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NMR analysis of a methylated non-pectic polysaccharide from water soluble yellow mustard mucilage

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ABSTRACT

Detailed structure information on non-pectic polysaccharides (NPP) from yellow mustard mucilage was investigated in the present study. Prior to Nuclear Magnetic Resonance (NMR) experiments, NPP was methylated in order to improve the resolution of NMR spectra by increasing the sample solubility.

^{1}H, ^{13}C, ^{1}H–^{1}H and ^{1}H–^{13}C NMR spectra revealed that NPP possessed a β-1,4 linked glucose backbone, with β-mannose 1,6 linked to the backbone chain, and the β-galactose 1,2 linked to the glycosidic backbone chain. The methyl groups were substituted to the 2, 3, 6 positions of the glucose residues and the 2, 3 positions of mannose and galactose residues. Ethyl group was also detected but no linkage was observed to the glucose residue.

1. Introduction

Non-pectic polysaccharide (NPP) from water soluble yellow mustard mucilage was isolated as described in our previous paper (Wu, Cui, Eskin, & Goff, 2009a). This fraction exhibited unique physical, chemical and rheological properties such as synergistic interactions with galactomannans (GMs), acid resistance, and stable gelling properties in wide temperature ranges (Wu et al., 2009a; Wu, Cui, Eskin, & Goff, 2009b). In previous studies, the water-soluble mucilage was fractionated using CTAB (cetyltrimethylammonium bromide) precipitation followed by DEAE-high capacity cellulose ion exchange chromatography (Cui, 1993; Cui, Eskin & Biliaderis, 1993). Two polysaccharide fractions were obtained using CTAB: CTAB-precipitants and CTAB-soluble fractions. Each of the fractions was further fractionated into five sub-fractions by ion exchange chromatography. Four of the 10 sub-fractions were composed of mainly glucose, and their solubility varied greatly. Among the four sub-fractions, only one soluble fraction was structurally analyzed using methylation analysis and NMR spectroscopy. The structure analysis revealed that this fraction consisted of a β-1,4 linked D-glucose backbone with occasional substitutions of ethyl and/or propyl groups at the 2, 3, or 6 positions of the glucose residue (Cui, Eskin & Biliaderis, 1995). However, it is still unclear how the other monosaccharide units, e.g. mannose and galactose, are linked to the backbone. In the current study, the non-pectic fraction was obtained by a new procedure (Wu et al., 2009a). In the new procedure pectins in the mucilage were hydrolyzed by pectinase leaving all the non-pectic polysaccharides remaining in solution. Therefore, this method was considered to be more efficient to obtain the total non-pectic fractions compared to the previous procedure (Cui, 1993; Cui et al., 1993). The NPP fraction in the current study could be the combination of the four sub-fractions reported by Cui (1993). The physicochemical properties of the non-pectic fraction from current study was different from the soluble fraction reported by Cui et al. (1995), which was only a small portion of the non-pectic fraction. As demonstrated in our previous studies (Wu et al., 2009a), NPP can form a weak gel even at very low concentrations, e.g., 0.1%. Gel formation, aggregation or poor solubility will result in poor resolution of the NMR spectra. This phenomenon was also mentioned by Parfondry and Perlin (1977) that cellulose derivatives of high molecular weight usually gave dilute and high viscous solutions which led to very long experimental runs and broad signals in NMR study. A method aimed at improving the resolution of NMR spectra of NPP was developed in the present study. NPP was methylated before being subjected to NMR examination. The methylated NPP (Me-NPP) was soluble in organic solvents and suitable for NMR analysis. Therefore, the objective of the current study was to produce high quality NMR data by methylating the

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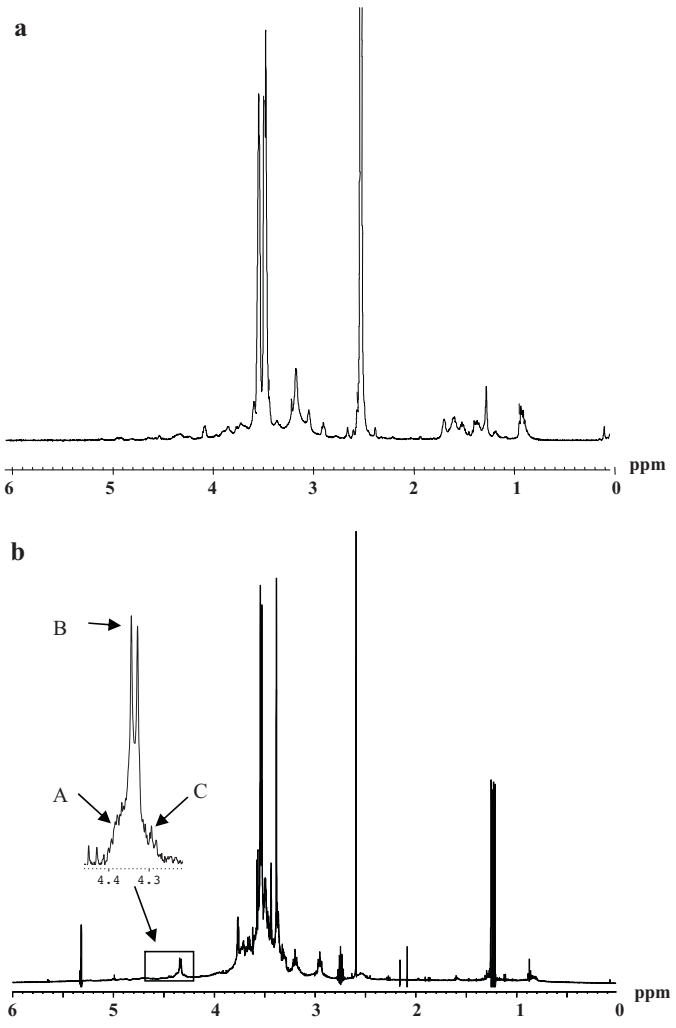

Fig. 1. ^{1}H spectrum of methylated and non-methylated NPP.

polysaccharide followed by NMR analysis to provide more detailed structural information on NPP.

2. Materials and methods

2.1. Sample preparation

NPP was isolated from water soluble yellow mustard mucilage as described by Wu et al. (2009a).

2.2. Methylation of NPP

The dried sample was dissolved in anhydrous DMSO (dimethyl sulfoxide) at 85 °C for 2 h with constant stirring, and sonicated for 4 h to ensure a complete dissolution. Dry sodium hydroxide powder was added to the mixture and stirred for 3 h at room temperature (22 °C). The mixture was stirred for an additional 2.5 h after adding methyl iodide (CH_3I). The methylated polysaccharide was then extracted with methylene chloride (CH_2Cl_2). The methylene chloride extract was passed through a sodium sulphate column (0.5 cm × 15 cm) to remove water. Methylated NPP was recovered by evaporating the solvent under a stream of nitrogen. The resulting residue was dissolved in deuterized methylene chloride (CD_2Cl_2) at a concentration of 2% for NMR analysis.

2.3. NMR spectroscopy

NMR spectra were recorded on a Bruker AMX500 spectrometer. Tetramethylsilane (TMS) was used as a chemical shift reference. Homonuclear ^{1}H–^{1}H correlation (COSY) spectra were recorded with F2 time domains of 1024 points and F1 time domains of 512 points. Total ^{1}H–^{1}H Correlation Spectroscopy (TOCSY) spectra were recorded at mixing time of 100 ms and with F2 time domains of 1024 points and F1 domains of 512 points. Heteronuclear Multiple-Quantum ^{1}H–^{13}C Coherence spectra (HMQC) were recorded with F2 time domain of 1024 points and F1 time domain of 256 points. Heteronuclear Multiple Bond ^{1}H–^{13}C Correlation spectra (HMBC)

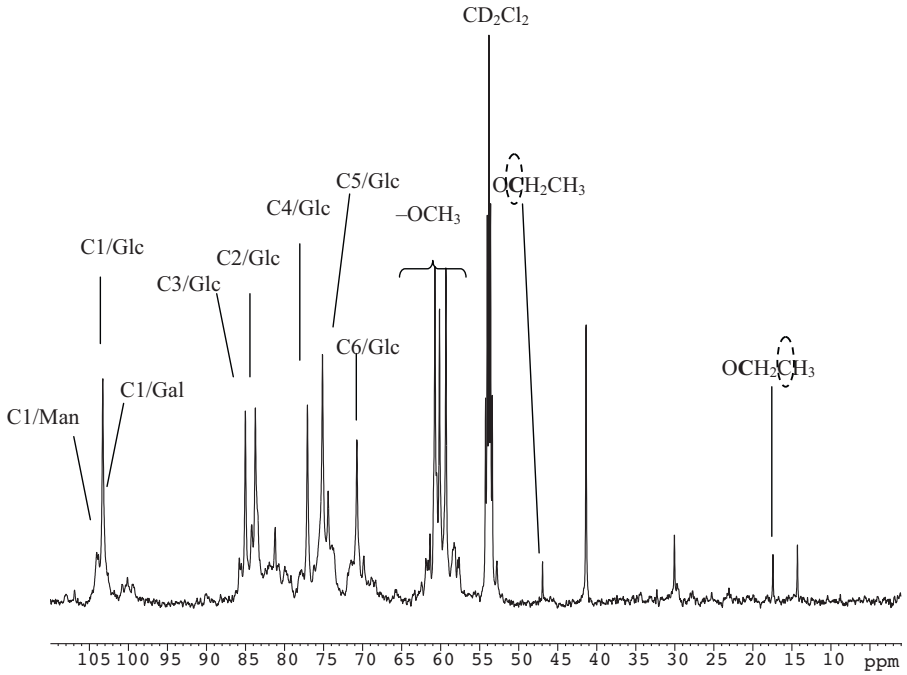

Fig. 2. ^{13}C NMR spectrum of methylated NPP.

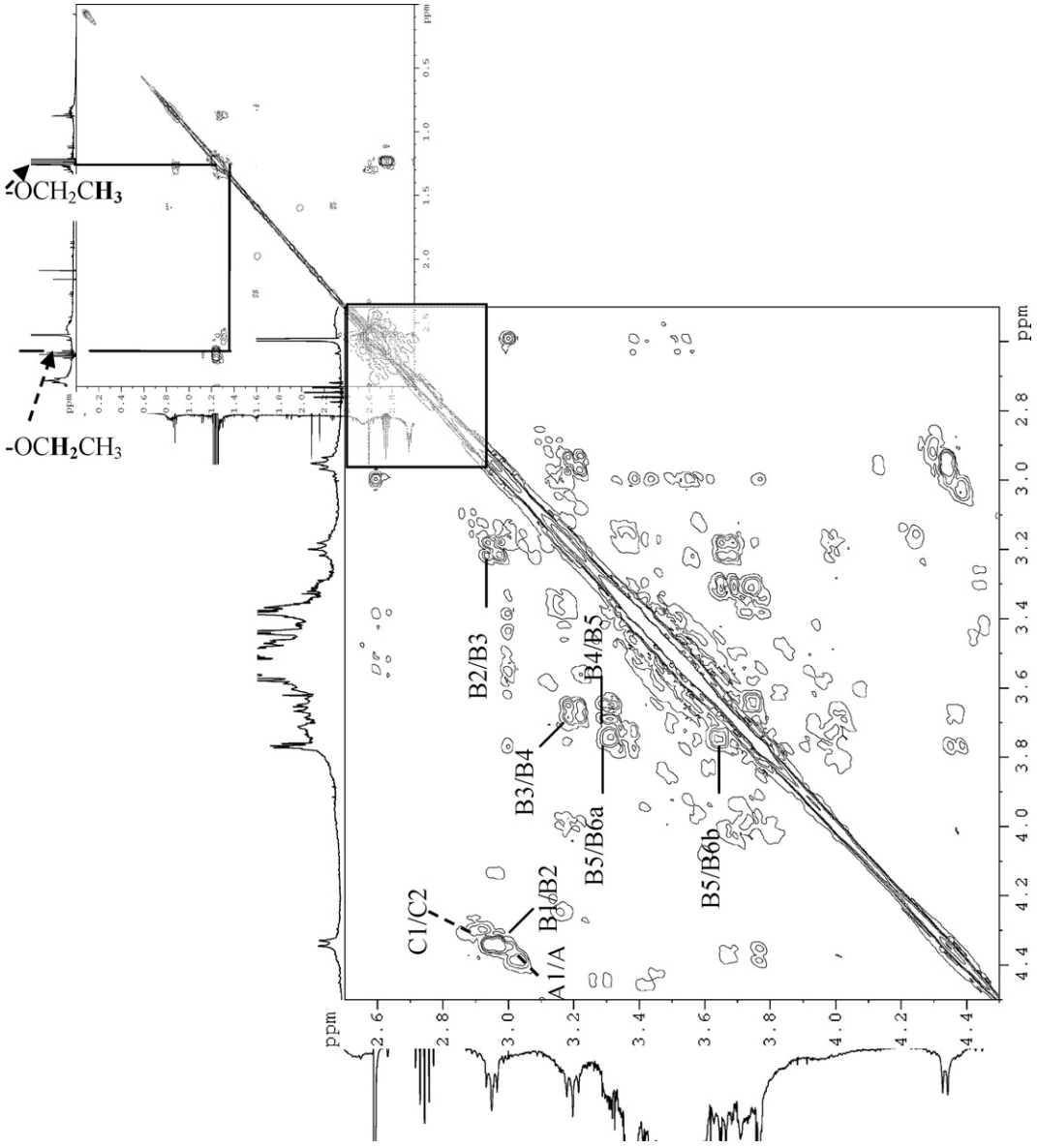

Fig. 3. Homonuclear shift correlated spectrum (COSY) of methylated NPP.

were recorded with F2 time domain of 1024 points and F1 time domain of 526 points.

3. Results and discussions

3.1. Methylation of NPP

NPP can form a weak gel at a low concentration of 0.1% (Wu et al., 2009a). This produced difficulties in obtaining NMR spectra with good resolutions, since usually the concentration of the polysaccharide required is at least 2%. It is worth mentioning that the cellulose-like fraction obtained by Cui et al. (1995) could be readily dissolved in water and the solution prepared for NMR was as high as 4%. We believe that the NPP investigated in the present study shared similar properties with that analyzed by Cui et al. (1995), e.g. the β-1,4 linked glycosidic backbone and chemical composition, however their molecular weight and linkage patterns might be very different because their physicochemical properties varied and the isolation methods were different (Wu et al., 2009a). The NPP in the current study may possess higher molecular weight and larger amount of unsubstituted region through which hydrogen bonding could be easily formed leading to the weak gel structure under low concentrations. NPP was dissolved in deutarized DMSO (1%) and ^{1}H spectrum was obtained (Fig. 1a), however, the resonance from the ^{13}C spectra and ^{1}H–^{1}H COSY, TOCSY, and ^{1}H–^{13}C HMQC and HMBC experiments were very weak. Through methylation, the –OH groups on sugar rings can be replaced by $–OCH_3$ groups to prevent the formation of hydrogen bonds among the molecules and improve solubility of NPP in an appropriate solvent. Methylation is widely used for modification of polysaccharides either for structural analysis (Cui, 2001) or for production purposes (Renard & Jarvis, 1999). In the present study, methylated NPP (Me-NPP) exhibited good solubility in CD_2Cl_2 and the solution concentration could be easily prepared up to 2%. The solvent CD_2Cl_2 was chosen because the Me-NPP was extracted using CH_2Cl_2 after methylation and this solvent could be easily evaporated to recover the sample. NMR experiments were performed with sound resolution (Figs. 1–6).

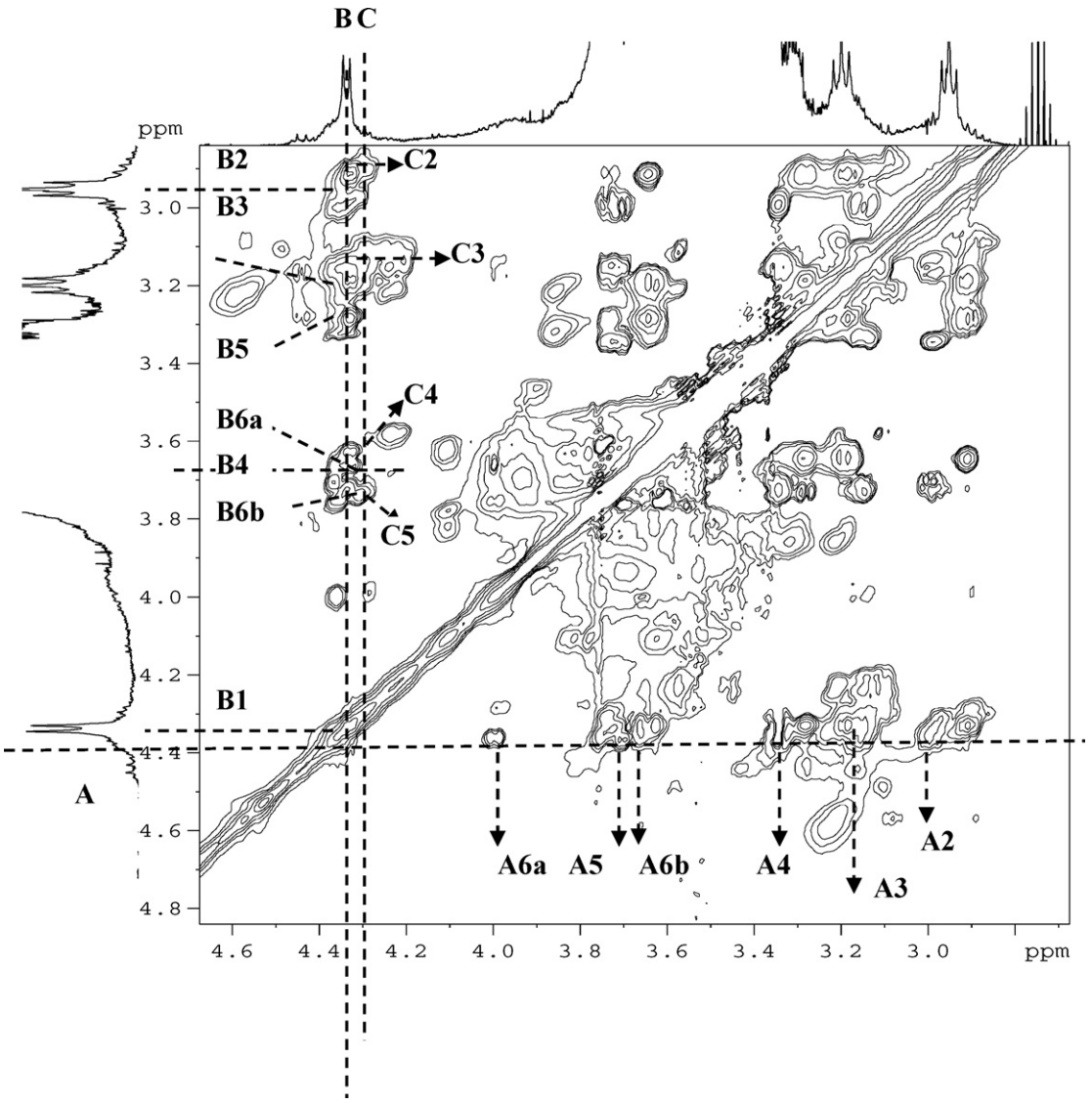

Fig. 4. Total correlation spectroscopy (TOCSY) of methylated NPP.

3.2. Structural elucidation of Me-NPP from NMR spectra

The ^{1}H and ^{13}C NMR spectra of Me-NPP are shown in Figs. 1b and 2 respectively.

Fig. 1a and b are ^{1}H NMR spectra of NPP. Fig. 1a is the non-methylated NPP and Fig. 1b is the Me-NPP respectively. One strong signal was observed at 3.48 ppm on Fig. 1b and was identified as methyl group (Bernlind, Oscarson, & Widmalm, 1994; Cui et al., 1995; Jansson, Kenne, & Kolare, 1994), which was not shown in Fig. 1a. The ^{1}H NMR spectrum (Fig. 1b) contained 3 major anomeric protons, all appearing in the range of 4.2–4.4 ppm, and labelled as A at 4.38 ppm, B at 4.33 ppm and C at 4.30 ppm. The coupling constant of the B and C anomeric protons are in the same range of 7.5–8.0 Hz, therefore, they were identified as β-configurations (Iwata, Azuma, Okamura, Muramoto, & Chun, 1992). Due to the overlapping of A with B, it is hard to determine the coupling constant of A. Some weak signals can also be observed from the ^{1}H NMR spectrum, at 5.0 ppm and 5.65 ppm respectively, indicating the existence of some α-configurations and uronic acids, however, the signals are too weak for the continued identification of these residues.

Monosaccharide analysis revealed that NPP was composed of 61% of glucose, 13.8% of galactose, 13% of mannose, 7.5% of xylose, 4.5% of rhamnose and 7% of uronic acid (Wu et al., 2009a). Among the three anomeric protons, the B proton yielded the strongest signal, indicating the abundance of this proton in the sample. Since D-glucose was the most abundant residue in NPP, B was identified as H1 from glucose. The comparison of the observed ^{1}H NMR chemical shifts of the three anomeric protons with those reported in the literature (Hannuksela & Hervé du Penhoat, 2004) allowed the identification of A as H1 from β-Man and C as H1 from β-Gal.

The ^{1}H NMR spectrum (Fig. 1a and b) also showed some signals in the range of 1–3 ppm, with more signals appearing in Fig. 1a than in Fig. 1b. Cui et al. (Cui et al., 1995; Cui, 2001) mentioned that some signals appeared in the similar region, which they believed were attributed to the ethyl and propyl groups.

The ^{13}C NMR spectrum of Me-NPP is presented as Fig. 2. The carbohydrate carbon signals are distributed in the range of 60–110 ppm. Other than the carbohydrate carbon signals, some resonances appeared in the range of 0–70 ppm. The strong signal at 54 ppm was identified as the solvent signal (CD_2Cl_2). The signals appearing around 60 ppm were attributed to the $-OCH_3$ group. The different substitution positions led to the difference in chemical shifts of the methyl groups, and some of the minor peaks present indicated that the material was not fully methylated (Parfondry &

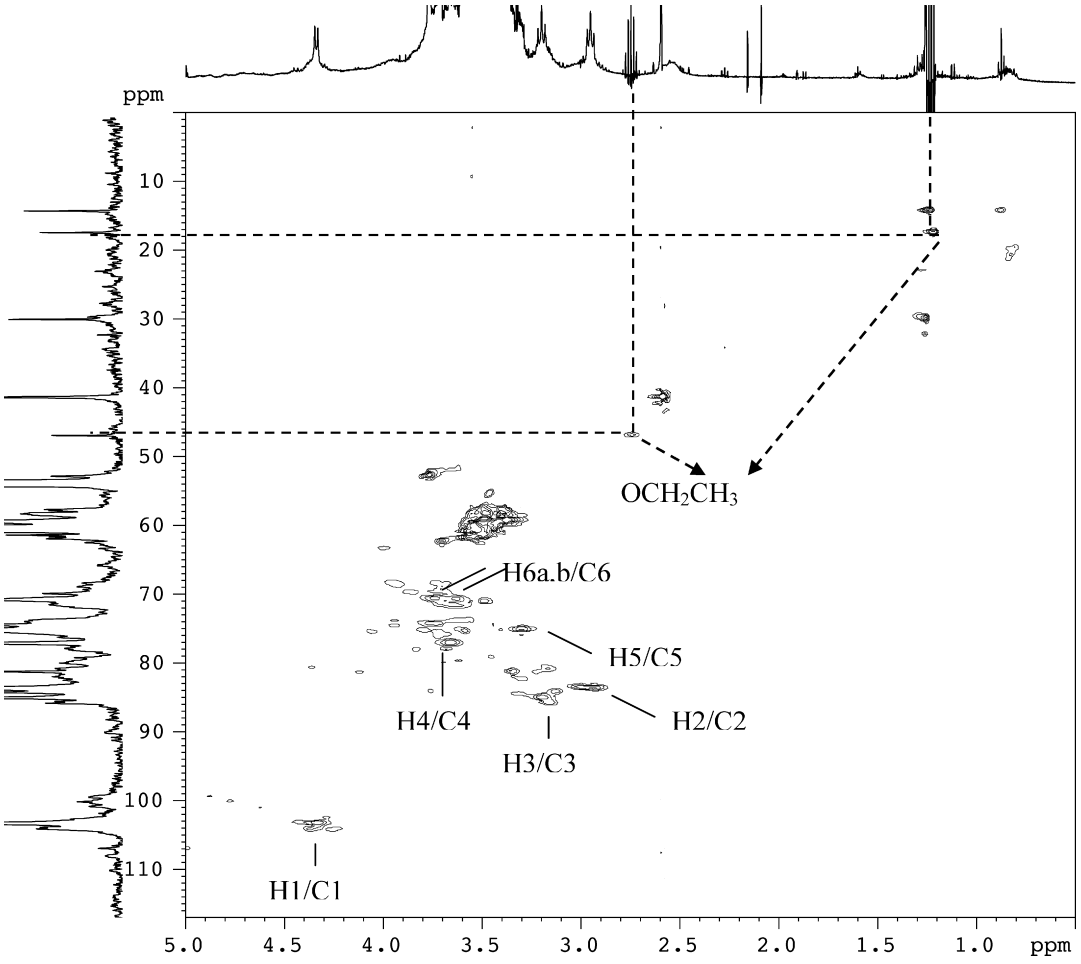

Fig. 5. ^{1}H–^{13}C heteronuclear multiple-quantum coherence (HMQC) spectrum of methylated NPP.

Perlin, 1977). Some of the signals in the non-carbohydrate carbon region were attributed to the ethyl groups originally substituted to the NPP, which is in agreement with previous observations (Cui et al., 1995).

^{1}H–^{1}H COSY (Fig. 3) was performed to obtain the information on the connectivity of protons by following the connectivity pattern H1–H2–H3–H4–H5–H6. In Fig. 3, each connective peak of the residue B was provided. The H1/H2 connective peaks of residues A and C were also provided. The complete proton assignment of each sugar residue was further confirmed by TOCSY (Fig. 4).

From ^{1}H–^{1}H COSY, other than the three anomeric protons, the protons from each pyranose ring (H2 to H6) overlapped in the region of 3.0–4.0 ppm, which produced difficulty in accurately assigning protons to each sugar residue. ^{1}H–^{1}H TOCSY can overcome this disadvantage. In TOCSY, all protons on the same sugar ring will have a correlation with all other protons on the same ring but not with protons on different rings. The protons belonging to one pyranose ring can be clearly observed and identified (Fig. 4). According to the proton assignment, ^{1}H–^{13}C correlation of Me-NPP (HMQC) (Fig. 5) gave the complete assignment of proton and carbon of the three monosaccharides correspondingly (Table 1). The connective peaks of residue B were marked as an example (Fig. 5). This assignment of glucose was supported by Parfondry and Perlin's results (Parfondry & Perlin, 1977) who studied ^{13}C NMR spectroscopy of cellulose ethers. They also methylated cellulose with the degree of substitution of 2.8 and dissolved the sample in deuteriochloroform ($CDCl_3$). The pattern of chemical shifts of the six carbons from our results is in good agreement with that from their study.

^{1}H–^{13}C HMBC spectrum showed long range connectivity of the ^{1}H–^{13}C correlation, hence the linkage information was obtained as shown in Fig. 6. The cross peak of H1/C4 (4.34 ppm/77.0 ppm) of glucose residue further confirmed the β-1,4 linked glucose in large quantity; a resonance at 3.75 ppm/103.6 ppm (H6 of glucose/C1 of mannose) indicated that mannose was 1–6 linked to the glucose. A resonance at 2.96 ppm/103.0 ppm (H2 of glucose/C1 of galactose) indicated galactose was 1, 2 linked to the glucose backbone. There might exit some other types of linkages that the present study cannot reveal due to the ambiguous resonance signals. Also, the substitution pattern of mannose and galactose, e.g. in terms of the ratio between main chain residue and side group residue, the distance between the two side groups, are still unclear.

From the ^{1}H–^{13}C HMQC spectrum (Fig. 5), the methyl group formed strong resonances around 3.48 ppm (^{1}H) and 60 ppm (^{13}C), which indicated the abundance of this group. The ^{1}H–^{13}C HMBC spectrum (Fig. 6) indicated that the methyl groups are densely substituted at C2 and C3 positions of glucose, mannose and galactose

Table 1
Assignment of resonances (ppm) of ^{1}H and ^{13}C spectra of methylated NPP.

Residue	H/C	1	2	3	4	5	6a/6b
Glucose	H	4.34	2.96	3.21	3.66	3.30	3.75/3.65
	C	103.2	84.0	86.0	77.0	75.0	71.0
Mannose	H	4.38	3.04	3.17	3.37	3.71	3.98/3.66
	C	103.6	83.5	80.5	81.2	69.5	75.0
Galactose	H	4.30	2.92	3.13	3.62	3.75	–/–
	C	103.0	84.2	80.0	75.7	74.0	73.0

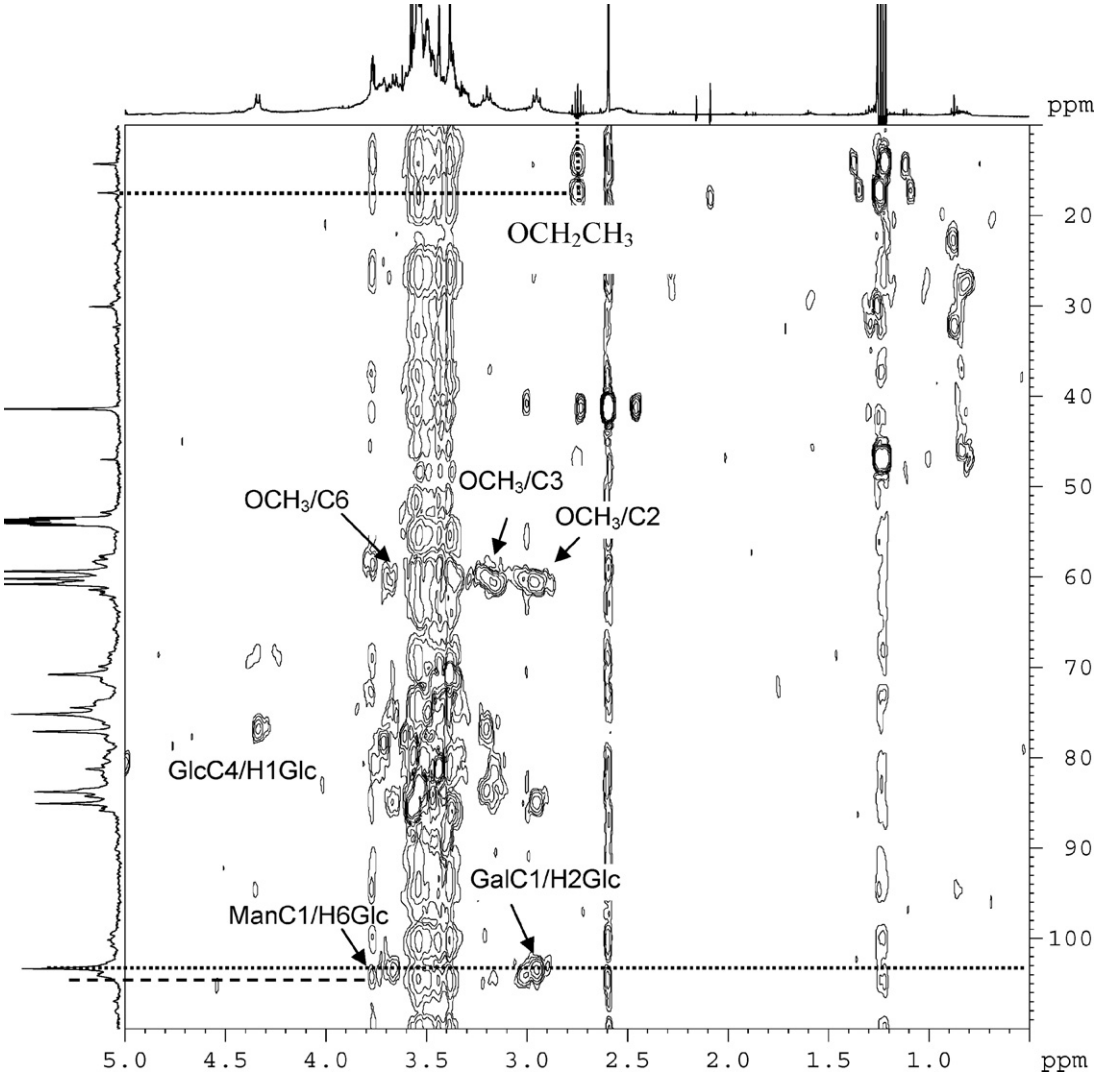

Fig. 6. ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) spectrum of methylated NPP.

residues. The methyl substitution at C6 position of glucose was also observed from the HMBC but with less density. The substitution trend was in agreement with that summarized by Gorin (1981) that the substitution rate of methyl groups to sugar rings follow the order: C2 > C3 ≫ C6.

In addition to the methyl group, ethyl group (–O-CH_2-CH_3) was also identified from ^{1}H–^{1}H COSY and HMBC spectra. The chemical shifts (^{1}H/^{13}C) for ethyl groups are 2.75/47.0 ppm for –CH_2– and 1.24/17.5 ppm for –CH_3. As revealed by Cui et al. (1995), the non-sugar signals in the range of 1–2.5 ppm on the ^{1}H spectrum were attributed to the –OCH_2CH_3 and the –OCH_3 groups. Cui et al. (1995) reported that the methyl groups were identified mostly attached to the C2 position and the ethyl groups mostly attached to the C3 position of the main chain. The existence of those groups can partially explain the superior emulsification behaviour of yellow mustard mucilage reported by Cui (2001). The methylation process may replace the ethyl groups with methyl groups since ethyl groups were detected in the sample but no linkage to the sugar ring was observed.

The dense substitution of methyl groups to the C2 and C3 positions of the sugar residues improved the solubility of NPP in CD_2Cl_2. Meanwhile, the possible replacement of originally substituted groups, e.g. methyl groups and ethyl groups, with the introduced methyl groups interfered with understanding of the original substitution groups and patterns. Quantitative and qualitative substitution pattern of the methyl and ethyl groups should be investigated using an alternative approach.

4. Conclusions

Methylation of NPP was successfully applied in the current study to obtain high quality NMR spectra. After methylation, ^{1}H, ^{13}C and ^{1}H–^{1}H, ^{1}H–^{13}C NMR spectra with good resolution were obtained. More detailed structural information on Me-NPP was derived based on the NMR spectra. The result revealed that Me-NPP possessed a β-1,4 linked glycosidic backbone, with mannose β-1,6 linked to the glycosidic backbone and galactose β-1,2 linked to the glycosidic backbone. The methyl groups substituted to the C2, C3 and C6 positions of the glucose residues. The C2 and C3 positions of the side groups, mannose and galactose residues, were also substituted with methyl groups. Ethyl groups were detected in the sample solution although no linkage to the sugar residues was observed.

References

Bernlind, C., Oscarson, S., & Widmalm, G. (1994). Synthesis, NMR, and conformational studies of methyl [alpha]-mannopyranoside 2-, 3-, 4-, and 6-monophosphates. *Carbohydrate Research*, *263*(2), 173–180.

Cui, S. W. (1993). Characterization of the structure and physical properties of yellow mustard (*Sinapis alba* L.) polysaccharides. Ph.D Thesis, University of Manitoba.

Cui, S. W. (2001). Yellow mustard gum. In S. W. Cui (Ed.), *Polysaccharide gums from agricultural products: Processing, structure and functionality* (pp. 1–59). CRC Press.

Cui, W., Eskin, N. A. M., & Biliaderis, C. G. (1993). Water-soluble yellow mustard (*Sinapis alba* L.) polysaccharides: Partial characterization, molecular size distribution and rheological properties. *Carbohydrate Polymers*, *20*(3), 215–225.

Cui, W., Eskin, N. A. M., & Biliaderis, C. G. (1995). NMR characterization of a water-soluble 1,4-linked [beta]-glucan having ether groups from yellow mustard (*Sinapis alba* L.) mucilage. *Carbohydrate Polymers*, *27*(2), 117–122.

Gorin, P. A. J. (1981). Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. In R. S. Tipson, & H. Derek (Eds.), *Advances in carbohydrate chemistry and biochemistry* (pp. 13–104). Academic Press.

Hannuksela, T., & Hervé du Penhoat, C. (2004). NMR structural determination of dissolved O-acetylated galactoglucomannan isolated from spruce thermomechanical pulp. *Carbohydrate Research*, *339*(2), 301–312.

Iwata, T., Azuma, J.-I., Okamura, K., Muramoto, M., & Chun, B. (1992). Preparation and n.m.r. assignments of cellulose mixed esters regioselectively substituted by acetyl and propanoyl groups. *Carbohydrate Research*, *224*, 277–283.

Jansson, P.-E., Kenne, L., & Kolare, I. (1994). NMR studies of some (1→6)-linked disaccharide methyl glycosides. *Carbohydrate Research*, *257*(2), 163–174.

Parfondry, A., & Perlin, A. S. (1977). 13C-n.m.r. spectroscopy of cellulose ethers. *Carbohydrate Research*, *57*, 39–49.

Renard, C. M. G. C., & Jarvis, M. C. (1999). Acetylation and methylation of homogalacturonans 1: Optimisation of the reaction and characterisation of the products. *Carbohydrate Polymers*, *39*(3), 201–207.

Wu, Y., Cui, W., Eskin, N. A. M., & Goff, H. D. (2009a). Fractionation and partial characterization of non-pectic polysaccharides from yellow mustard mucilage. *Food Hydrocolloids*, *23*(6), 1535–1541.

Wu, Y., Cui, W., Eskin, N. A. M., & Goff, H. D. (2009b). Rheological investigation of synergistic interactions between galactomannans and non-pectic polysaccharide fraction from water soluble yellow mustard mucilage. *Carbohydrate Polymers*, *78*(1), 112–116.