



Homogalacturonan from squash: Characterization and tau-binding pattern of a sulfated derivative

Yu Zhang^{a,b,c}, Panhang Liu^{a,b,c}, Chunyu Wang^{d,e,h}, Fuming Zhang^{f,h},
Robert J. Linhardt^{d,e,f,g,h}, David Eliezerⁱ, Quanhong Li^{a,b,c,*}, Jing Zhao^{a,b,c,*}

^a College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

^b China National Engineering Research Center for Fruit and Vegetable Processing, Beijing 100083, China

^c Key Laboratory of Fruit and Vegetable Processing, Ministry of Agriculture, Beijing 100083, China

^d Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY, United States of America

^e Department of Biological Sciences, Rensselaer Polytechnic Institute, Troy, NY, United States of America

^f Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States of America

^g Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, United States of America

^h Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, United States of America

ⁱ Department of Biochemistry, Program in Structural Biology, Weill Cornell Medical College, New York, United States of America

ARTICLE INFO

Keywords:

Binding pattern
Homogalacturonan
Squash
Sulfated polysaccharide
Tau K18 protein

ABSTRACT

A pectic polysaccharide (WAP) was isolated from squash and identified as a homogalacturonan with a molecular mass of 83.2 kDa by GPC, monosaccharide composition analysis, FT-IR and NMR spectra. Sulfation modification of WAP was carried out and a sulfated derivative (SWAP) was obtained with a substitution degree of 1.81. The NMR spectrum indicated that the sulfation modification mainly occurred at the C-2 and C-3 positions of galacturonan residues. The binding pattern of SWAP to tau K18 protein was observed in 2D ¹H–¹⁵N HSQC spectra of tau, which resembled the tau-heparin interaction, with R2 domain as the major binding region. These results suggest that SWAP has the potential to act as a heparin mimic to inhibit the transcellular spread of tau; thus natural polysaccharide from squash may be developed into therapies for AD and related tauopathies.

1. Introduction

Squash, belonging to genus *Cucurbita* and family *Cucurbitaceae*, has been cultivated worldwide for thousands of years. Squash is abundant in nutrients and active ingredients, among which polysaccharides are receiving more and more attention due to their beneficial health properties (Huang, Zhao, et al., 2021). Pectin is a complex polysaccharide rich in galacturonic acid commonly found in the cell walls of fruits and vegetables (Zhang et al., 2021). There are many types of pectins due to their different structures and components. Pectins are divided into high-methoxylated (HM) pectin and low-methoxylated (LM) pectin according to degree of methoxylation (DM) (Maxwell et al., 2012). The pectin macromolecular structure consists of a “smooth” region (homogalacturonic acid, HG) and a “hairy” region (Rhamnogalacturonan type I, RG-I; Rhamnogalacturonan type II, RG-II and Xylogalacturonan, XGA) (Holck et al., 2011). A variety of pectins have been extracted from squash pulp and skin, with varying structures and monosaccharide

compositions (Jun et al., 2006; Kost'aloova et al., 2013). Squash pectin has cell protection and antioxidant properties with its characteristics of highly branched of RG-I region, RG-II region and a high content of galacturonic acid (Torkova et al., 2018). In certain circumstances, natural polysaccharides are chemically modified to enhance the functionality (Cao et al., 2020). Sulfated polysaccharides, which have drawn considerable research interest over the years in nutraceutical, cosmetic and pharmaceutical applications, have been shown to enhance antioxidant, anti-obesity, anti-viral, anti-inflammatory, hypoglycemic, anti-cancer and other active health effects (Chen, Huang, 2019a, 2019b; Lu et al., 2021; Xie et al., 2020; Zhu et al., 2021). Many polysaccharides show a significant increase in biological activity through sulfation modification (Caputo et al., 2019; Wang et al., 2018). In addition, sulfated pectin also has a variety of active functions, such as anti-coagulation and anti-inflammatory, etc. (Cipriani et al., 2009; Fan et al., 2012; Sabater et al., 2019). Tau protein is a microtubule-associated protein that can promote the assembly and stability of

* Corresponding authors at: Qinghuadong Road No. 17, Haidian District, Beijing 100083, China.

E-mail addresses: quanhong.li@hotmail.com (Q. Li), zhaojing.cau@hotmail.com (J. Zhao).

<https://doi.org/10.1016/j.carbpol.2022.119250>

Received 28 October 2021; Received in revised form 8 February 2022; Accepted 9 February 2022

Available online 15 February 2022

0144-8617/© 2022 Published by Elsevier Ltd.

microtubules. It is mainly expressed in the central nervous system. Neurofibrillary tangles, mainly composed of tau protein, is a pathological hallmark of Alzheimer's disease (AD) (Zhao, Huvent, et al., 2017; Huang, Zhu, et al., 2021). The intercellular spread of tau is considered to be one of the main pathological pathways of AD (Yang et al., 2021). It has been reported recently that cellular uptake of tau, a critical step in the prion-like spread of tau pathology, can be inhibited by heparin, which is a highly sulfated glycosaminoglycan (Zhao et al., 2019). Thus, heparin analogs are promising inhibitors of tau spread.

In this study, a natural squash polysaccharide was isolated and characterized and then modified by a sulfation. The binding pattern of the sulfated squash polysaccharide to tau was investigated by nuclear magnetic resonance spectroscopy (NMR) and compared to the interaction of tau with heparin. Intriguingly, our data demonstrate that sulfated squash polysaccharides might be a potential candidate compound to inhibit the transcellular spread of tau protein.

2. Materials and methods

2.1. Material and chemicals

The fresh mature fruit of squash (*Cucurbita moschata*) was cleaned with tap water thoroughly. The skins were peeled and seeds were discarded. Squash pulp was then cut into small pieces and freeze-dried before use. The lyophilized samples were crush into powder using a miller and sieved through a 60 mesh sieve for the subsequent experiment (Zhao, Zhang, et al., 2017). Squash was purchased from a local supermarket (US). Tau K18 protein was obtained from Weill Cornell Medical College. All the reagents were of analytical grade.

2.2. Extraction and purification of polysaccharide

The squash powder (100 g) was mixed with distilled water at a ratio of 1/30 (w/v) and placed in an 80 °C water bath for 4 h with constant stirring. The supernatant was obtained by centrifugation at 8000 ×g for 15 min. After centrifugation, the supernatant was concentrated to 300 mL by evaporation at 40 °C. Proteins in the concentrated solution were removed using the Sevag reagent (Sevag et al., 1938). Absolute ethyl alcohol was then added to the solution (final concentration: 80%) and the mixture was allowed to stand at 4 °C for 12 h. The precipitated crude polysaccharide was collected by centrifugation at 8000 ×g for 25 min, dissolved in water and dialyzed (membrane cut-off of 1000 Da) and lyophilized.

The crude polysaccharide was prepared into a solution with a concentration of 10 mg/mL and applied to a diethylaminoethyl (DEAE)-Sephacrose Fast Flow column (2.5 × 8 cm), which was eluted with different concentrations of NaCl solution (0, 0.1, 0.2, 0.3, 0.5 M) for three column volumes at a flow rate of 1.0 mL/min. Then, the main fraction (0.2 M NaCl) was collected and precipitated at 4 °C for 12 h with the alcohol (final concentration: 50%). The water-extracted acidic polysaccharide (WAP) was dialyzed (membrane cut-off of 1000 Da) and lyophilized for further determination (Zhao, Zhang, et al., 2017).

2.3. Homogeneity and molecular mass analysis

The homogeneity and molecular mass of WAP were determined by the high performance gel permeation chromatography (HPGPC, Shimadzu Scientific Instruments, JPN) with a Shimadzu LC-10A HPLC system equipped with a BRT102 column (7.8 mm × 30.0 cm) and a refractive index detector (RID) (Li, Wei, et al., 2021). The dextran standard (5 kDa, 12 kDa, 25 kDa, 50 kDa and 100 kDa) and sample at a concentration of 5 mg/mL were accurately prepared and injected with a flow rate of 0.8 mL/min at 40 °C. Data were analyzed by LCsolution Software (Shimadzu Scientific Instruments, JPN).

2.4. Monosaccharide composition and degree of esterification (DE) analysis

For this analysis, the method of Huang, Zhao, et al. (2021) was applied with some modifications. WAP (1 mg) was hydrolyzed with 1 mL 2 M trifluoroacetic acid (TFA) for 6 h at 105 °C in sealed ampoule bottle. Then, evaporated under reduced pressure with methanol and the hydrolyzed product was derivatized with methanol-1-phenyl-3-methyl-5-pyrazolone (PMP) solution under an alkaline condition. The sample was detected by a high performance liquid chromatography (HPLC, Agilent Technologies Inc., US) system using an Agilent XDB-C18 column (4.6 × 250 nm) and connecting with ultraviolet detector. The mobile phase was a mixture of 0.1 mol/L KH₂PO₄ and acetonitrile (83:17). The flow rate was 1.0 mL/min and column temperature was 35 °C. The absorbance of samples was detected at 245 nm. Glucose, L-rhamnose, D-xylose, L-arabinose, D-mannose, L-fucose, D-galactose, and D-galacturonic acid were derivatized and used as standards. The DE of WAP was determined by the titrimetric method (USP 26 NF 21, 2003) with some modifications. DE was calculated by the Eq. (1):

$$DE = V_2 / (V_1 + V_2) \quad (1)$$

where V₁ and V₂ represent the volume of sodium hydroxide consumed for the first and second time, respectively.

2.5. Preparation of sulfated polysaccharide

The methods of sulfation modification was according to the Chen et al. (2019) with some modifications. Under stirring, chlorosulfonic acid (4 mL) was slowly added to a three-necked flask containing 10 mL of pyridine. The whole process was cooled in an ice bath and completed within 30 min. WAP (0.5 g) was dissolved in 30 mL dimethylformamide (DMF) and stirred in boiling water at constant temperature for 1 h. After cooling to the room temperature, the solution was adjusted to neutral with 0.5 mol/L NaOH and precipitated, dialyzed (membrane cut-off of 1000 Da) and lyophilized to obtain sulfated WAP (SWAP).

2.6. Sulfate content and degree of substitution of SWAP

The sulfate radical content was determined by the barium chloride-gelatin colorimetric method (Kawai et al., 1969). SWAP (5 mg) was sealed in 1 M hydrochloric acid solution and hydrolyzed at 100 °C for 6 h. The solution was cooled, spin-dried, and the residue was dissolved in 1 mL of deionized water for further determination. Barium chloride-gelatin solution (0.5%) and 100 µg/mL potassium sulfate standard solution were prepared. Potassium sulfate was taken 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL successively, and hydrolysate was taken 0.2 mL, and then added deionized water to 1.0 mL. Next, 0.5 mL of 3% trichloroacetic acid solution and 0.5 mL of 0.5% barium chloride-gelatin solution were added. The absorbance at 360 nm was measured. Standard curve with the sulfate content as the abscissa and the absorbance value as the ordinate was drew. The sulfate radical content S% was obtained according to the standard curve. Degree of substitution (DS) was calculated by the Eq. (2) (Chen and Huang, 2019a):

$$DS = (1.62 \times S\%) / (32 - 1.02 \times S\%) \quad (2)$$

2.7. FT-IR spectroscopy

The FTIR spectra (Perkin Elmer Spectrum One FT-IR Spectrometer, Perkin Elmer, USA) were collected for WAP and SWAP. Characterize samples were scanned by infrared absorption spectrometer using a KBr dispersion method at a wavenumber range of 4000–500 cm⁻¹. Spectra had 16 scans with a resolution of 4 cm⁻¹. The background interference was removed and scanned by using a polysaccharide-free sample.

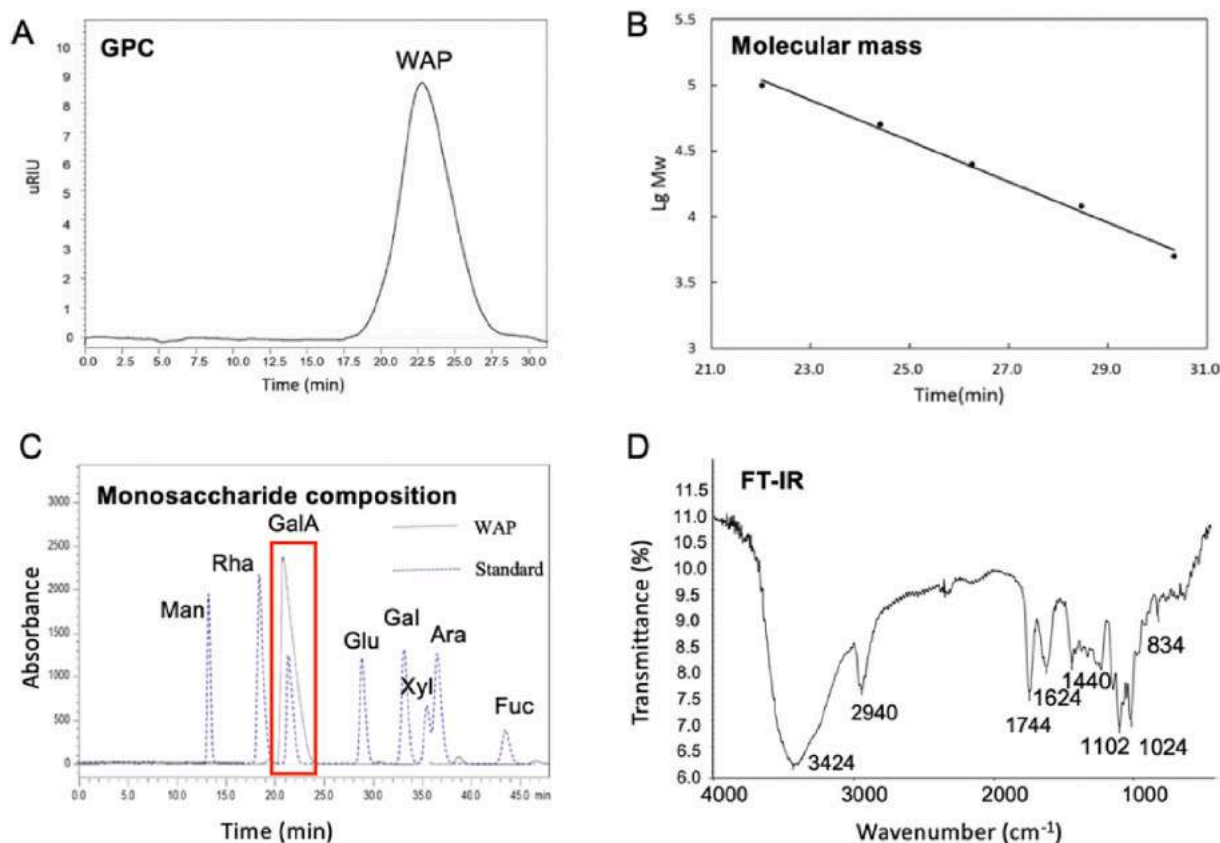


Fig. 1. HPGPC chromatography of WAP (A); Calibration curve of standards with different molecular mass (B); Monosaccharide composition of WAP (C); FT-IR spectrum of WAP (D).

2.8. Characterization of WAP by NMR spectroscopy

NMR analysis was performed according to the methods of Zhao, Zhang, et al. (2017). Briefly, polysaccharide (20 mg) was dissolved into 0.5 mL of D₂O with constant stirring and then lyophilized. After repeating the operation three times, the sample was dissolved in 0.5 mL of D₂O. A Bruker 800 MHz (18.8 T) standard-bore NMR spectrometer (Bruker Avance II 800, GER) equipped with a ¹H/²H/¹³C/¹⁵N cryoprobe with z-axis gradients was used to record the 1D (¹H, ¹³C) and two-dimensional (2D) correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) NMR spectra of the WAP and SWAP. Data of NMR were analyzed using Topspin 2.1.6 (Bruker, GER) and MestRe Nova 5.3.0 (Mestrelab Research S.L.) software.

2.9. Characterization of SWAP-tau binding by NMR

¹⁵N-labeled tau K18 was dissolved in 100 mM NaCl, 10 mM Na₂HPO₄, and 4 mM dithiothreitol at pH 7.4 in 90/10% H₂O/D₂O (Barre & Eliezer, 2013). The ¹H¹⁵N HSQC spectrum was collected on a 0.1 mM tau sample by an 800 MHz nuclear magnetic resonance instrument (Bruker, GER) equipped with a cryogenic probe at 10 °C. Data were processed by Topspin 2.1.6 (Bruker, GER) and Sparky software. The SWAP was added to 0.1 mM tau K18 solution (the molar ratio of SWAP to tau K18 was 1:1), and ¹H¹⁵N HSQC spectra were collected on an 800 MHz nuclear magnetic resonance instrument (Bruker, GER) at 10 °C. Heparin replaces SWAP as a control. The weighted average chemical shift perturbations (CSPs) of each amino acid of tau K18 were calculated by the Eq. (3):

$$\Delta\delta = \sqrt{100\Delta H^2 + \Delta N^2} \quad (3)$$

Where ΔH and ΔN were the chemical shift differences of amide ¹H and ¹⁵N of the bound and free tau K18, respectively.

2.10. Statistical analysis

The data were exhibited as means \pm standard deviations of at least triplicate determinations and the values were analyzed using SPSS (version 20.0, Chicago, USA). Origin Pro 8.6 program (Origin Lab Inc., USA) was used for data processing. Significant differences were determined at $P < 0.05$ levels.

3. Results and discussion

3.1. Isolation and homogeneity of WAP

Following hot water treatment of squash and ethanol precipitation, the 0.2 M NaCl eluted fraction from a DEAE-Sepharose Fast Flow column corresponded to a homogenous polysaccharide, namely WAP, with a yield of 1.3%. The homogeneity of WAP was confirmed by a single symmetrical elution peak at 22.77 min by HPGPC (Fig. 1A). As shown in Fig. 1B, the standard curve was derived from dextran (5 kDa, 12 kDa, 25 kDa, 50 kDa and 100 kDa). According to the standard curve equation ($\log M = -0.1551 \times X + 8.4528$, $R^2 = 0.9934$, where M is the molecular weight and X is the retention time), the molecular weight of WAP was calculated to be 8.32×10^4 Da.

3.2. Monosaccharide composition of WAP

Based on the pre-column derivatization by reversed phase (RP)-HPLC, WAP was mainly composed of galacturonic acid (Fig. 1C), which accounted for 99.4% of the total peak area. It is inferred that the WAP is

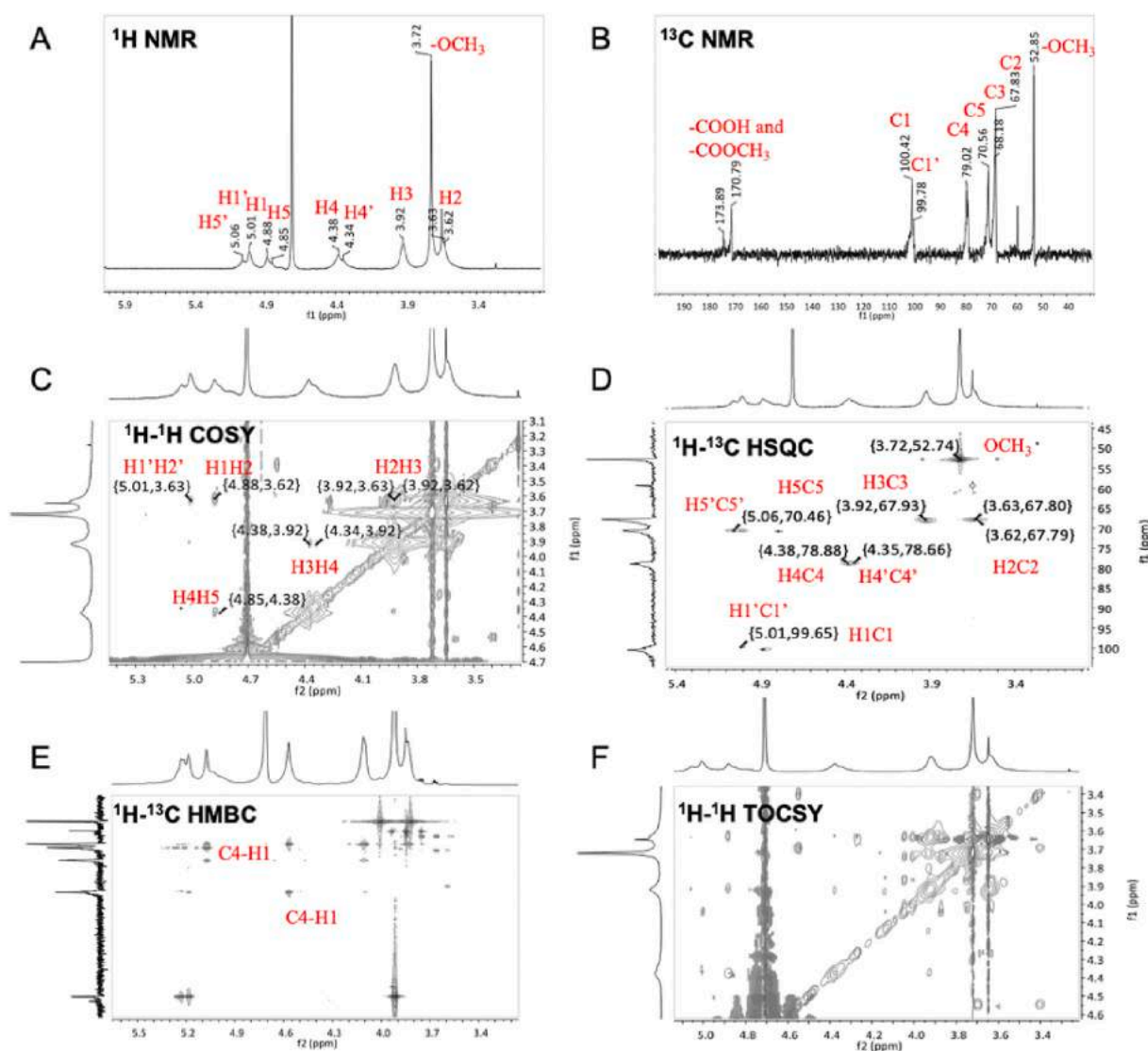


Fig. 2. ^1H spectrum (A), ^{13}C spectrum (B), COSY spectrum (C), HSQC spectrum (D), HMBC spectrum (E) and TOCSY spectrum (F) of WAP.

a homogalacturonan (HG). Previous studies showed diverse structural compositions of squash polysaccharides, which may be caused by the different extraction and purification methods, thereby affecting its structural properties and biological activity (Chaves et al., 2019; Chen et al., 2019; Li, Zhao, et al., 2021; Shen et al., 2017). For pectin, the carboxyl group of galacturonic acid may exist in the form of methoxyl ester. The degree of methyl esterification of pectin is defined by DE, which is the ratio of esterified galacturonic acid to total galacturonic acid (Ridley et al., 2001). DE has a major influence on the solubility, gelation and other functional properties of pectin (Nguemazong et al., 2012; Sila et al., 2009). The DE of WAP was measured to be 67.91%, indicating WAP to be a highly methoxylated pectin.

3.3. Fourier transform-infrared (FT-IR) analysis of WAP

Chemical groups in polysaccharides can be characterized by infrared spectroscopy based on their vibrational frequencies (Zhang & Zhang, 2019). It can be seen from Fig. 1D that the broad peak at 3424 cm^{-1} corresponds to the characteristic absorption peak of O—H bond stretching vibration. Since polysaccharide molecules have many hydroxyl groups, the formation of intramolecular and intermolecular hydrogen bonds cause the peak to be particularly broad, which is one of the typical characteristics of polysaccharide substances. The band at

2940 cm^{-1} is attributed to the stretching vibration of the C—H bond (Jeff et al., 2013). The larger absorption peak at 1744 cm^{-1} is the characteristic absorption peak of methoxy group in pectin polysaccharides, which again demonstrated the presence of COOH or COOCH₃ groups in WAP (Chylinska et al., 2016). Peaks occurring at 1624 cm^{-1} and 1440 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of the carboxyl group, respectively, confirming the presence of uronic acid in WAP (Nejatzadeh-Barandozi & Enferadi, 2012). The absorption peak of methoxy group is greater than the absorption peak of carboxyl group, indicating that the degree of methyl esterification of WAP was higher. The signal in the range of $1000\text{--}1200\text{ cm}^{-1}$ result from the vibration of the sugar ring backbone C—O and C—C. Polysaccharides with different monosaccharide compositions had different spectral shapes (Liu et al., 2016). Band at 834 cm^{-1} indicated the existence of the α configuration pyranose (Yuen et al., 2009). Finally, the main peaks assigned to the asymmetric and symmetric stretching vibration of CH₂ of pyranose ring for WAP ($2940, 1370, 1235\text{ cm}^{-1}$) (Muhidinov et al., 2020).

3.4. NMR spectrum of WAP

The ^1H , ^{13}C , COSY, TOCSY, HSQC and HMBC NMR spectra of WAP were recorded on an 800 MHz NMR spectrometer. In ^1H spectrum, there

Table 1
 ^1H and ^{13}C NMR chemical shift data of WAP.

Residue	Atom	$^{13}\text{C}/\text{ppm}$	$^1\text{H}/\text{ppm}$	
GalpA	Non esterified 1,4- α -GalpA-	1	100.42	4.88
		2	67.83	3.62
		3	68.18	3.92
		4	79.02	4.38
		5	70.56	4.85
		6	173.89	–
Esterified 1,4- α -GalpA-		1	99.78	5.01
		2	67.83	3.63
		3	68.18	3.92
		4	78.66	4.34
		5	70.56	5.06
		6	170.79	–
		O-CH ₃	52.74	3.72

are mainly 6 group of peaks, corresponding to H-1, H-2, H-3, H-4, H-5 and methoxy H, respectively (Fig. 2A). The resonance peak at 3.72 ppm is much higher than other peaks, which corresponds to the signal of methyl proton (-CH₃) in the methyl esterified carboxyl group (-COOCH₃). The splitting of other hydrogen signals is caused by the methyl esterification of some galacturonic acid in the chain. The ^{13}C NMR of WAP is shown in Fig. 2B. The resonance peaks at 170.79 ppm and 173.89 ppm in the low field are methylated carboxyl carbon and non-methylated carboxyl carbon, respectively (Gao et al., 2013; Panda et al., 2015). On the basis of the one-dimensional NMR spectra and two-dimensional NMR spectra (COSY, HSQC, HMBC, TOCSY) the chemical shifts of all carbon atoms and hydrogen atoms in the WAP could be assigned (Fig. 2C-F; Table 1). The resonance signals of hydrogen and adjacent carbons are shown in the ^1H - ^1H COSY spectra (Fig. 2C). The complete signals of all hydrogens on one sugar ring can be obtained through sequential assignment. According to previous studies, the terminal hydrogen H-1 of unesterified galacturonic acid is located at 4.88 ppm and exhibits an α configuration (Bedouet et al., 2003). The cross

peaks at δ 4.88/3.62 ppm indicate that the chemical shift of H-2 was 3.62 ppm. In a similar fashion, the chemical shifts of H-3, H-4, H-5 were established to be 3.92, 4.38 and 4.85 ppm, respectively. The shift of the H-4 and H-5 signals to a lower field may be caused by the carboxyl group at the C-6 position of galacturonic acid. For methoxylated galacturonic acid, the chemical shift of each proton of the sugar ring was also shifted due to the introduction of the methoxy group. Due to the electron withdrawing effect of the methoxy group, the closer it was to the carboxyl group, the greater the offset effect. Therefore, the offsets of C-1, C-4 and C-5 were relatively large, and C-2 and C-3 were relatively small. The terminal hydrogen H'-1 of the esterified galacturonic acid showed a chemical shift of 5.01 ppm. The chemical displacement of H'-2, H'-3, H'-4 and H'-5 were 3.63, 3.92, 4.34 and 5.06 ppm, respectively. According to the ^1H - ^{13}C HSQC spectrum (Fig. 2D), the C-1 ~ C-5 chemical shifts of the unesterified and esterified galacturonic acid could be assigned by the hydrocarbon correlation peak signal on the same carbon. From the δ 100.42/4.88, 67.83/3.62, 68.18/3.92, 79.02/4.38, and 70.56/4.85 ppm, the chemical shifts of C-1 ~ C-5 of the unesterified galacturonic acid were 100.42, 67.83, 68.18, 79.02 and 70.56 ppm, respectively. In a similar way, the chemical shifts of C-1 ~ C-5 of esterified galacturonic acid were 99.78, 67.83, 68.18, 78.66 and 70.56 ppm, respectively. The sharp peak at \sim 60 ppm in Fig. 2B could be assigned to the residual glycerol in the sample which might be from the spin column we used for the sample concentration. In the ^1H - ^{13}C HMBC spectrum (Fig. 2E), the correlation peaks between galacturonic acid H-1 and C-4 and the correlation peaks between C-1 and H-4 proved that galacturonic acid was between the α -1,4-glycosidic linkage. Previous studies showed the similar result of NMR (Petersen, Meier, Duus & Clausen, 2008; Wang et al., 2014).

3.5. Proposed structure of WAP

According to all the above results, a structure of WAP can be proposed (Fig. 3). WAP is an acidic polysaccharide with a molecular weight

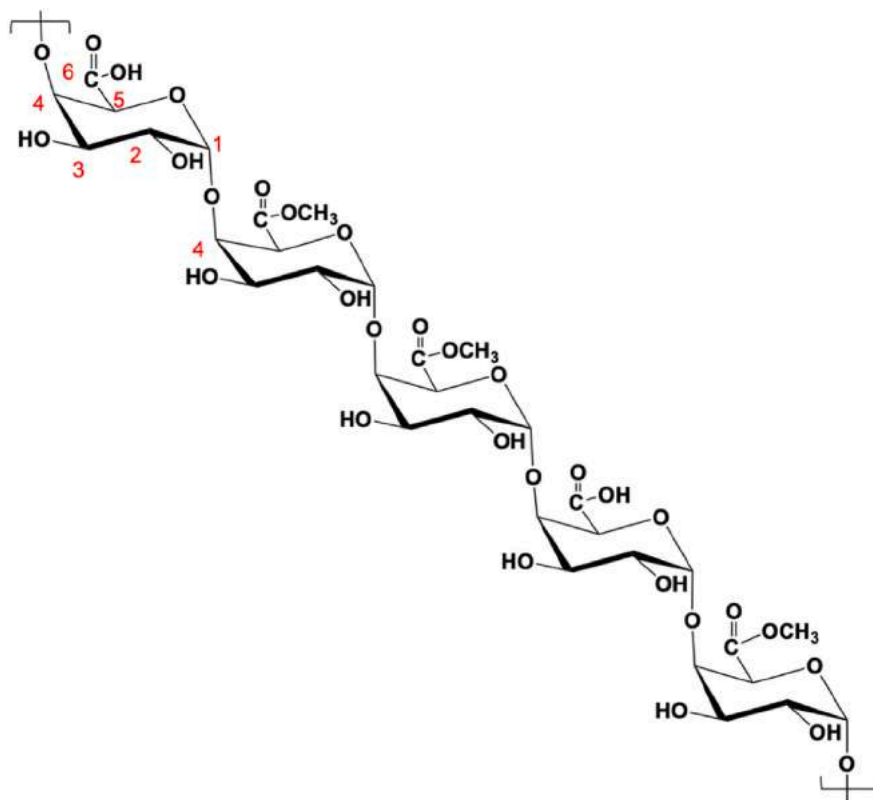


Fig. 3. Proposed characteristic structure of WAP.

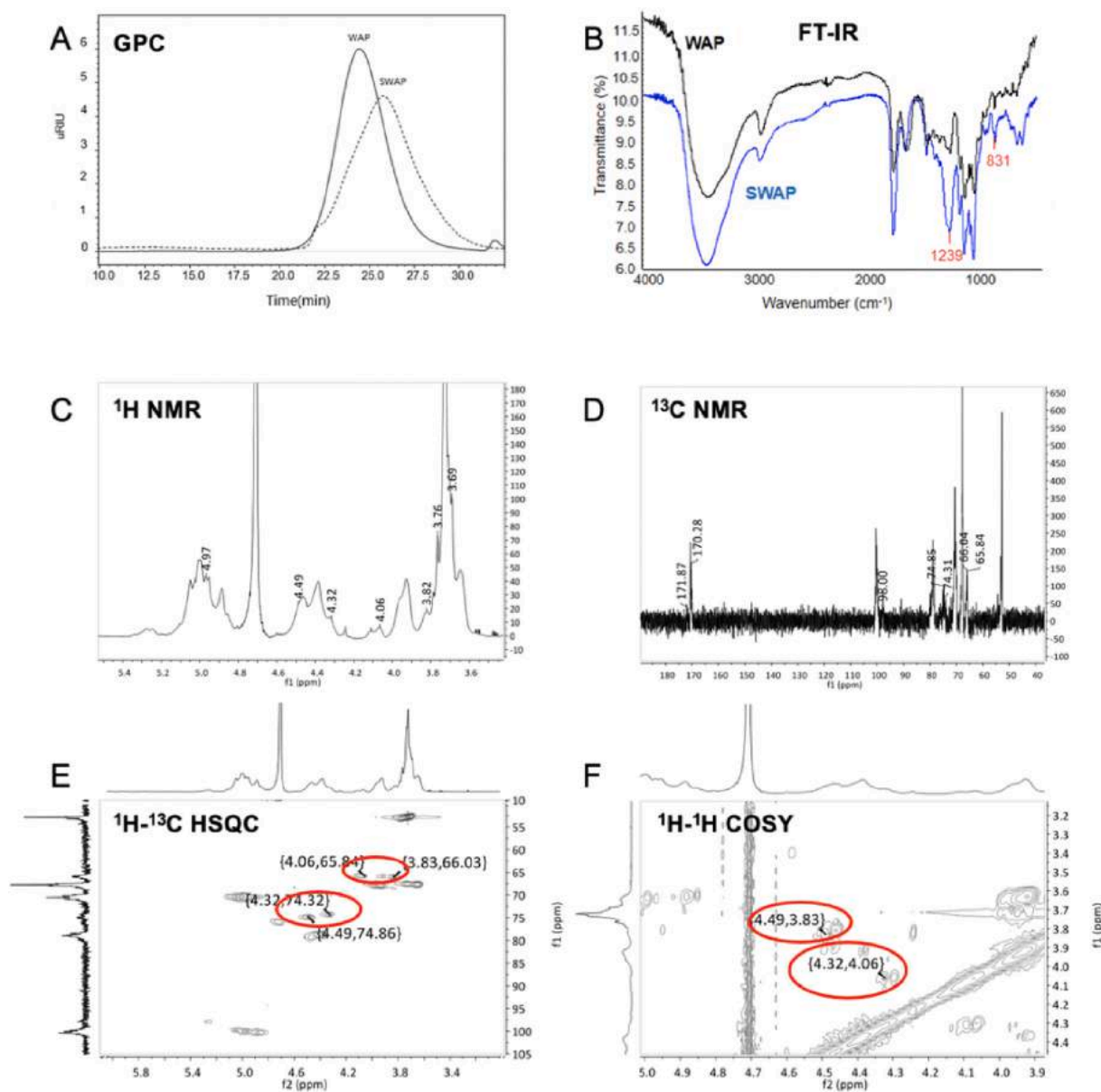


Fig. 4. HPGPC chromatography of SWAP (A); FT-IR spectrum of SWAP (B); ^1H spectrum (C), ^{13}C spectrum (D), HSQC spectrum (C) and COSY spectrum (D) of SWAP.

of about 83.2 kDa. The monosaccharide composition is galacturonic acid. The chain of WAP was a linear structure composed of galacturonic acid connected by α -(1-4)-glycosidic bonds. It is a pectin acid polysaccharide containing only homogalacturonic acid (HG) domains.

3.6. Modification of WAP by sulfation

Using the barium sulfate-gelatin method, a sulfated derivative of WAP (SWAP) was obtained with a sulfate content of 16.7%. The degree of substitution of SWAP was measured to be 1.81. The molecular weight of SWAP was calculated to be 2.47×10^4 Da (Fig. 4A), which is smaller compared to WAP. This may be caused by the degradation of some sugar chains during the sulfation process (Hu et al., 2015). In the FT-IR spectrum (Fig. 4B), SWAP showed a large absorption peak at 1239 cm^{-1} , assigned to the stretching vibration of S—O (Xiong et al., 2020). In addition, the absorption peak at 831 cm^{-1} is assigned to the bending vibration of the sulfo group C—O—S (Li et al., 2015; Mao et al., 2008). These results confirm the presence of sulfate groups in SWAP.

3.7. NMR spectrum of SWAP

1D NMR spectra (^1H and ^{13}C) and 2D spectra (HSQC and COSY) of SWAP were collected to further validate the presence of sulfate in SWAP (Fig. 4C-F). After sulfation modification, the ^1H spectrum and ^{13}C spectra of SWAP were more complex than before, and many new peaks appeared. There were new resonance peaks at 5.03, 4.97, 4.49, 4.32, 4.24, 4.06, 3.82, 3.76 and 3.69 ppm in the ^1H spectrum (Fig. 4C). In the ^{13}C spectrum (Fig. 4D), new resonance peaks at 171.87, 170.28, 98.00, 74.85, 74.31, 66.04 and 65.84 ppm appeared. The appearance of the new resonance peak was due to the influence of the sulfate group on the carbon/hydrogen atoms of the sugar ring. Due to the electron withdrawing effect of the sulfate acid group, the chemical shift of the carbon atom directly connected to the sulfate acid group often moved to a higher field, and the indirect carbon atom often moved to a lower field (Li et al., 2014; Wang et al., 2010). It can be inferred that the peaks at 170.28 and 98.00 ppm were the resonance peaks of C-6 and C-1 in the sulfated modified sugar ring moving towards the higher field. Therefore, it was indirectly connected to C-6 and C-1, which is consistent with the

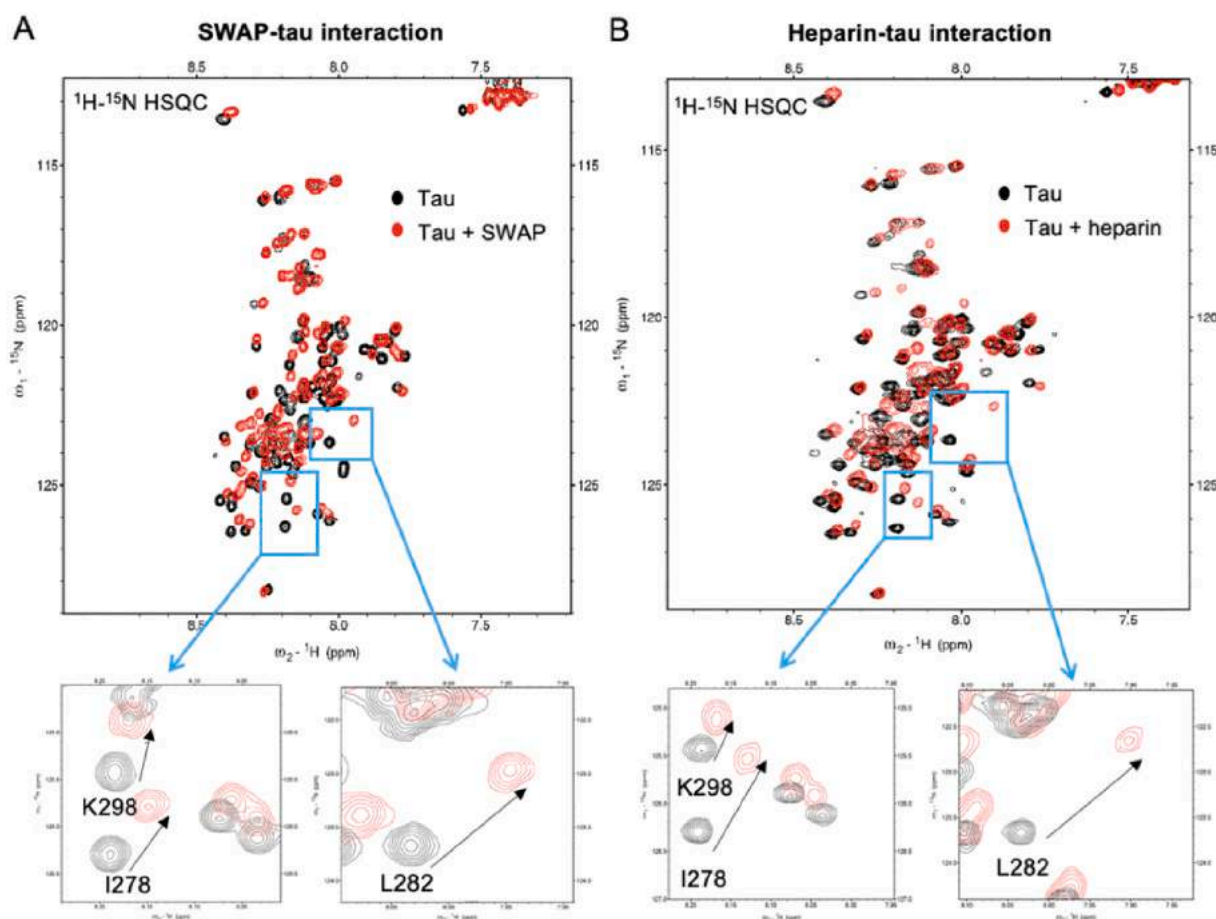


Fig. 5. Overlay of HSQC spectrum of tau K18 with (red) and without (black) SWAP (A) and heparin (B). Regions around residues I278, L282 and K298 with big CSPs were zoomed in and showed below the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

steric hindrance caused by sulfation modification (Yang et al., 2005). In the HSQC spectrum (Fig. 4E), the new resonance peaks with stronger signals in the carbon spectrum and hydrogen spectrum had four main correlation peaks, which were δ 4.49/74.86, 4.32/74.32, 4.06/65.84 and 3.83/66.03 ppm, respectively. It further proved the influence of the introduced sulfo group on the sugar ring of SWAP. The two sets of related peaks in the COSY spectrum (Fig. 4F) were δ 4.49/3.83 and 4.32/4.06 ppm. We speculate that the sulfated groups are located on adjacent carbon atoms, presumably at the C-2 and C-3 positions, similar to a previous study on citrus pectin (Hu et al., 2015). The main chain of pectin was 1–4 linked galacturonic acid, and the C-6 position was substituted by a carboxyl group or a methoxylated carboxyl group. Therefore, C-2 and C-3 were the two positions with relatively low steric hindrance which may be more prone to sulfation modification.

3.8. Characterization of SWAP-tau interaction

The repeating domains of tau in the form of tau K18 are widely used in the research of tau aggregation and interaction (Kaur et al., 2020; Liu et al., 2019; Zhao, Zhang, et al., 2017). SWAP was added into tau K18 solution (molar ratio 1:1) and the $^1\text{H}^{15}\text{N}$ HSQC spectrum of the complex was recorded to better understand the binding of SWAP to tau (Fig. 5A). Heparin, a known tau binder, was used as a positive control (Fig. 5B). Natural heparin is a heterogenous polysaccharide composed of uronic acid and glucosamine with sulfation substitution likely on the 3-OH, 6-OH and -NH of the glucosamine residue, and the 2-OH of the uronic acid residue. SWAP is also charge-wise heterogenous because the sulfation may occur to the 2-O or 3-O position, or both. Since we are using heparin

as a positive control, we allowed the charge-wise heterogenous of SWAP by sparing the ion-exchange step in its purification. Significant chemical shift perturbation (CSPs) of tau K18 were observed upon the addition of SWAP. The weighted average CSPs of each amide group were shown in Fig. 6, and the total CSPs were 25.01 and 21.80 ppm, respectively.

The largest CSPs caused by SWAP occurred in the R2 domain of tau K18, especially the I277-D283 region at the front of R2. I278 and L282 had the largest CSP, which may be due to the conformational rearrangement of tau K18, exposing the side chains of hydrophobic amino acids. Studies had shown that the hexapeptide $^{275}\text{VQIINK}^{280}$, which overlapped with this peptide, was the key site that triggers the aggregation of tau and the binding to microtubules (MT), as well as the main site that binds to heparin (Smet et al., 2004). It is worth mentioning that many basic amino acids (mainly lysine) exhibited larger CSPs, including K254, K280, K281, K290, K294, K298, K340, K343 and K347. The isoelectric point PI of lysine is 9.74. In neutral solutions, it is positively charged and can easily interact with the negatively charged sulfate groups on SWAP, confirming the electrostatic interaction between SWAP and the tau K18. The above-mentioned binding characteristics of SWAP-tau interaction closely resemble those of heparin binding to tau. However, there were still differences in local CSPs. SWAP caused more pronounced CSP for residue Q276 and I297, compared with heparin, which may be related to the slightly different conformational change of tau K18 caused by SWAP compared to heparin. Other amino acids with significant CSPs include E264, N265, S285, N286, L357, L253, L344, L315, E338, T263, D345, etc. In contrast to heparin, SWAP seems to have affected a wider range of amino acids residues within K18.

Here we show that, as a chemically modified natural polysaccharide,

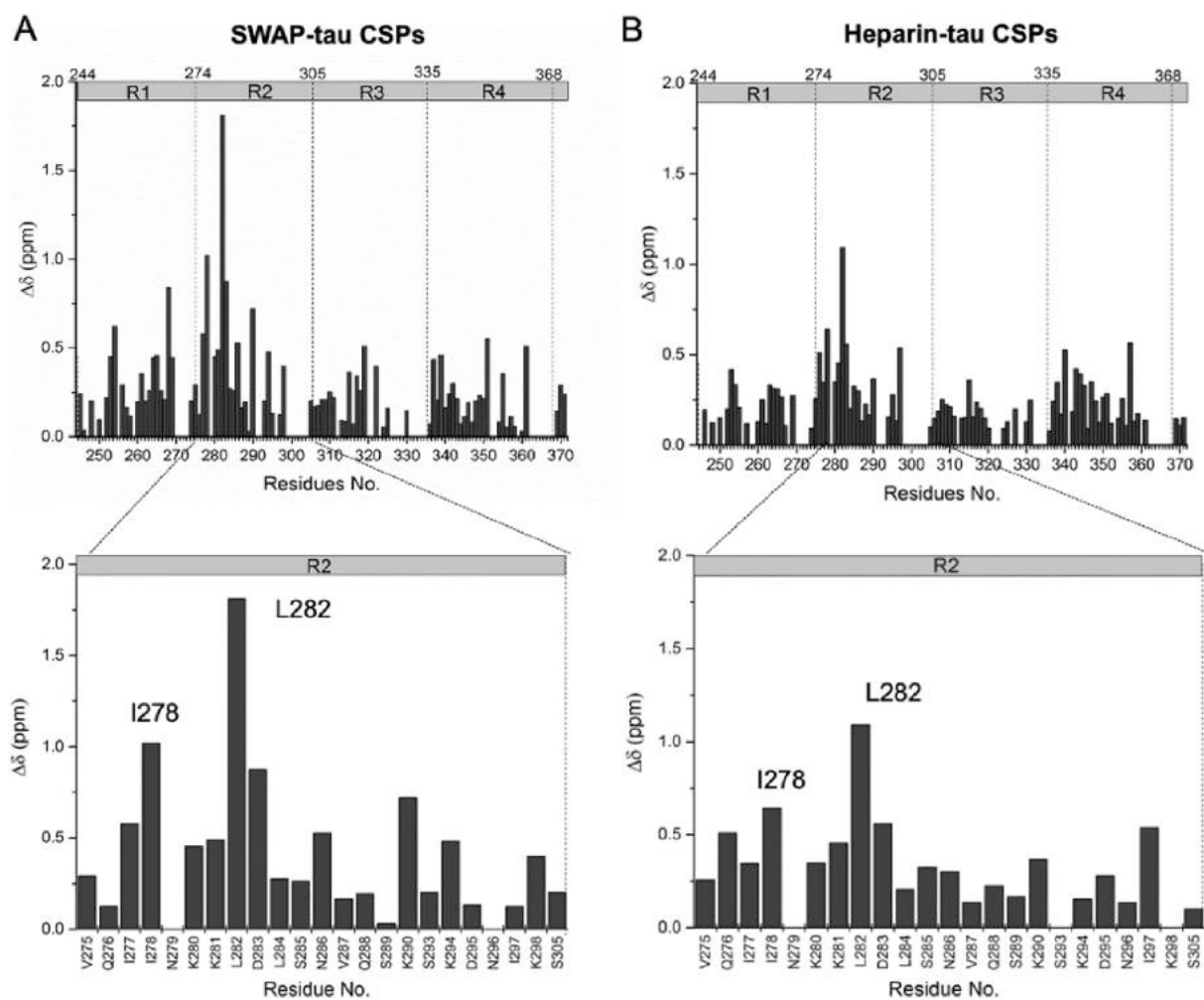


Fig. 6. Bar graph of ^1H - ^{15}N residue CSPs of tau K18 titrated with SWAP (A) and heparin (B). Zoom-in graphs of the R2 region are shown below the figure.

SWAP mimicked heparin binding to tau, indicating the potential of SWAP in reducing cellular uptake of tau and the spread of tau pathology. SWAP and heparin share some similarities in structure. For example, they are both in a chain conformation which is quite flexible in solution and they both contain uronic acid residues and a similar sulfation degree (~ 1.8). As the tau-glycan interaction was mainly driven by electrostatic interactions (Zhao, Zhang, et al., 2017), WAP well resembles heparin-tau binding after its sulfated modification. To exclude the influence of carboxyl group in WAP, the tau binding capacity of WAP was tested on a tau-immobilized SPR chip and compared with SWAP (Supplementary Fig. 1). The binding capacity of SWAP is significantly higher (~ 3 folds) than that of WAP, indicating that sulphation is crucial for its interaction with tau. However, detailed structural diversities between SWAP and heparin lead to the different behaviors when they bind to tau. SWAP has a higher MW than heparin, indicating a longer chain, which might explain the wider range of amino acids residues affected upon SWAP binding. Besides, heparin is composed of glucuronic acid, iduronic acid and glucosamine, with sulfation substitution on the 3-OH, 6-OH and -NH of the glucosamine residue, and the 2-OH of the uronic acid residue. In contrast, SWAP is composed of galacturonic acid with sulfation substitution on the 2-OH and 3-OH position. These sulfation pattern variations may also lead to conformational changes in the tau-glycan interface. Moreover, as a pectic polysaccharide, whether the methylation level of SWAP affected its tau binding character remains a mystery, which needs more investigations in the future.

4. Conclusion

In this paper, a pectic polysaccharide (WAP) with pure HG domain was prepared from squash with a molecular weight of 83.2 kDa and DE of 67.91%. The detailed structure of WAP was confirmed by monosaccharide composition, FT-IR and NMR spectra as a homogalacturonan with α -1,4-glycosidic. To enhance the bioactivity of WAP, a sulfated derivative of WAP (SWAP) was prepared, with sulfo groups primarily attached to the C-2 and C-3 position. The binding capacity of SWAP to tau K18 was comparable to that of heparin through 2D HSQC NMR. The results indicated that SWAP closely resembles heparin binding to tau K18, which has important implications for the modification of natural polysaccharide and its utilization in treating AD and related tauopathies.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carbpol.2022.119250>.

CRediT authorship contribution statement

Yu Zhang: Formal analysis, Writing – original draft. **Panhang Liu:** Formal analysis, Writing – original draft. **Chunyu Wang:** Conceptualization, Resources. **Fuming Zhang:** Conceptualization, Resources. **Robert J. Linhardt:** Conceptualization, Resources. **David Eliezer:** Resources. **Quanhong Li:** Supervision, Resources, Writing – review & editing. **Jing Zhao:** Conceptualization, Data curation, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest

Acknowledgments

This work was supported by a fund for agro-scientific research in the public interest (No. 201303112) to Q. Li, NIH funding (AG066493) to D. Eliezer, and NIH funding (AG069039) to C.Wang.

References

- Barre, P., & Eliezer, D. (2013). Structural transitions in tau k18 on micelle binding suggest a hierarchy in the efficacy of individual microtubule-binding repeats in filament nucleation. *Protein Science*, 22(8), 1037–1048.
- Bedouet, L., Courtois, B., & Courtois, J. (2003). Rapid quantification of O-acetyl and O-methyl residues in pectin extracts. *Carbohydrate Research*, 338(4), 379–383.
- Cao, Y. Y., Ji, Y. H., Liao, A. M., Huang, J. H., Thakur, K., Li, X. L., ... Wei, Z. J. (2020). Effects of sulfated, phosphorylated and carboxymethylated modifications on the antioxidant activities in-vitro of polysaccharides sequentially extracted from *Amanita edulis*. *International Journal of Biological Macromolecules*, 146, 887–896.
- Caputo, H. E., Straub, J. E., & Grinstaff, M. W. (2019). Design, synthesis, and biomedical applications of synthetic sulphated polysaccharides. *Chemical Society Reviews*, 48(8), 2338–2365.
- Chaves, P. F. P., Iacomini, M., & Cordeiro, L. M. C. (2019). Chemical characterization of fructooligosaccharides, inulin and structurally diverse polysaccharides from chamomile tea. *Carbohydrate Polymers*, 214, 269–275.
- Chen, L., & Huang, G. L. (2019a). Antioxidant activities of sulfated squash polysaccharides. *International Journal of Biological Macromolecules*, 126, 743–746.
- Chen, L., & Huang, G. L. (2019b). The antioxidant activity of derivatized cushaw polysaccharides. *International Journal of Biological Macromolecules*, 128, 1–4.
- Chen, S., Khan, B. M., Cheong, K. L., & Liu, Y. (2019). Pumpkin polysaccharides: Purification, characterization and hypoglycemic potential. *International Journal of Biological Macromolecules*, 139, 842–849.
- Chylinska, M., Szymanska-Chargot, M., & Zdunek, A. (2016). FT-IR and FT-Raman characterization of non-cellulosic polysaccharides fractions isolated from plant cell wall. *Carbohydrate Polymers*, 154, 48–54.
- Cipriani, T. R., Gracher, A. H. R., de Souza, L. M., Fonseca, R. J. C., Belmiro, C. L. R., Gorin, P. A. J., ... Iacomini, M. (2009). Influence of molecular weight of chemically sulfated citrus pectin fractions on their antithrombotic and bleeding effects. *Thrombosis and Haemostasis*, 101(5), 860–866.
- Fan, L. H., Gao, S., Wang, L. B., Wu, P. H., Cao, M., Zheng, H., ... Zhou, J. P. (2012). Synthesis and anticoagulant activity of pectin sulfates. *Journal of Applied Polymer Science*, 124(3), 2171–2178.
- Gao, X. G., Zhi, Y., Sun, L., Peng, X. X., Zhang, T., Xue, H. T., ... Zhou, Y. F. (2013). The inhibitory effects of a Rhamnogalacturonan I (RG-I) domain from ginseng pectin on Galectin-3 and its structure-activity relationship. *Journal of Biological Chemistry*, 288(47), 33953–33965.
- Holck, J., Hjerno, K., Lorentzen, A., Vignsnaes, L. K., Hemmingsen, L., Licht, T. R., ... Meyer, A. S. (2011). Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after in vitro fermentation. *Process Biochemistry*, 46(5), 1039–1049.
- Hu, Y. Q., Ye, X. Q., Yin, X. Z., & Chen, S. G. (2015). Sulfation of citrus pectin by pyridine-sulfurtrioxide complex and its anticoagulant activity. *LWT-Food Science and Technology*, 60(2), 1162–1167.
- Huang, L. L., Zhao, J., Wei, Y. L., Yu, G. Y., & Li, Q. H. (2021). Characterization of a neutral polysaccharide from squash (*Cucurbita moschata* Duch) with potential immunomodulatory activity. *International Journal of Biological Macromolecules*, 188, 729–739.
- Huang, R., Zhu, Z. J., Wu, Q. P., Bekhit, A., El-Din, A., Wu, S. J., ... Ding, Y. (2021). Whole-plant foods and their macromolecules: Untapped approaches to modulate neuroinflammation in Alzheimer's disease. In *Critical reviews in food science and nutrition* (pp. 1–19).
- Jeff, I. B., Li, S. S., Peng, X. X., Kassim, R. M. R., Liu, B. F., & Zhou, Y. F. (2013). Purification, structural elucidation and antitumor activity of a novel mannogalactoglucan from the fruiting bodies of *Lentinus edodes*. *Fitoterapia*, 84, 338–346.
- Jun, H. I., Lee, C. H., Song, G. S., & Kim, Y. S. (2006). Characterization of the pectic polysaccharides from squash peel. *LWT-Food Science and Technology*, 39(5), 554–561.
- Kaur, J., Giri, A., & Bhattacharya, M. (2020). The protein-surfactant stoichiometry governs the conformational switching and amyloid nucleation kinetics of tau K18. *European Biophysics Journal with Biophysics letters*, 49(6), 425–434.
- Kawai, Y., Seno, N., & Anno, K. (1969). A modified method for chondrosulfatase assay. *Analytical Biochemistry*, 32(2), 314–321.
- Kost'aloza, Z., Hromadkova, Z., & Ebringerova, A. (2013). Structural diversity of pectins isolated from the Styrian oil-squash (*Cucurbita pepo* var. *styriaca*) fruit. *Carbohydrate Polymers*, 99, 831.
- Li, F., Wei, Y. L., Liang, L., Huang, L. L., Yu, G. Y., & Li, Q. H. (2021). A novel low-molecular-mass squash polysaccharide: Structural characterization, antioxidant activity, and hypoglycemic potential. *Carbohydrate Polymers*, 251, 117090.
- Li, F., Zhao, J., Wei, Y. L., Jiao, X., & Li, Q. H. (2021). Holistic review of polysaccharides isolated from pumpkin: Preparation methods, structures and bioactivities. *International Journal of Biological Macromolecules*. <https://doi.org/10.1016/j.ijbiomac.2021.10.037>
- Li, H. F., Guo, S. B., Man, S. L., Fan, Y. Y., Wang, T. T., Li, X., & Gao, W. Y. (2015). Graded ethanol precipitation method on physicochemical properties and antioxidant activities of polysaccharides extracted from *Astragalus Radix*. *Zhongguo Zhong yao zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica*, 40(11), 2112–2116.
- Li, X. L., Xiao, J. J., Zha, X. Q., Pan, L. H., Asghar, M. N., & Luo, J. P. (2014). Structural identification and sulfated modification of an antiglycation Dendrobium huoshanense polysaccharide. *Carbohydrate Polymers*, 106, 247–254.
- Liu, X. C., Zhu, Z. Y., Tang, Y. L., Wang, M. F., Wang, Z., Liu, A. J., & Zhang, Y. M. (2016). Structural properties of polysaccharides from cultivated fruit bodies and mycelium of *Cordyceps militaris*. *Carbohydrate Polymers*, 142, 63–72.
- Liu, Y. Y., Liu, Z. Y., Wei, M. Y., Hu, M. X., Yue, K. X., Bi, R. B., ... Liu, Z. Q. (2019). Pharmacodynamic and urinary metabolomics studies on the mechanism of *Schisandra* polysaccharide in the treatment of Alzheimer's disease. *Food & Function*, 10(1), 432–447.
- Lu, W. J., Yang, Z. F., Chen, J., Wang, D., & Zhang, Y. (2021). Recent advances in antiviral activities and potential mechanisms of sulfated polysaccharides. *Carbohydrate Polymers*, 272, 118526.
- Mao, W. J., Fang, F., Li, H. Y., Qi, X. H., Sun, H. H., Chen, Y., & Guo, S. D. (2008). Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*. *Carbohydrate Polymers*, 74(4), 834–839.
- Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin - an emerging new bioactive food polysaccharide. *Trends in Food Science & Technology*, 24(2), 64–73.
- Muhidinov, Z. K., Bobokalonov, J. T., Ismoilov, I. B., Strahan, G. D., Chau, H. K., Hotchkiss, A. T., & Liu, L. S. (2020). Characterization of two types of polysaccharides from *Eremurus hissaricus* roots growing in Tajikistan. *Food Hydrocolloids*, 105, 105768.
- Nejatzadeh-Barandozi, F., & Enferadi, S. T. (2012). FT-IR study of the polysaccharides isolated from the skin juice, gel juice, and flower of *Aloe vera* tissues affected by fertilizer treatment. *Organic and Medicinal Chemistry Letters*, 2(1), 33.
- Ngouemazong, D. E., Kabuye, G., Fraeye, I., Cardinaels, R., Van Loey, A., Moldenaers, P., & Hendrickx, M. (2012). Effect of debranching on the rheological properties of Ca²⁺-pectin gels. *Food Hydrocolloids*, 26(1), 44–53.
- Panda, B. C., Mondal, S., Devi, K. S. P., Maiti, T. K., Khatua, S., Acharya, K., & Islam, S. S. (2015). Pectic polysaccharide from the green fruits of *Momordica charantia* (Karela): Structural characterization and study of immunoenhancing and antioxidant properties. *Carbohydrate Research*, 401, 24–31.
- Ridley, B. L., O'Neill, M. A., & Mohnen, D. A. (2001). Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, 57(6), 929–967.
- Sabater, C., Molina-Tijeras, J. A., Vezza, T., Corzo, N., Montilla, A., & Utrilla, P. (2019). Intestinal anti-inflammatory effects of artichoke pectin and modified pectin fractions in the dextran sulfate sodium model of mice colitis. Artificial neural network modelling of inflammatory markers. *Food & Function*, 10(12), 7793–7805.
- Sevag, M. G., Lackman, D. B., & Smolens, J. M. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *The Journal of Biological Chemistry*, 1(124), 42–49.
- Shen, W. X., Chen, C. H., Guan, Y. Y., Song, X. W., Jin, Y. H., Wang, J. F., ... Zhong, L. (2017). A pumpkin polysaccharide induces apoptosis by inhibiting the JAK2/STAT3 pathway in human hepatoma HepG2 cells. *International Journal of Biological Macromolecules*, 104, 681–686.
- Sila, D. N., Van Buggenhout, S., Duvetter, T., Fraeye, I., De Roeck, A., Van Loey, A., & Hendrickx, M. (2009). Pectins in processed fruit and vegetables: Part II - structure-function relationships. *Comprehensive Reviews in Food Science and Food Safety*, 8(2), 86–104.
- Smet, C., Leroy, A., Sillen, A., Wieruszkeski, J. M., Landrieu, I., & Lippens, G. (2004). Accepting its random coil nature allows a partial NMR assignment of the neuronal Tau protein. *Chembiochem*, 5(12), 1639–1646.
- Torkova, A. A., Lisitskaya, K. V., Filimonov, I. S., Glazunova, O. A., Kachalova, G. S., Golubev, V. N., & Fedorova, T. V. (2018). Physicochemical and functional properties of *Cucurbita maxima* squash pectin and commercial citrus and apple pectins: A comparative evaluation. *PLoS One*, 13(9), Article e0204261.
- USP 26 NF 21. (2003). *The United States pharmacopeia-the national formulary* (pp. 1401–1402). Rockville, MD: United States Pharmacopeial Convention.
- Wang, J. L., Guo, H. Y., Zhang, J., Wang, X. F., Zhao, B. T., Yao, J. A., & Wang, Y. P. (2010). Sulfated modification, characterization and structure-antioxidant relationships of *Artemisia sphaerocephala* polysaccharides. *Carbohydrate Polymers*, 81(4), 897–905.
- Wang, Z. J., Xie, J. H., Shen, M. Y., Nie, S. P., & Xie, M. Y. (2018). Sulfated modification of polysaccharides: Synthesis, characterization and bioactivities. *Trends in Food Science & Technology*, 74, 147–157.
- Xie, L. M., Shen, M. Y., Hong, Y. Z., Ye, H. D., Huang, L. X., & Xie, J. H. (2020). Chemical modifications of polysaccharides and their anti-tumor activities. *Carbohydrate Polymers*, 229, 115436.
- Xiong, Q. P., Hu, Y. D., Ye, X. Y., Song, Z. Y., Yuan, J., Xiong, B. Y., ... Zhou, L. (2020). Extraction, purification and characterization of sulfated polysaccharide from *Bellamy quadrata* and its stabilization roles on atherosclerotic plaque. *International Journal of Biological Macromolecules*, 152, 314–326.
- Yang, J. H., Du, Y. M., Huang, R. H., Wan, Y. Y., & Wen, Y. (2005). The structure-anticoagulant activity relationships of sulfated lacquer polysaccharide - effect of carboxyl group and position of sulfation. *International Journal of Biological Macromolecules*, 36(1–2), 9–15.

- Yang, Z. J., Zhou, D. D., Huang, S. Y., Fang, A. P., Li, H. B., & Zhu, H. L. (2021). Effects and mechanisms of natural products on Alzheimer's disease. *Critical Reviews in Food Science and Nutrition*. <https://doi.org/10.1080/10408398.2021.1985428>
- Yuen, S. N., Choi, S. M., Phillips, D. L., & Ma, C. Y. (2009). Raman and FTIR spectroscopic study of carboxymethylated non-starch polysaccharides. *Food Chemistry*, 114(3), 1091–1098.
- Zhang, S. K., He, Z. Y., Cheng, Y., Xu, F. Z., Cheng, X. X., & Wu, P. (2021). Physicochemical characterization and emulsifying properties evaluation of RG-I enriched pectic polysaccharides from *Cerasus humilis*. *Carbohydrate Polymers*, 264, 118007.
- Zhang, Y., & Zhang, G. Z. (2019). Starch content and physicochemical properties of green wheat starch. *International Journal of Food Properties*, 22(1), 1463–1474.
- Zhao, J., Huvent, I., Lippens, G., Eliezer, D., Zhang, A. Q., Li, Q. H., ... Wang, C. Y. (2017). Glycan determinants of heparin-tau interaction. *Biophysical Journal*, 112(5), 921–932.
- Zhao, J., Zhang, F. M., Liu, X. Y., Ange, K. S., Zhang, A. Q., Li, Q. H., & Linhardt, R. J. (2017). Isolation of a lectin binding rhamnogalacturonan-I containing pectic polysaccharide from pumpkin. *Carbohydrate Polymers*, 163, 330–336.
- Zhao, J., Zhu, Y. N., Song, X. H., Xiao, Y. Y., Su, G. W., Liu, X. Y., ... Wang, C. Y. (2019). 3-O-Sulfation of heparan sulfate enhances tau interaction and cellular uptake. *Angewandte Chemie-International Edition*, 59(5), 1818–1827.
- Zhu, Z. J., Han, Y. H., Ding, Y., Zhu, B. W., Song, S., & Xiao, H. (2021). Health effects of dietary sulfated polysaccharides from seafoods and their interaction with gut microbiota. *Comprehensive Reviews in Food Science and Food Safety*, 20(3), 2882–2913.