according to Ciucanu and Kerek (1984) with minor modifications. Briefly, the polysaccharide sample (~1 mg) was dissolved in dry DMSO (0.50 mL), followed by the addition of a DMSO/sodium hydroxide slurry (0.50 mL), left stirred for 2 h at room temperature. Two separate methyl iodide (0.25 mL) was added with stirring for 30–40 min. The reaction liquid was cooled down and the partially methylated polysaccharide was partitioned between chloroform and water. The partially methylated sample in the chloroform layer was dried and then hydrolyzed by 4 M trifluoroacetic acid at 100 °C for 4 h. The resultant aldoses were reduced to their corresponding alditols by sodium borodeuteride ($NaBD_4$). The partially methylated alditols were then acetylated with a pyridine:acetic anhydride (1:1) solution at 100 °C for 1 h. The partially methylated alditol acetates were dissolved in dichloromethane and analyzed by GC–MS on an Agilent 6890 instrument using a HP-1 MS column. The column was controlled at 50 °C for 2 min, increased to 170 °C at 30 °C/min, and to 250 °C at 5 °C/min and held for 15 min.

2.4.5. NMR and IR spectroscopy

For NMR analysis, the polyhexNAc sample (10 mg) was lyophilized with D_2O (99.8%) twice before final dissolution in 500 μL high-quality D_2O (99.96%) containing 0.1 μL acetone. ^1H NMR analysis was carried out at 600 MHz and ^{13}C NMR at 150 MHz at room temperature on a Bruker AVANCE III 600 spectrometer with Topspin 3.0 software for data processing. The observed ^1H chemical shifts were reported using acetone as an internal reference (2.23 ppm). COSY, HMBC and HMQC experiments were also carried out at 25 °C.

Infrared (IR) spectrum was taken on a Perkin-Elmer 1600 at room temperature. The transmittance spectra were computed from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution with the triangular apodization function in the Spectrum 6.1 software. Symmetrical interferograms on 64 scans were co-added for each spectrum. All spectra were baseline corrected.

2.5. Antioxidant activity tests

The antioxidant activities of purified EPS fractions were determined using two chemical assays, the Trolox equivalent antioxidant capacity (TEAC) and the ferric reducing ability of plasma (FRAP) assay, and a cytoprotection test using H_2O_2-induced cell injury.

The TEAC and FRAP assays have been described in details by Leung et al. (2009). In brief, the TEAC assay measures the ability of a compound to eliminate or scavenge $ABTS^{\bullet+}$ radicals using Trolox as a response reference (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). The $ABTS^{\bullet+}$ radicals were generated from potassium persulphate ($K_2S_2O_8$) oxidation of ABTS. A polysaccharide sample solution in water was mixed with the $ABTS^{\bullet+}$ solution for 20 min at room temperature, followed by measurement of the absorbance at 734 nm. Radical scavenging activity was represented by the percentage of radical reduction as given by $(1 - A/A_0) \times 100$, where A and A_0 are the absorbance values of $ABTS^{\bullet+}$ solution in the presence and absence of the polysaccharide sample, respectively. The activity was converted to TEAC (μmol Trolox/g sample) by the calibration with Trolox from 0 to 30 μM.

The FRAP assay was performed according to Benzie and Strain (1996). The FRAP reagent was freshly prepared by mixing 3 M acetate buffer (pH 3.6), 0.1 M 2,4,6-Tris(2-pyridyl)-s-triazine in 0.4 M HCl and 0.2 M ferric chloride hexahydrate $FeCl_3 \cdot 6(H_2O)$ at 10:1:1 volume ratio. The FRAP reagent was warmed to 37 °C and mixed with a polysaccharide sample solution for 15 min at room temperature. The absorbance of solution was measured at 593 nm and converted to a FRAP activity (μmol Fe(II)/g sample) by the calibration with ferrous sulphate from 0 to 30 μM.

The cytoprotective activity of polysaccharides against oxidative cell damage was tested in rat pheochromocytoma PC12 cell culture, subjected to peroxide H_2O_2 treatment as reported by Li et al. (2003). The polysaccharide samples were pre-dissolved in phosphate buffered saline (PBS) at 10 mg/L. The H_2O_2 solution for the test was freshly prepared at 100 mM in PBS. The PC12 cell culture was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in a CO_2 incubator at 37 °C. The cells were seeded into a 96 well-plate at 5×10^4 cells per mL and 100 μL/well, and incubated for 24 h and then 80 μM H_2O_2 (final concentration) was added to the wells together with the polysaccharide solution at the desired concentrations (0.001–200 μg/mL) or with an equal volume of PBS as the negative control. The cell viability was measured by the MTT assay and represented in percentage relative to the native culture (N) without treatments.

3. Results and discussion

3.1. Fractionation and composition of EPS-2

The EPS-2 fraction was separated with the Superdex 75 column into five fractions (OF-I, II, III, IV and V) as detected by RI (Fig. 1(a)) and were collected for protein and monosaccharide analysis (Table 1). Fractions OF-I, II and III having very low protein contents were probably composed mainly of carbohydrates; OF-IV and V having high protein contents were probably composed of glycopeptides. In terms of the monosaccharide composition, most of these fractions contained both neutral and amino sugars (Table 1). Fraction OF-III, in particular, contained two amino sugars at relatively high molar ratios ($ManNH_2$ 1.0:$GalNH_2$ 1.1:Gal 0.3). This fraction was of special interest as amino polysaccharides had rarely been isolated in contrast to the many neutral and acidic polysaccharides documented in the literature. This fraction was further purified by ion exchange chromatography (Fig. 1(b)) and desalting through a Bio-Gel P2 column, yielding the polyhexNAc. PolyhexNAc exhibited a single peak on HPGPC (Fig. 1(c)), which was calibrated to

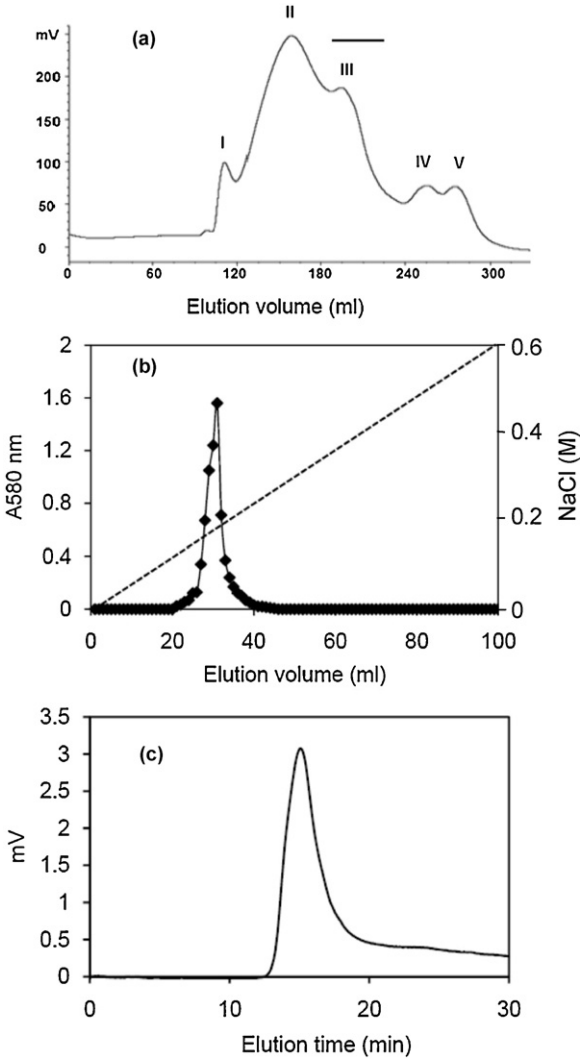

Fig. 1. Chromatographic profiles of EPS fractions: (a) fractionation of the low-MW exopolysaccharide EPS-2 by gel filtration chromatography on a Superdex 75 preparative column (2.6 cm × 100 cm) with RI detection; (b) isolation and purification of fraction OF-III from (a) by ion-exchange chromatography on a DEAE A-52 column, yielding the polyhexNAc; (c) HPGPC profile of polyhexNAc on a TSK3000 column.

an average molecular weight of 6.0 kDa. The molecular composition of polyHexNAc was close to that of OF-III (Table 1).

3.2. IR spectrum of polyhexNAc

Fig. 2 shows the FT-IR spectrum of polyhexNAc in the region of 4000–1800 cm^{-1}. The peaks at 3482 cm^{-1} and 2987 cm^{-1} are characteristic of O—H and C—H stretching vibrations from the GalNAc and ManNAc, respectively. Between 1800 and 400 cm^{-1} are the characteristic bands of amino sugars, i.e. the peak at 1650.9 cm^{-1} assigned to amide I band, the peak at 1560.7 cm^{-1} to amide group II vibration, and the peak at 1419.0 cm^{-1} due to C—N vibration of the N-acetyl group (Foot & Mulholland, 2005; Gaudiana & Conley, 1970). The peak at 1054 cm^{-1} is attributed to C—O—C stretching vibration, and 973 cm^{-1} to C—C stretching vibration with C—C—H deformation. The molecular structure and composition of polyhexNAc derived from the IR analysis was consistent with the above monosaccharide analysis shown in Table 1. In addition, no peak appeared at 1250 cm^{-1} and 850 cm^{-1}, indicating no sulfation in the polyhexNAc molecule.

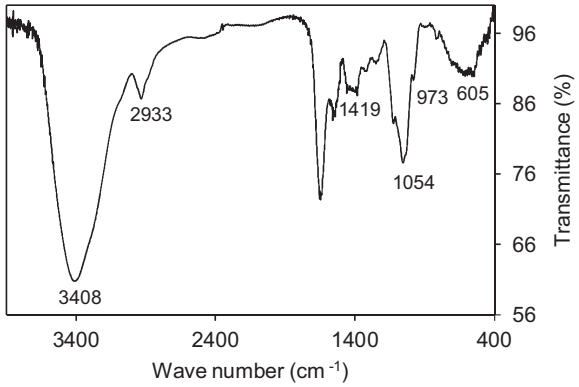

Fig. 2. FT-IR spectrum of polyhexNAc.

Table 2
MS data of polyhexNAc.

Observed ions[a] $[M+Na]^+$	$[M+3H]^{3+}$	Assignment molal mass	Composition
3697.5	1225.5	3675.5	$HexNAc_{18}$
	1279.3	3856.4	$Gal.HexNAc_{18}$
3901.6	1293.2	3878.5	$HexNAc_{19}$
	1333.2	3996.6	$Gal_2.HexNAc_{18}$
4104.8	1360.8	4082.6	$HexNAc_{20}$
	1400.9	4199.7	$Gal_2.HexNAc_{19}$
4308.1	1428.5	4285.1	$HexNAc_{21}$
	1468.8	4402.4	$Gal_2.HexNAc_{20}$
	1482.5	4453.1	$Gal.HexNAc_{21}$
4511.4	1495.6	4488.4	$HexNAc_{22}$
	1536.2	4605.7	$Gal_2.HexNAc_{21}$
4714.4	1564.3	4691.4	$HexNAc_{23}$
4917.6	1631.9	4894.8	$HexNAc_{24}$
	1700.3	5097.9	$HexNAc_{24}$

[a] $[M+Na]^+$ ions obtained from MALDI-TOF-MS and $[M+3H]^{3+}$ ions from ESI-MS.

3.3. Sequence analysis of polyhexNAc by MS

Table 2 shows the series of singly and triply charged ion clusters observed from the mass spectra of polyhexNAc (Supplemental materials Fig. 1) and the assigned molar masses and sugar residues.

Table 1
Protein and monosaccharide contents of EPS fractions attained by gel filtration chromatography on a Superdex 75 column.

Fraction	Protein content (%)[a]	MW (kDa)	Molar ratio[b]					
			$ManNH_2$	Man	Glc	GlcA	Gal	$GalNH_2$
OF-I	3.4	30	–	0.2	1.0	–	0.1	–
OF-II	4.3	7	–	1.0	0.05	–	1.0	0.11
OF-III	6.1	6	1.0	–	–	–	0.3	1.1
OF-IV	30.1	13	–	0.1	–	–	2.1	1
OF-V	50.5	6	–	1.0	3.2	1.5	1.0	0.96
PolyhexNAc	3.2	6	1.0	–	–	–	0.2	1.1

[a] Determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).
[b] –, undetectable.

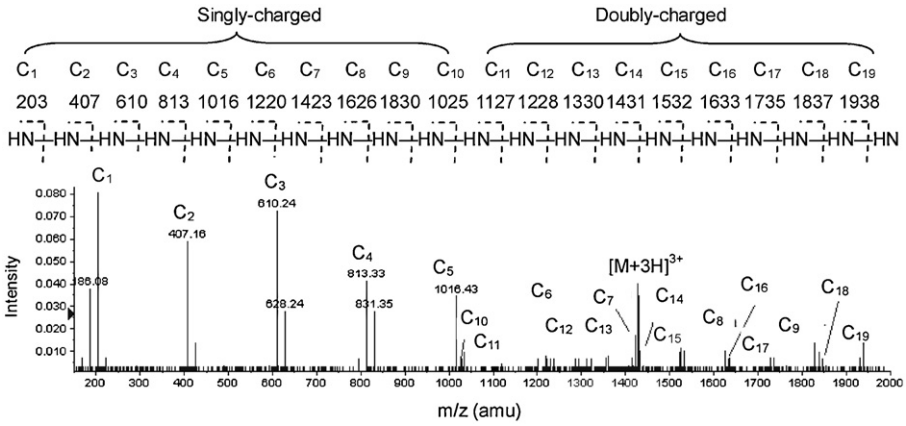

Fig. 3. Positive CID-MS-MS spectrum of a fragment with 1428.3 *m*/*z* from the ES-MS spectrum of polyhexNAc (HN: N-acetylhexosamine residues).

The mass difference between two adjacent singly charged ion clusters was about *m*/*z* 203 and that between the adjacent triply charged ion clusters was *m*/*z* 67.7, both of which are characteristic of N-acetylhexosaminosyl residues. Therefore, both sets of mass data confirmed that the polysaccharide molecule was composed mainly of N-acetylhexosaminosyl sugars. A series of triply charged ion clusters with a *m*/*z* 122.5 also appeared on the ES-MS but not on the MALDI-TOF-MS, which is indicative of a disaccharide-GalhexNAc group present randomly in the glycan chain. The MS results altogether suggested that polyhexNAc was composed mainly of N-acetyl hexosaminosyl residues, which were randomly substituted by Gal.

3.4. ES-CID-MS–MS spectrum

Based on the results from MALDI-TOF-MS and ES-MS, the positive ES-CID-MS/MS spectrum of a triply charged fragment selected from the serial cluster ions at *m*/*z* 1428 on ES-MS was acquired (Fig. 3). On the spectrum, the series of singly charged product ions with a difference between adjacent ions *m*/*z* 203–204 is assigned to C_1—C_9, and the series of doubly charged product ions with *m*/*z* 102 is assigned C_{10}—C_{19}. A full set of B/C ions on the spectrum suggests that the fragments were linear, un-branched oligosaccharides. However, those fragments containing a Gal unit showed very low intensity which could not be assigned by ES-CID-MS/MS were analyzed by methylation analysis and NMR.

3.5. Structure data from methylation analysis

The results from methylation analysis (Table 3) suggests that the polysaccharide fraction contained terminal ManNAc and Gal, 3-linked GalNAc, 4-linked ManNAc and 3,4-linked ManNAc at a mole ratio of 0.13:0.25:1.23:1.0:0.20. The variation in the ManNAc linkage in the polysaccharide chain was indicative of the heterogeneity in the substitution of the ManNAc residues by the Gal, which was consistent with the results from the ES-MS.

3.6. Structure data from NMR

The ^{1}H NMR spectrum of polyhexNAc (Fig. 4(a)) shows only one proton signal (4.90 ppm, d, J = 6.68) in the anomeric region (4.5–5.5 ppm), while ^{13}C NMR (Fig. 4(b)) reveals two obvious anomeric signals at 98.1 and 102.1 (with a proportion of 1.3:1). The NMR results together with those from methylation analysis suggested that the two spin systems be attributed to ManNAc (β) and GalNAc (β). The signals at 2.01 ppm (s) on ^{1}H NMR and 24.8 on ^{13}C NMR were from the N-acetyl group of both GalNAc and ManNAc. The composition was also consistent with that from the monosaccharide analysis.

Full assignments of ^{1}H and ^{13}C chemical-shifts (Table 3) of sugar units of hexNAc as well as Gal were made from 2D NMR analysis (Supplemental materials Fig. 2). The COSY spectrum showed cross-peaks between anomeric protons H-1 and H-2 of sugar units,

Table 3
Structual data from methylation and NMR analysis of polyhexNAc.

GC/GC–MS of alditol acetate derivatives from methylated polyhexNAc

Methylated sugar	RT (min)[b]	Molar ratio	Mass fragment (*m*/*z*)	Type of linkage
2,3,4,6-Tetra-*O*-Me-GalNAc[a]	1.00	0.13	159,161,203,205	GalNAc-(1→
2,3,6-Tri-*O*-Me-ManNAc	1.13	1.0	159,233	→4)-MalNAc-(1→
2,4,6-Tri-*O*-Me-GalNAc	1.25	1.23	159,163,275	→3)-GalNAc-(1→
2,6-DI-*O*-Me-GalNAc	1.31	0.20	159	→3,4)-ManNAc-(1→
2,3,4,6-Tetra-*O*-Me-Gal	0.83	0.23	118,161,162,205	Gal-(1→

NMR data of polyhexNAc[c]

Residue	H_1 (C_1)	H_2 (C_2)	H_3 (C_3)	H_4 (C_4)	H_5 (C_5)	H_6 (C_6)	CH_3	C=O
ManNAc(β)	4.90, J = 7.3 (98.0)	4.18 (50.3)	4.03 (66. 8)	4.00 (76.4)	3.49 (66.9)	3.77 (61.5)	2.01 (24.8)	(177.49)
GalNAc(β)	4.91, J = 7.2 (102.1)	4.03 (50.1)	4.02 (80.8)	3.57 (73.4)	3.90 (70.1)	4.09 (69.4)	1.98 (24.3)	(174.49)
Gal(α)	5.23, J = 3.6 (98.73)	3.78 (76.23)	3.92 (69.4)	–	–	–		

[a] 2,3,4,6-Tetra-*O*-Me-GalNAc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-GalNAc.
[b] Retention time (RT) of sugars relative to that of 2,3,4,6-tetra-*O*-Me-GalNAc.
[c] Chemical shifts in ppm and coupling constants (J) in Hz.

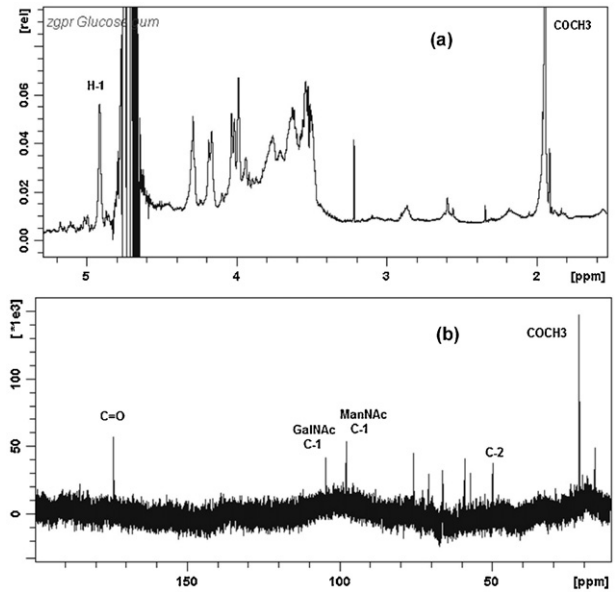

Fig. 4. NMR spectra of polyhexNAc: (a) ^{1}H NMR; (b) ^{13}C NMR.

e.g. M1/M2 for ManNAc. The TOCSY spectrum helped to assign the remaining proton signals, e.g. H-3, H-4 and H-6 in Table 3. M-1 at 4.90 ppm was the anomeric signal from ManNAc, H2-H6 were identified as 4.18, 4.03, 4.00, 3.49 and 3.77 ppm. Based on the assignment of the protons, the carbon signals were assigned by the HMQC, which shows the correlation signal of proton and carbon in the same bonds. It can be deduced from the obvious downshifts in the C-3 of GalNAc and the C-4 of ManNAc that the two sugars should be 3-linked and 4-linked, respectively. The presence of the correlation-relevant signals G1/M4 and M4/G1 in the NOESY also clearly indicates the linkage of GalNAc and ManNAc at the C-4 position of each other. The β anomeric configuration of both ManNAc and GalNAc can be deduced from the H1/H2 coupling constants 7.3 and 7.2 Hz, respectively (Table 1). Together with the results from the MS and methylation analysis and according to IUPAC nomenclature (Dixon, 1982), the complete sequence for the polyhexNAc repeating unit is represented by

[4)- β-D-ManNAc-(1→3)-β-D-GalNAc-(1→)$_n$ (n=9-13, m=1-2)

3
↑
1

α-D-Galp_m

3.7. Antioxidant activity

Table 4 shows the activities of four EPS fractions determined by the TEAC assay for scavenging ABTS$^{\bullet+}$ radicals and the FRAP assay for the ferrous-reducing power. All three indexes showed a consistent trend of activity increase with the increase in protein content (Table 1) from OF-I to OF-V. The strong correlation of antioxidant activity to the protein content of various EPS fractions isolated from the Cs-HK1 mycelial culture has also been observed in our previous study (Leung et al., 2009). In comparison of the TEAC and FRAP values, the novel polyhexNAc (OF-III) was more active than the deproteined and high-MW EPS fractions. The antioxidant function of polyhexNAc as well as other ESP fractions (OF-I to V) was also found in the cell culture test (Supplemental Fig. 3) with a dose-dependent protecting effect against the H_2O_2-induced cell viability loss. In the concentration range of 10–200 mg/mL, the protective effect was statistically significant at $p < 0.05$.

Table 4
Antioxidant activities of EPS fractions attained by gel filtration chromatography on a Superdex 75 column.

Fraction	IC50 on ABTS$^{\bullet+}$ (mg/mL)	TEAC (μmol Trolox/g)	FRAP (μmol Fe^{2+}/g)
OF-I	0.373	154.8	9.5
OF-II	0.295	194.4	13.8
PolyhexNAc[a]	0.174	330.6	45.7
OF-V	0.035	1183.8	611.1

[a] PolyhexNAc was purified from OF-III by DEAE chromatography.

4. Conclusions

A novel polyhexNAc polysaccharide (oligosaccharide) with an average MW 6.0 kDa was isolated and purified from the low MW fraction of EPS precipitated with ethanol from the liquid fermentation medium of a *C. sinensis* fungus Cs-HK1. The complete molecular structure has been established through several experimental and analytical approaches including various MS techniques, methylation analysis and NMR. The polyhexNAc also exhibited notable antioxidant activity. Compared to the poly-N-acetylhexosamine polysaccharides previously found in the bacterium cell walls, the polyhexNAc was an exopolysaccharide produced and released into the liquid medium by the Cs-HK1 fungus and was much easier to recover. In other words, the liquid fermentation of medicinal fungi can be explored for efficient production and recovery of novel and bioactive amino-polysaccharides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.12.067.

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