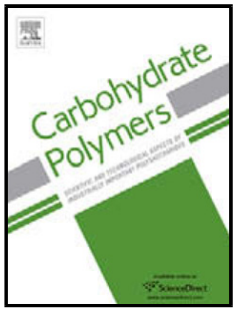

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Purification, structural characterization and bioactivity evaluation of a novel proteoglycan produced by *Corbicula fluminea*

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Highlights

- A novel proteoglycan, named CFPS-11, was isolated from *Corbicula fluminea*
- The structural feature of CFPS-11 was elucidated by physical and chemical analyses
- CFPS-11 exhibited significant antioxidant activity in a dose-dependent manner
- CFPS-11 showed remarkable inhibitory activities against α-amylase and α-glucosidase
- CFPS-11 could be explored as a potent food additive or nutritional supplement

Abstract

A novel proteoglycan, named CFPS-11, was isolated from *Corbicula fluminea*, which is a food source of freshwater bivalve mollusk. CFPS-11 had an average molecular weight of 807.7 kDa and consisted of D-glucose and D-glucosamine in a molar ratio of 12.2:1.0. The protein moiety (~5%) of CFPS-11 was covalently bonded to the polysaccharide chain in O-linkage type through both serine and thereonine residues. The polysaccharide chain of CFPS-11 was composed of (1→4)-α-D-glucopyranosyl and (1→3,6)-α-D-glucopyranosyl residues, which branched at O-6. The branch chain consisted of (1→)-α-D-glucopyranosyl

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and (1→)-α-D-N-acetylglucosamine residues. CFPS-11 exhibited significant antioxidant activity in a dose-dependent manner and remarkable inhibition activities against α-amylase and α-glucosidase by *in vitro* assays. These findings indicated that the CFPS-11 from *C. fluminea* has the potential for development as a health food ingredient.

Keywords: *Corbicula fluminea*; Proteoglycan; Purification; Structural characterization; Antioxidant activity

1. Introduction

Nowadays, natural polysaccharides (PS) have been recognized as an important class of biomacromolecules because of their useful properties. A major area of application of natural PS is in the food industry as food additives or nutraceutical supplements because of their distinct functional properties and numerous health benefits (Giavasis, 2014; Lovegrove et al., 2017). Proteoglycans are a class of protein- or peptide-PS complexes (PSPs) with the protein/peptide chains being covalently bound to the PS side-chains in certain positions, and the total carbohydrate content is significantly higher than that of protein (Murray et al., 2012; Schwartz, 2009). Proteoglycans can have more prominent biological functions and therapeutic properties, such as immunoregulation, antitumor, antioxidant, anticoagulant, hpyerglyceric, and hypolipidemic, than PS, thereby endowing potential applications in functional foods and pharmaceuticals (Fu et al., 2007; Pan et al., 2013; Schwartz, 2009). Over the last few decades, a large number of proteoglycans with various structures and properties have been isolated and purified from various natural resources, including animals, plants, and microorganisms. Some of these proteoglycans include the acidic PSP from pumpkin (Fu et al., 2007), a glycosaminoglycan-like PS from abalone *Haliotis discus hannai* Ino (Li et al., 2011), an arabinogalactan protein from *Lycium ruthenicum* (Peng et al., 2012), a poly-N-acetylhexosamine from *Cordyceps sinensis* (Chen et al., 2013), a proteoglycan from *Ganoderma lucidum* fruiting bodies (Pan et al., 2014), and a PSP from *Herba Epimedii* (Chi et al., 2017). There is a continued interest in further research and development of novel proteoglycans with remarkable bioactivities and nutritional values from natural resources.

Asian clam (*Corbicula fluminea*) belongs to a class of freshwater bivalve mollusk and is widely distributed in China and some other Asian countries. It is consumed as a favorite food because of good taste and nutrition and is also rich in functional ingredients beneficial to health. *C. fluminea*, is also used in traditional Chinese medicine for a broad spectrum of therapeutic effects, such as improved appetite and eyesight, dieresis, liver disease and measles treatment, fever abatement, cough relief, reduced sputum, and antialcoholism (Lin et al., 2012; Zhu, Lin, Wu & Lin, 2004). Modern pharmacological studies have demonstrated that PS and PSPs of *C. fluminea* exhibit antitumor and antioxidant activities (Liao et al., 2013, 2015, 2016; Zhu et al., 2004). For example, Liao et al. (2016) reported a novel protein-bound PS, named CFPS-1 isolated from *C. fluminea* could effectively and inhibit breast cancer MCF-7 and MDA-MB-23 cells. Recently, our research group attained a proteoglycan (CFPS) from *C. fluminea* by three-phase partitioning (TPP) technique and the partially purified CFPS shows notable radical scavenging and antioxidant activities *in vitro* (Yan, Wang, Qiu & Shao, 2017). The biological activities of PS and PSPs depend mainly on chemical composition and structural characteristics, such as monosaccharide composition, molecular weight (MW), glycosidic bond, and chain conformation (Zhang, Cui, Cheung, & Wang, 2007). However, to the best of our knowledge, no or little attention has been devoted to investigate the purification, structural elucidation, and bioactivity evaluation of the CFPS isolated from *C. fluminea* by using TPP approach.

The present work was on the purification and bioactivity assessment of a novel proteoglycan, named CFPS-11 isolated from *C. fluminea* through column chromatographies. CFPS-11 was structurally characterized by the combination of physical and chemical methods. Additionally, the antioxidant and α-amylase, and α-glucosidase inhibition activities of CFPS-11 were evaluated by *in vitro* assays.

2. Materials and methods

2.1. Materials and chemicals

The CFPS was obtained from *C. fluminea* by the three-phase partitioning (TPP) method which was devloped in our previsous study (Yan et al., 2017). In brief, the TPP was

performed with 20% (w/v) mass fraction of $(NH_4)_2SO_4$, 9.8 mL of *t*-butanol at 35.3°C, extraction time of 30 min, and pH 6.0. The CFPS recovered from the lower aqueous phase was then dialyzed, concentrated, and lyophilized to yield the partially purified CFPS. Acarbose, 1-phenyl-3-methyl-5-pyrazolone (PMP), porcine pancreatic α-amylase, α-glucosidase, p-nitrophenyl-α-D-glucopyranoside (PNPG), 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DEAE-Sepharose Fast Flow and Superdex^{TM} 200pg were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other chemicals and solvents were of laboratory grade and used without further purification.

2.2. Isolation and purification of CFPS-11

CFPS (1.0 g) was completely dissolved in 20 mL of deionized water, centrifuged at 15000 rpm for 30 min, and loaded onto a pre-equilibrated DEAE Sepharose Fast Flow chromatography column (2.0×40 cm). Afterward, CFPS was eluted successively with deionized water and NaCl aqueous solution (0.1 and 0.2 M) at a flow rate of 2.0 mL/min. Each fraction (8.0 mL) was collected and separately detected by phenol-sulfuric acid method at 490 nm (Dubois et al., 1956) and spectrophotometer method at 280 nm. The main fraction was combined, concentrated, dialyzed (MWCO: 8-12 kDa), and lyophilized to obtain two PS fractions, namely, CFPS-1 and CFPS-2 (Fig. 1a). CFPS-1 was further applied to a Superdex^{TM} 200 pg gel filtration chromatography column (1.5×60 cm) by using deionized water as elute at a flow rate of 2.0 mL/min. The purified PS named CFPS-11 was obtained (Fig. 1b) and subsequently used for structural characterization and bioactivity evaluation in this work.

2.3. General physicochemical properties analysis

The total carbohydrate content, and protein content of CFPS-11 were determined by phenol-sulfuric acid method using D-glucose as a standard, and Bradford method using bovine serum albumin (BSA) as a standard, respectively (Dubois et al., 1956; Bradford,

1976). Optical rotation was determined for the CFPS-11 dissolved in deionized water on a Perkin-Elmer 341 digital polarimeter at 589 nm and 20 °C. UV-visible spectrum of CFPS-11 (1.0 mg/mL) was recorded in a Varian Cary 100 spectrophotometer (Varian Co., USA) from 190 nm to 400 nm at 25 °C.

2.4. Homogeneity and MW determination

The homogeneity and M_w of CFPS-11 was determined by high-performance gel permeation chromatography (HPGPC). HPGPC was performed on a Waters 1515 isocratic pump and a Waters 2414 refractive index detector equipped with two ultrahydrogel columns (250 and 2000, 7.8 mm × 300 mm, Waters Corp., Milford, MA, USA) at 50 °C and eluted with deionized water at flow rate of 0.6 mL/min. CFPS-11 (2.0 mg/mL) was dissolved in deionized water and filtered through 0.45 μm membranes (Millipore, USA) before injection. Dextran MW standards ranging from 5.2 kDa to 1482 kDa (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used for calibration. Data were collected and analyzed by the online Breeze software package (Waters Corp., Milford, MA, USA).

2.5. Monosaccharide composition and amino acid analysis

Monosaccharide composition was analyzed by the PMP-HPLC method according to our previous report (Chen et al., 2013). Briefly, CFPS-11 was treated through a series of procedures, including hydrolysis, derivation, incubation, neutralization, and chloroform extraction. The resultant aqueous layer was analyzed by HPLC on an Agilent 1100 instrument with an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6×150 mm) at 25 °C with UV detection at 250 nm. The mobile phase was potassium phosphate-buffered saline (PBS, 0.05 M, pH 6.9) containing 15% acetonitrile (solvent A) and 40% acetonitrile (solvent B) at a flow rate of 1.0 mL/min. D-arabinose (D-Ara), D-glucose (D-Glc), D-glucosamine (D-GlcN), D-galactose (D-Gal), D-galacturonic acid (D-GalA), D-mannose (D-Man), L-rhamnose (L-Rha) and D-xylose (D-Xyl) were used as monosaccharide standards for the identification and quantification of the corresponding peaks.

For amino acid composition, CFPS-11 (2.0 mg) was completely hydrolyzed with 1.0 mL

of HCl (6 M) at 110 °C for 24 h in a sealed tube. The hydrolysate was dried by evaporation under reduced pressure and redissolved in 100 μL of citrate buffer. Amino acid composition was recorded on an automated amino acid analyzer (HITACHI L-8800, Amino Acid Analyzer, Japan).

2.6. Methylation analysis

Freeze-dried CFPS-11 (10 mg) was methylated three times according to the reported method with some modifications (Needs & Sevendran, 1993). The permethylated CFPS-11 was successively subjected to depolymerization by 90% formic acid at 100 °C for 6 h, hydrolysis by TFA (2 M) at 100 °C for 4 h, reduction with $NaBH_4$, and acetylation with acetic anhydride. The resulting methylated alditol acetate was dissolved in chloroform and analyzed by GC-MS on an Agilent 6890 instrument with 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.

2.7. FT-IR and NMR spectroscopic analyses

The FT-IR spectrum of the CFPS-11 was performed with a Nexus 670 FT-IR spectrometer (Thermo Nicolet Co., USA) in the wavenumber range of 500–4000 cm^{-1} with KBr pellets and referenced against air. For NMR analysis, 30 mg of freeze-dried CFPS-11 was dissolved in 0.5 mL of D_2O. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AVANCEIII 600 MHz spectrometer (Bruker, Rheinstetten, Germany) at 25 °C. All chemical shifts were expressed in reference to DDS (δ ^{1}H 0.00 ppm, δ ^{13}C 0.00 ppm) as an external standard.

2.8. β-Elimination reaction

CFPS-11 was subject to β-elimination reaction to analyze the linkage types between protein and PS according to the reported method with minor modifications (Chen et al., 2008). Briefly, CFPS-11 (5.0 μg/mL) was dissolved in 0.2 mol/L NaOH containing 1.0 mol/L $NaBH_4$ and incubated at 25 °C for 2 h. Afterward, the resultant solution was measured by a

Varian Cary 100 spectrophotometer (Varian Co., USA) in the wavelength range of 210–270 nm. The CFPS-11 aqueous solution (5.0 μg/mL) without alkaline treatment was used as a control.

2.9. In vitro antioxidant activity

The *in vitro* antioxidant activities of the CFPS-11 were evaluated through DPPH and hydroxyl free radicals scavenging abilities. The CFPS-11 was previously dissolved in deionized water at different concentrations (0.5-5.0 mg/mL). Vitamin C (V_c) was used as a positive antioxidant reference. Details of the operation conditions and methods were described as previously reported (Wang, Chen, Luo & Yan, 2016).

2.10. α-Amylase and α-glucosidase inhibitory activity of CFPS-11

2.10.1. α-Amylase inhibitory activity

The α-amylase inhibitory activity of CFPS-11 was determined through the reported methods with slight modifications (Lordan et al., 2013). In this test, PBS (100 mM, pH 6.9) was used as solvent. CFPS-11 was previously dissolved in PBS at various concentrations (0.5–5.0 mg/mL). First, 500 μL of porcine pancreatic α-amylase (1 U/mL) was added in the CFPS-11 solution and incubated at 37 °C for 10 min. Subsequently, 500 μL of potato starch solution (1%, w/w) was mixed and continued to incubate for 10 min at 37 °C. The reaction was terminated by addition of 1.0 mL of dinitrosalicylic acid reagent and boiling at 100 °C for 5 min. The mixture was diluted by 10 mL of deionized water. Absorbance was measured at 520 nm by a UV spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd., China). The α-amylase inhibitory activity (%) was estimated by the following equation: Inhibitory activity (%) = $(1 - A_1/A_0) \times 100\%$, where A_1 and A_0 are the absorbance values of the solution with sample and without sample, respectively. Acarbose was used as a positive reference, and deionized water was used as the blank control.

2.10.2. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity of CFPS-11 was investigated according to previous

reports with minor modifications (Lordan et al., 2013). PBS (100 mM, pH 6.9) was used as solvent in this assay. CFPS-11 was completely dissolved in PBS at different concentrations (0.5–5.0 mg/mL). Approximately 100 μL of the sample solution was mixed with 10 μL of α-glucosidase solution (5.7 U/mL) and incubated at 37 °C for 10 min. Subsequently, 200 μL of PNPG (6 mM) was added in the reaction mixture and kept warm at 37 °C for 20 min. Afterward, 1.0 mL of Na_2CO_3 solution (1.0 M) was used for cessation reaction. Absorbance was measured at 400 nm by a UV spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd., China). The α-glucosidase inhibitory activity (%) was calculated according to the following formula: Inhibitory activity (%) = $(1 - A_1/A_0) \times 100\%$, where A_1 and A_0 are the absorbance values of the solution with sample and without sample, respectively. Acarbose was used as a positive reference, and deionized water was used as the blank control.

2.11. Statistical analysis

All experiments are conducted in three replicates and the mean ±standard deviation (SD) is used in the analysis. The statistical analysis was performed by Student's *t*-test and analysis of variance (ANOVA) using OriginPro Software Version 8.0 (OriginLab Corp., MA, USA). $P<0.05$ indicated statistically significant differences.

3. Results and discussion

3.1. Isolation, purification, and physicochemical properties of CFPS-11

The CFPS attained by the TPP method was furhter fractionate by ion exchange chromatography on a DEAE Sepharose Fast Flow column. As shown in Fig. 1a, the CFPS was separated into two fractions (CFPS-1 and CFPS-2), which were eluted with deionized water and NaCl aqueous solution (0.1 M), respectively. Two peaks showed simultaneous absorbances at 490 and 280 nm. The yields of CFPS-1 and CFPS-2 were 32.30% and 19.05%, respectively, and the fraction CFPS-1 was considered as the main component. Given its high yield and convenient preparation, CFPS-1 was subsequently subjected to further purification by using gel filtration chromatography on a Superdex^{TM} 200 pg column (Fig. 1b). The purified CFPS-11 was collected, concentrated, dialyzed, and lyophilized for further structural

characterization and bioactivety evaluation. The preliminary results obtained from column chromatographies demonstrated that the CFPS-11 was a proteoglycan.

The purified CFPS-11 appeared as white fluffy material after lyophilization. The carbohydrate and protein contents were 95.4%±1.5% and 4.5%±0.2%, respectively (Table 1). Moreover, two characteristic bands were observed in the UV visible spectrum of the CFPS-11 (data not shown) at approximately 205 and 280 nm; these bands were attributed to carbohydrates and proteins, respectively. These results suggested that the purified CFPS-11 was a proteoglycan. In this work, HPGPC was used to determine the homogeneity and M_w of CFPS-11, and the obtained results are shown in Fig. 1c and Table 1. CFPS-11 showed a single and symmetrical sharp peak, which indicated that CFPS-11 was a homogeneous proteoglycan. According to the calibration equation derived by linear regression of the calibration curve with standard dextrans, the M_w of CFPS-11 was around 807.7 kDa (Table 1). The M_w of CFPS-11 obtained in this study was considerably higher than that described by Liao et al. (2013, 2016); the difference may be due to the variations in raw materials and separation protocols. In addition, the optical rotation of CFPS-11 was $[\alpha]_D^{20}$ = +32.3° (c 0.2, H_2O) (Table 1), which indicated that CFPS-11 may mainly contain α-type glycosidic linkages in its structure.

The HPLC-PMP derivatization method was applied to analyze the monosaccharide composition of the CFPS-11, and the results are summarized in Table 1. Comparison with the monosaccharide standards concluded that CFPS-11 was composed of D-Glc and D-GlcN in a molar ratio of 12.2:1.0. Liao et al. (2013) found that a papain-released PS (CFPS-2) isolated from *C. fluminea* was composed mainly of glucose and fucoseat a molar ratio of 0.68:0.86 and also minor contents of galactose, glucosamine, and galactosamine. Later, Liao et al. (2016) reported a novel protein-bound PS (CFPS-1) isolated from *C. fluminea* which was composed mainly of mannose and glucose in a molar ratio of 3.1: 12.7. In comparison, CFPS-11 exhibited a distinct monosaccharide composition. Additionally, CFPS-11 was also different from proteoglycans obtained from other mollusks, such as *Anodonta anodonta* (Volpi & Maccari, 2005) and *H. discus hannai* Ino (Li et al., 2011).

As illustrated in Table 2, analysis of amino acids in CFPS-11 indicated that the protein composition of CFPS-11 consisted of 17 kinds of amino acids, among which aspartic acid (1.38 mg/g), threonine (1.20 mg/g), serine (1.21 mg/g), glutamic acid (1.64 mg/g), leucine (1.40 mg/g), lysine (5.48 mg/g), and methionine (1.83 mg/g) were the main components. The relatively high contents of threonine (1.20 mg/g) and serine (1.21 mg/g) revealed the possible existence of O-glycosidic linkage between PS and protein in CFPS-11 (Chen et al., 2008). Similar phenomena were also observed in previous studies (Li et al., 2011; Pan et al., 2014, 2015; Peng et al., 2012). However, He et al. (2013) found that a novel protein-bound PS (HS002-II) purified from the submerged cultures of *Hirsutella sinensis* Liu, Guo, Yu, and Zeng consisted of 57.9% PS and 42.1% protein with N-type carbohydrate–protein linkage. Liao et al. (2016) detected 15 amino acids in the protein-bound PS CFPS-1, and glutamic acid, serine, and threonine were the main components. In addition, the existence of aspartic acid (1.38 mg/g) and glutamic acid (1.64 mg/g) in CFPS-11 indicated that the CFPS-11 may be an acidic proteoglycan. Linkages between PS chain and protein moiety in the proteoglycan strucutures are divided into two types according to their stability against alkali, that is, O-glycosidic linkages and N-glycosidic linkages (Matsunaga et al., 1998). In the present study, Fig. 1d shows that β-elimination of CFPS-11 occurred after alkaline treatment due to the distinct absorbance at around 240 nm. Such observation implied that the protein and PS were covalently linked by an O-glycosidic linkages in CFPS-11. This result was in a good agreement with the results of amino acid analysis.

3.2. Structural characteristics of CFPS-11

The FT-IR spectrum of CFPS-11 is shown in Fig. 2a. A strong and wide absorption peak at approximately 3386 cm^{-1} corresponded to the stretching vibrations of O–H and N–H. A weak absorption peak at about 2928 cm^{-1} was attributed to the stretching vibration of C–H. The characteristic absorption peak at around 1647 cm^{-1} was ascribed to the bending vibration of amide or amino group ($–NH_2$). The absorption peak at about 1543 cm^{-1} was due to the secondary –CONH– group of proteins (Liao et al., 2016; Zhang, 1999), which indicated the presence of protein in CFPS-11. The absorption bands between 934 and 1153 cm^{-1} were due

to the stretching vibrations of pyranose rings (Liao et al., 2013). The three stretching peaks at about 1153, 1059, and 1025 cm^{-1} further implied the presence of pyranoside in CFPS-11. The peak at around 839 cm^{-1} indicated the presence of α-glycosidic bonds in the PS fraction (Zhang, 1999). This finding obtained from FT-IR spectroscopy indicated that the CFPS-11, which contained α-glycosidic bonds, was a proteoglycan. Such result was in good agreement with the results presented in Fig. 1 and Table 1.

The specific interglycosidic linkages between the monosaccharide residues of PS are commonly elucidated by methylation analysis. In this work, CFPS-11 was methylated, hydrolyzed, converted to alditol acetates, and analyzed by GC-MS. Table 3 presents the identification and proportions of the methylated alditol acetates of CFPS-11 according to relative literature and the standard data from the Complex Carbohydrate Research Center (CCRC, University of Georgia, USA) Spectral Database for PMAA (Sassaki et al., 2005; Wang, He & Huang, 2007). Results showed that CFPS-11 was mainly composed of 2,3,4,6-Me_4-Glcp, 2,3,4,6-Me_4-GlcNAc, 2,3,6-Me_3-Glcp, and 2,4-Me_2-Glcp in a molar ratio of 1.96:1.00:7.12:2.86, which indicated the presence of Glcp(1→, GlcNAc(1→, →4)Glcp(1→, and →3,6)Glcp(1→ residues in the main chain of CFPS-11. This result also suggested that the →4)Glcp(1→ residue might be regarded as the main consecutive repeating unit of CFPS-11 due to its high proportion, and the →3,6)Glcp(1→ residue might be the branching linkage in the CFPS-11 structure. Furthermore, the Glcp(1→ and GlcNAc(1→ might be considered as terminal residues attached to the O-6 position of →3,6)Glcp(1→ residue. This result showed a good correlation between terminal and branched residues. Moreover, the methylation analysis result well agreed with that of monosaccharide composition (Table 1).

NMR spectroscopy was used for further structural characterizations of the CFPS-11, including monosaccharide composition, anomeric configuration, and the types and sequences of the glycosidic linkages according to the available data reported in literature (Agrawal, 1992; Yu & Yang, 1999; Zhang, 1999). As shown in the ^1H NMR spectrum (Fig. 2b), four anomeric proton signals at 5.29, 5.32, 5.14, and 4.89 ppm were observed; these signals

indicated that the repeating unit of CFPS-11 was composed of four residues, namely, α-D-Glcp(1→, →4)-α-D-Glcp(1→, →3,6)-α-D-Glcp(1→, and α-D-GlcNAc(1→ residues, which were designated as A, B, C, and D residues, respectively; these results were well consistent with the methylation analysis results. The chemical shifts from δ 3.0 ppm to δ 4.2 ppm were assigned to the H-2 to H-6 protons. In addition, the resonance peaks observed in the range of δ0.5–2.8 ppm were assigned to amino acid signals (Banfi & Patiny, 2008), among which chemical shift at δ 1.97 ppm was ascribed to the N-acetyl group of GlcNAc (Agrawal, 1992). This result was also in agreement with that from monosaccharide composition analysis. As shown in the ^{13}C NMR spectrum of CFPS-11 (Fig. 2c), four anomeric carbon signals at δ 99.40, 99.09, 95.79, and 91.98 ppm were observed and attributed to the C-1 of the →4)-α-D-Glcp(1→, α-D-Glcp(1→, →3,6)-α-D-Glcp(1→, and α-D-GlcNAc(1→ residues in the CFPS-11 structure, respectively. Therefore, the strong anomeric carbon signal at δ 99.40 ppm was ascribed to the C-1 in (1→4)-α-D-Glcp, and the signals at 71.18, 73.24, 76.59, 72.43, and 60.26 ppm were assigned to C-2, C-3, C-4, C-5, and C-6 of the (1→4)-α-D-Glcp residues, respectively. Moreover, the anomeric signal observed at δ 95.79 ppm was attributed to (1→3,6)-α-D-Glcp. Other carbon signals from C-2 to C-6 within 60–78 ppm in the ^{13}C NMR spectrum were assigned according to the reference data (Zhang, 1999). Resonances at δ 91.98, 54.61, 72.30, 71.82, 72.20, and 60.97 ppm were assigned to C-1, C-2, C-3, C-5, and C-6 of α-D-GlcNAc(1→ residues, respectively. In particular, a weak resonance at δ 176.82 ppm corresponded to the –CONH group of protein, further indicating that CFPS-11 was a proteoglycan; this result was consistent with those UV-vis results and FT-IR spectra. The presence of carbon signal at 21.42 ppm belonged to the N-acetylated methyl group (Agrawal, 1992; Chen al., 2013; Pan et al., 2015). The entire assignment of the ^{1}H and ^{13}C chemical shifts of CFPS-11 were achieved on the basis of the ^{1}H and ^{13}C NMR spectra, and they are summarized in Table 4.

In summary, on the basis of the results of acid hydrolysis, methylation analysis, HPLC, GC-MS, FT-IR, and NMR measurements, the CFPS-11 isolated from *C. fluminea* was concluded as a novel water-soluble proteoglycan comprised of D-Glc and D-GlcN in a molar

ratio of 12.2:1.0 with a M_w of 807.7 kDa. The protein moiety was linked covalently to the PS chain of CFPS-11 in O-linkage type through both serine and threonine residues. The CFPS-11 might possess a backbone of (1→4)-α-D-Glcp (55.02%) and (1→3,6)-α-D-Glcp (22.10%) residues, which branched at O-6. The branch consisted of (1→)-α-D-Glcp (15.15%) and (1→)-α-D-GlcNAc (7.73%) residues. Hence, on the basis of these data, the possible chemical structure of repeating unit of the CFPS-11 was predicted and displayed in Fig. 2d. The structural features of CFPS-11 were different from those of protein-bound PS (CFPS-1) reported by Liao et al. (2016). The CFPS-1 possessed a backbone of 1,6-linked and 1,4,6-linked-α-D-Glcp, which was terminated with a 1-linked-α-D-Manp residue at the O-4 position of 1,4,6-linked-α-D-Glcp in a molar ratio of 3:1:1. Regarding the other mollusks, Volpi & Maccari (2005) reported that glycosaminoglycans from the body of the large freshwater mollusk bivalve *A. anodonta* are composed of chondroitin sulfate (~38%), nonsulfated chondroitin (~21%), and heparin (41%). Li et al. (2011) also found a novel glycosaminoglycan-like sulfated PS (AAP) from the pleopods of *H. discus hannai* Ino, and the AAP containing a chondroitin-like bone is composed of galactosamine, glucuronic acid, fucose, and galactose with a ratio of 2.14:2.37:2.94:1; the sulfate content is 15.5%.

3.3. Antioxidant activity of CFPS-11

DPPH·, as a stable nitrogen atom-centered free radical, has been frequently used to evaluate the free radical scavenging capacity of antioxidants, such as plant extracts and natural and synthesized compounds in food science (Li et al., 2008). The scavenging ability of CFPS-11 on DPPH free radicals compared with vitamin C (V_c) as a positive reference is illustrated in Fig. 3a. Evidently, CFPS-11 showed an increased DPPH radical scavenging ability in a dose-dependent manner in the concentration range of 0.5–5.0 mg/mL. At 5.0 mg/mL CFPS-11, the scavenging ability on DPPH radical was 61.28%, which was considerably weaker than that of V_c (91.62%, 0.5 mg/mL). The EC_{50} value of CFPS-11 was 3.80 mg/mL, which was smaller than that of a papain-released PS (CFPS-2) (4.30 mg/mL) from *C. fluminea* (Liao et al., 2013). Moreover, the scavenging effect of CFPS-11 on DPPH radical was stronger than that of CFPS (58.91%, 5.0 mg/mL) obtained by TPP approach in

our previous study (Yan et al., 2017). This result indicated that CFPS-11 exhibited stronger DPPH radical scavenging capacity than those of CFPS and CFPS-2; this difference may be attributed to its hydrogen donation power to the free radicals, thereby effectively scavenging the DPPH radicals. As reported by Li et al. (2013), an antioxidant glycoprotein (Fraction AIV-2) of 27.2 kDa was purified from the ethanol-soluble protein hydrolysate of *Mustelus griseus* muscle. The DPPH radical scavenging activity of fraction AIV-2 was up to about 97% and was even higher than that of V_c at 5.0 mg/mL. Additionally, Xia et al. (2011) isolated a water-soluble protein-bound PS (POPPS-a) from the fruiting bodies of *Pleurotus ostreatus*, which exhibited potent radical scavenging activity in the three *in vitro* assays.

As the most active reactive oxygen, hydroxyl radical can induce severe damage to adjacent biomolecules in the body, which results in a variety of diseases, such as aging, cancer, and Alzheimer disease (Rollet-Labelle et al., 1998). Hence, scavenging on hydroxyl free radicals by antioxidants may be protective against tissue injury. As shown in Fig. 3b, the effects of CFPS-11 on hydroxyl radical scavenging ability were similar to those of DPPH radical scavenging ability at the tested concentration range (0.5–5.0 mg/mL). At 5.0 mg/mL, the scavenging ability of CFPS-11 on hydroxyl radical was 60.33%, which was slightly stronger than that of CFPS (47.10%, 5.0 mg/mL) reported by our research group (Yan et al., 2017); by contrast, this value was far lower than that of V_c (96.67%, 0.5 mg/mL). The EC_{50} value of CFPS-11 was 3.94 mg/mL. Zhang et al. (2013) reported that a mannoglucan sulfate (SF-2), which was isolated and purified from starfish, displayed significant antioxidant activity *in vitro*. The hydroxyl radical scavenging activity of SF-2 was up to 100% at the concentration of 10 mg/mL. Two fucosylated chondroitin sulfate (ACP and HOP) from sea cucumber *Acaudina molpadioidea* and *Holothuria nobilis*, showed 30% and 24.6% scavenging effects on hydroxyl radicals , respectively at 0.5 mg/mL (Zou, Pan, Dong, He, & Wang, 2016). The scavenging capacity on hydroxyl free radicals may be related with several factors, including monosaccharide composition, amino acid composition, MW, and chemical structure of proteoglycans. This finding suggested that CFPS-11 would be beneficial to a certain extent to protect against oxidative damage.

3.4. Inhibitory effects of CFPS-11 on α-Amylase and α-glucosidase

Nowadays, the hypoglycemic activity *in vitro* of bioactive compounds is commonly evaluated through inhibition assays of α-amylase and α-glycosidase activity (Lordan et al., 2013; Wu, Shi, Wang & Wang, 2016). The inhibition of α-amylase and α-glycosidase can evidently delay the conversion of glucose to blood sugar and reduce the postprandial content of blood glucose. Consequently, such inhibition can be regarded as one of the most effective approach for treating type 2 diabetes (Tundis, Loizzo & Menichini, 2010; Wu et al., 2016). Therefore, in the present work, the inhibitory effects of CFPS-11 on α-amylase and α-glycosidase activities were evaluated. As shown in Fig. 3c, the inhibitory effect of CFPS-11 on α-amylase dose-dependently increased with the increasing concentration of CFPS-11 at 0.5–5.0 mg/mL. At 5.0 mg/mL, the inhibitory ability of CFPS-11 on α-amylase was 51.18%, which was higher than that of the protein-PS PSP-I from pumpkin (*Cucurbita moschata*) seeds (Wang et al., 2017), but lower than that of the positive control acarbose (70.87%). Our result indicated that the CFPS-11 showed evident α-amylase inhibitory activity with an EC_{50} value of 4.86 mg/mL *in vitro*. Furthermore, the CFPS-11 also presented a concentration-dependent inhibitory effect on α-glycosidase activity in the determined concentration range (0.5–5.0 mg/mL) (Fig. 3d). The inhibitory effect of CFPS-11 on α-glycosidase was 65.74% at 5.0 mg/mL, which was less than that of the positive reference acarbose (92.78%). Result suggested that CFPS-11 exhibited excellent α-glycosidase inhibitory ability with an EC_{50} value of 3.92 mg/mL *in vitro*. Zhang et al. (2016) reported that water-soluble PS (GP90 and P90) extracted from the fruit of *Psidium guajava*, exhibited excellent α-glycosidase inhibition activity with an EC_{50} of 2.27 μg/mL and 0.18 mg/mL, respectively. The inhibitory activities of GP90 and P90 were 1379- and 17-times higher, respectively than the acarbose positive control (EC_{50} 3.13 mg/mL). Notably, CFPS-11 showed the stronger inhibitory ability against α-glycosidase than that of α-amylase under the same concentrations. This difference might be due to the different mechanisms of action between α-amylase and α-glycosidase, and a moderated inhibition of α-amylase and a stronger α-glycosidase inhibition should be preferred (Cho, Han & You, 2011). Similarly, Kim et al (2014) found that fucoidan extracted from *Ascophyllum nodosum* inhibited both

α-amylase (IC_{50} 0.12-4.64 mg/mL) and α-glycosidase (IC_{50} 0.013-0.047 mg/mL), but the quantities required for α-amylase inhibition were much higher than those required for α-glycosidase. In contrast, Li et al. (2017) reported that *Sargassum pallidum* PSs exhibited weaker inhibitory effects on α-glycosidase than α-amylase. The α-amylase and α-glycosidase inhibitory activities may be attributed to the structural characterizations of the CFPS-11, such as carbohydrate and protein contents, monosaccharide composition, amino acid composition, MW, functional group, chemical structure, chain conformation, and extraction and isolation methods. Therefore, these findings suggested that the CFPS-11 isolated from *C. fluminea* could be useful as a potential component of new mediation as a food additive or as a food supplement for an enzyme-targeted treatment of type 2 diabetes. Further studies on hypoglycemic activity *in vivo* and molecular mechanism for type 2 diabetes are currently underway in our laboratory.

4. Conclusions

A novel proteoglycan (CFPS-11) was isolated from *C. fluminea*, and its structural characterization and bioactivity were evaluated. Results showed that the CFPS-11 was composed of D-Glc and D-GlcN in a molar ratio of 12.2:1.0 with a M_w of 807.7 kDa. The protein moiety was linked covalently to the PS chain of CFPS-11 in O-linkage type through both serine and thereonine residues. The CFPS-11 structure consisted of a (1→4)-α-D-Glcp backbone, and the (1→3)-α-D-Glcp residues formed branches at the O-6 position with 1-linked-α-D-Glcp and 1-linked-α-D-GlcNAc terminal residues. The CFPS-11 possessed remarkable antioxidant activity and α-amylase and α-glycosidase inhibitory activities *in vitro*. Thus, these results suggested that the CFPS-11 isolated from *C. flumenia* can be futher explored as a potential functional food or nutritional supplement for improving human health.

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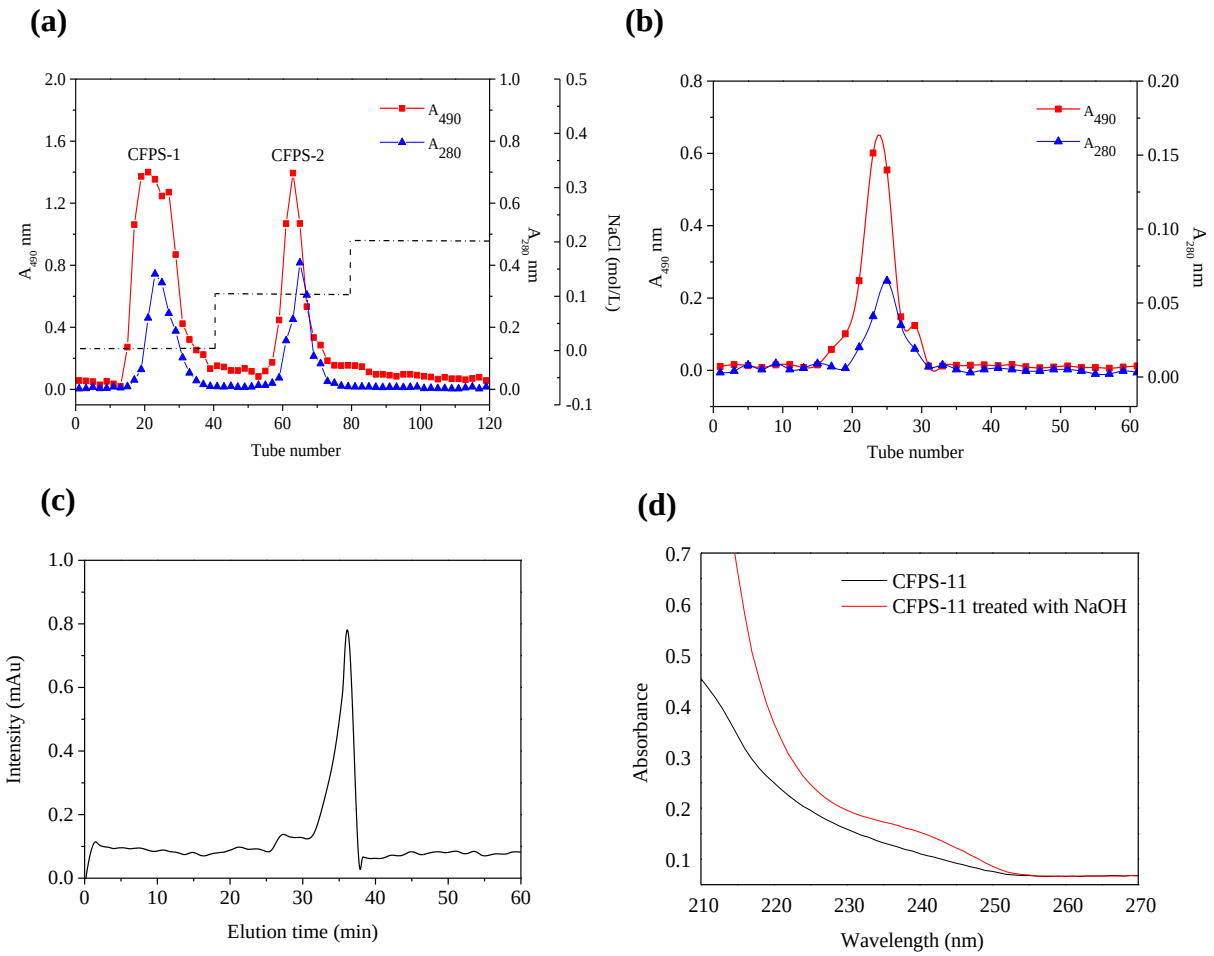

Fig. 1. (a) DEAE Sepharose Fast Flow chromatographic profile of CFPS eluted with deionized water and NaCl solution (0.1 and 0.2 M). (b) Superdex^{TM} 200 pg chromatographic profile of CFPS-1 eluted with deionized water. (c) High-performance gel permeation chromatography chromatogram of CFPS-11. (d) UV spectrum of CFPS-11 before and after β-elimination reaction.

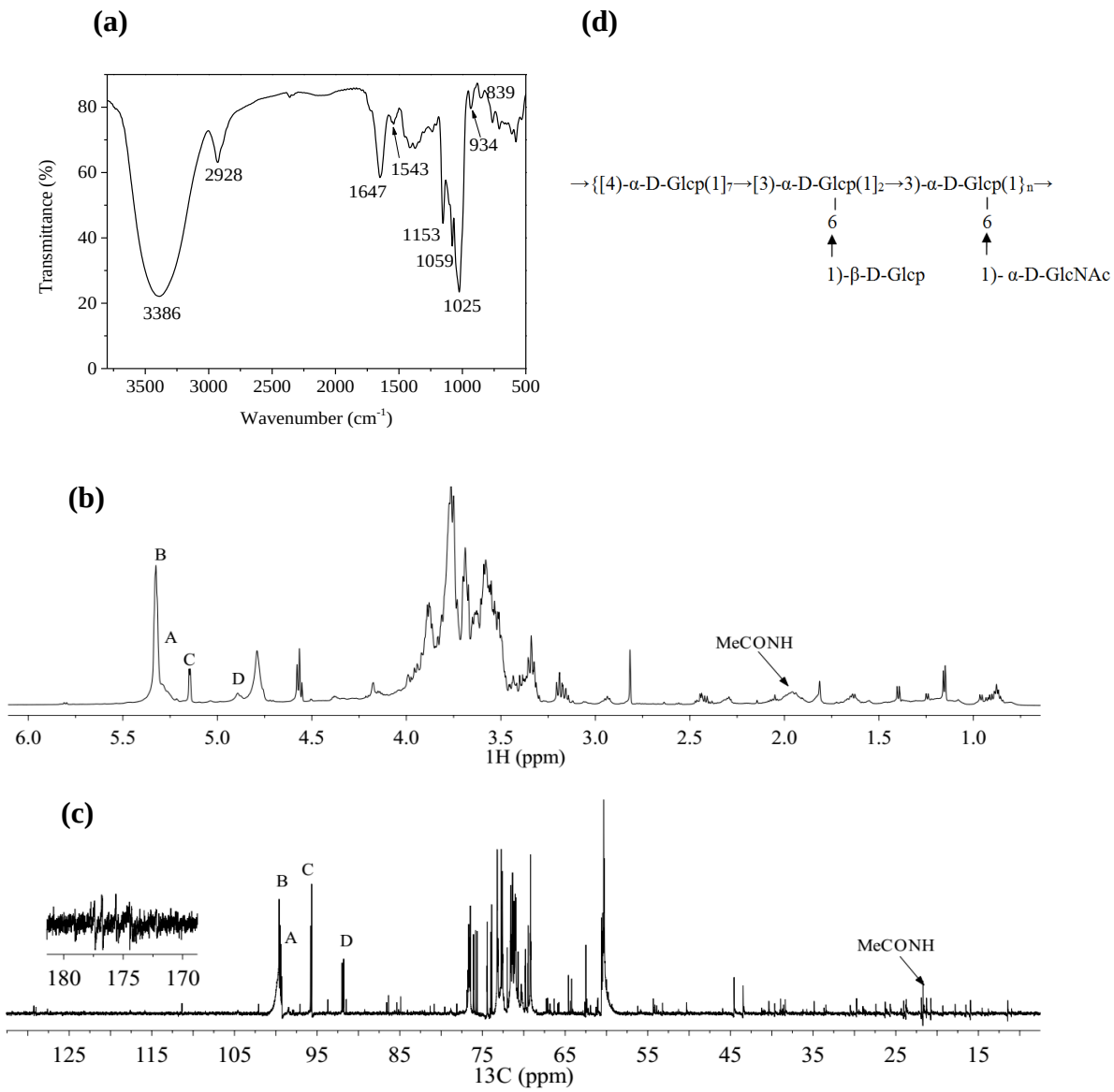

Fig 3. (a) Fourier transform infrared spectroscopy spectrum of CFPS-11. (b) ^{1}H nuclear magnetic resonance (NMR) spectrum of CFPS-11. (c) ^{13}C NMR spectrum of CFPS-11. (d) Possible chemical structure of repeating unit of CFPS-11.

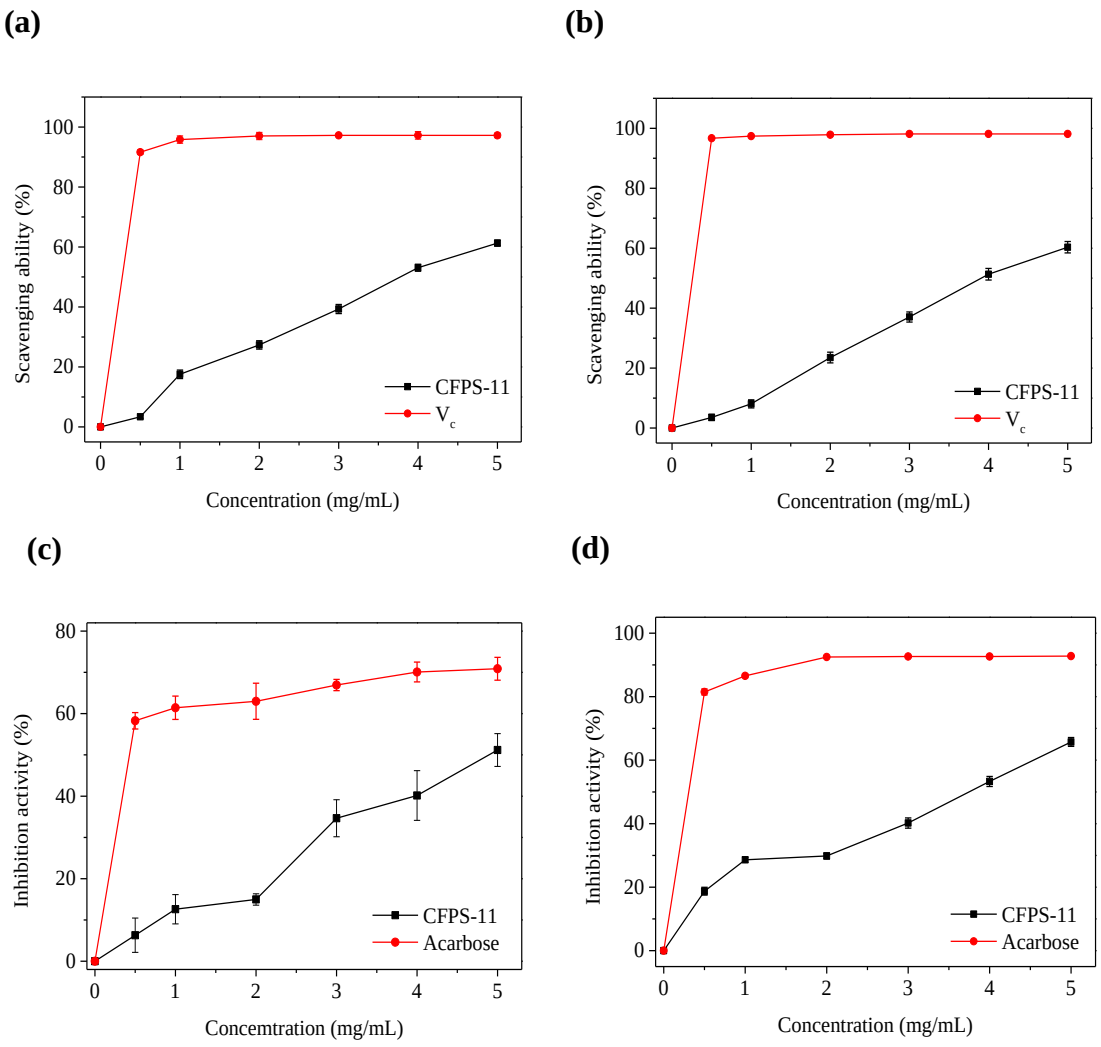

Fig 3. (a) Scavenging ability of CFPS-11 on DPPH radicals. (b) Scavenging ability of CFPS-11 on hydroxyl radicals. (c) Inhibitory effect of CFPS-11 on α-amylase activity. (d) Inhibition effect of CFPS-11 on α-glycosidase activity. Each value is expressed as mean ± standard error (n = 3).

Table 1. Preliminary physicochemical properties and structural characteristics of CFPS-11 isolated from *Corbicula fluminea*

Sample	Carbohydrate (%)	Protein (%)	$[\alpha]_D^{20}$ (º)	M_w (kDa)	Sugar composition (molar ratios) (mol %)	
					GlcN	Glc
CFPS-11	95.4±1.5	4.5±0.2	32.3	807.7	1.0	12.2

Table 2. Amino acid composition of the purified CFPS-11 from *C. fluminea*

Amino acids	Concentration (mg/g)	Amino acids	Concentration (mg/g)
Aspartic acid	1.38	Leucine	1.40
Threonine	1.20	Tyrosine	0.88
Serine	1.21	Phenylalanine	0.38
Glutamic acid	1.64	Lysine	5.48
Glycine	0.81	Methionine	1.83
Alanine	0.83	Histidine	1.04
Cysteine	0.04	Arginine	1.01
Valine	0.74	Tryptophan	n.d.[a]
Isoleucine	0.89	Proline	0.76

[a] Not determined.

Table 3. Gas chromatography-mass spectrometer results of the methylated products of CFPS-11

Methylation product	RT (min) [a]	Mass fragments (m/z)	Linkage type	Molar ratio
2,3,4,6-Me_4-Glcp	1.00	43,59,71,87,101,117,129,145,161,205	Glcp(1→	1.96
2,3,4,6-Me_4-GlcNAc	1.02	159,161,203,205	GlcNAc(1→	1.00
2,3,6-Me_3-Glcp	1.08	43,87,101,113, 117,129,131,233	→4)Glcp(1→	7.12
2,4-Me_2-Glcp	1.15	43,87,99, 129,161,189	→3,6)Glcp(1→	2.86

[a] Retention time (RT) of sugars relative to that of 2,3,4,6-Me_4-Glcp

Table 4. ^{1}H and ^{13}C NMR chemical shifts of CFPS-11 recorded in D_2O at 25 °C

Sugar residue	Chemical shifts (ppm)						
	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6	MeCONH
A α-D-Glcp(1→	99.09/5.29	74.30/3.19	76.93/3.52	70.74/3.42	76.29/3.44	64.47/3.73	
B →4)-α-D-Glcp(1→	99.40/5.32	71.18/3.69	73.24/3.75	76.59/3.77	72.43/3.89	60.26/3.59	
C →3,6)-α-D-Glcp(1→	95.79/5.14	70.58/3.53	76.76/3.70	72.74/3.58	75.83/3.99	69.46/3.67	
D α-D-GlcNAc(1→	91.98/4.89	54.61/3.84	72.30/3.73	71.82/3.50	72.20/3.94	60.97/3.81	176.82,21.42 /1.97