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Characterisation of cell wall polysaccharides from okra (*Abelmoschus esculentus* (L.) Moench)

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ABSTRACT

Okra pods are commonly used in Asia as a vegetable, food ingredient, as well as a traditional medicine for many different purposes; for example, as diuretic agent, for treatment of dental diseases and to reduce/prevent gastric irritations. The healthy properties are suggested to originate from the high polysaccharide content of okra pods, resulting in a highly viscous solution with a slimy appearance when okra is extracted with water. In this study, we present a structural characterisation of all major cell wall polysaccharides originating from okra pods. The sequential extraction of okra cell wall material yielded fractions of soluble solids extractable using hot buffer (HBSS), chelating agent (CHSS), dilute alkaline (DASS) and concentrated alkaline (CASS). The HBSS fraction was shown to be rich in galactose, rhamnose and galacturonic acid in the ratio 1.3:1:1.3. The degree of acetylation is relatively high (DA = 58) while the degree of methyl esterification is relatively low (DM = 24). The CHSS fraction contained much higher levels of methyl esterified galacturonic acid residues (63% galacturonic acid; DM = 48) in addition to minor amounts of rhamnose and galactose. The ratio of galactose to rhamnose to galacturonic acid was 1.3:1.0:1.3 and 4.5:1.0:1.2 for HBSS and CHSS, respectively. These results indicated that the HBSS and CHSS fractions contain rhamnogalacturonan type I next to homogalacturonan, while the latter is more prevailing in CHSS. Also the DASS fraction is characterised by high amounts of rhamnose, galactose, galacturonic acid and some arabinose, indicating that rhamnogalacturonan I elements with longer arabinose- and galactose-rich side chains were part of this fraction. Partial digestion of HBSS and CHSS by pectin methyl esterase and polygalacturonase resulted in a fraction with a lower Mw and lower viscosity in solution. These samples were subjected to NMR analysis, which indicated that, in contrast to known RG I structure, the acetyl groups in HBSS are not located on the galacturonic acid residues, while for CHSS only part of the acetyl groups are located on the RG I galacturonic acid residues. The CASS fraction consisted of XXXG-type xyloglucan and 4-methylglucuronoxylan as shown by their sugar (linkage) composition and enzymatic digestion.

1. Introduction

The okra plant, *Abelmoschus esculentus* (L.) Moench, a native plant from Africa, is now grown in many other areas such as Thailand, the Middle East and the southern states of the USA. The okra pod is often used as a vegetable. Its water extracts contain thick slimy polysaccharides and are used to thicken soups and stews.[1,2] The immature fruit is also used in folk medicine as a diuretic agent and for treatment of dental disease.[3] Okra polysaccharides are also used as egg white substitute,[4] fat substitute in chocolate bar cookies[5] and in chocolate frozen dairy dessert.[6]

The okra polysaccharide was found firstly as an acidic polysaccharide consisting of galactose, rhamnose and galacturonic acid.[7] Deters et al.[8] confirmed the findings as mentioned by Lengsfeld et al.[9] that okra polysaccharide consisted of the sugars rhamnose, galacturonic acid, galactose, glucose and glucuronic acid. Agarwal et al.[10] suggested that galacturonic acid in the okra polysaccharide could be in the L-configuration. The main structural elements of okra polysaccharide was described by Tomada et al.[11] who concluded that it contained a repeating unit of alternating α-(1→2)-linked rhamnosyl and α-(1→4)-linked galacturonic acid residues with a disaccharide side chain of β-(1→4)-linked galactosyl moieties attached to O-4 of about half the L-rhamnosyl residues. The acetyl content of the okra polysaccharide was determined to be 5.5% w/w while the precise position of the acetyl groups within the polysaccharides was not mentioned. Lengsfeld et al.[9] suggested

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from linkage analysis data that okra polysaccharide sub-fractions, which were extracted by water and fractionated by anion-exchange chromatography, contained more galacturonan than rhamnogalacturonan as the main structural elements.

In contrast to the chemical characteristics of okra pectin, the information about other polysaccharides like hemicelluloses is still lacking. In this study, we present the characterisation of all main cell wall polysaccharide in okra, which were extracted sequentially with different aqueous extractants of increasing strength, with emphasis on the detailed structures of the various pectin fractions.

2. Results and discussion

2.1. Specific parts of the okra pod

In order to have an impression on the proportion of individual parts of the fresh okra pods, the whole okra pod was separated into three parts; calyx, pulp and seed. The relative amounts of the different parts of fresh okra expressed as fresh weight, dry weight and alcohol-insoluble solids (AIS) are shown in Table 1. The pulp was the major part (~ca. 72 g/100 g) of fresh okra, whereas the calyx represented ~ca.15 g/100 g of fresh okra and the seed represented ~ca. 9 g/100 g of fresh okra. As can been seen from the figures, about 4% of the material was not recovered and this is probably due to some losses of the seed fraction. Since the okra pulp formed the major part of the okra pod, this part was subjected to further studies. The okra pulp yielded about 5.8 g/100 g fresh okra of alcohol-insoluble solid (AIS) representing cell wall materials.

2.2. Sugar composition and absolute configuration of okra AIS and okra AIS extracts

The sugar composition of okra AIS is shown in Table 2. Okra AIS consisted of mainly glucose (44 mol %), galactose (17 mol %) and galacturonic acid (16 mol %). In addition to polysaccharides the AIS also contained 15.8% of protein. The sugar composition suggests that okra AIS consisted of different types of polysaccharides including pectin, hemicelluloses such as xylan and xyloglucan, and cellulose. The sequential extraction of the cell wall material (AIS) provides information about the extractability of the different polysaccharides, namely pectins, hemicellulose and cellulose.[12] The okra AIS was therefore sequentially extracted with different aqueous extractants. Table 2 shows the sugar composition of the fractions obtained from sequential extraction of okra AIS of which the HBSS and DASS fractions were the main fractions.

The HBSS fraction consisted of 35 mol % of galacturonic acids, in addition to high amounts of rhamnose (26 mol %) and galactose (34 mol %). Galactose was found as the main neutral sugar in the HBSS fraction, and about 30% of all galactose present in the AIS was recovered in this fraction. The ratio of the main sugars presented in the HBSS fraction was 1.3:1.0:1.3 for galactose:rhamnose:galacturonic acid, respectively, which was rather similar to that reported by Tomada et al.[11] Lengsfeld et al.[9] reported that this ratio was 0.9:1.0:0.6 for the okra polysaccharide water extract. Compared to this result, our HBSS fraction contained slightly more galactose. This was also true when compared with the data for water-extracted okra polysaccharides as described by Deters et al.[8]

The CHSS fraction contained higher amounts of galacturonic acid content (63 mol %) and less galactose (17 mol %) and rhamnose (14 mol %) when compared to the HBSS fraction. The CHSS fraction contained also 3 mol % arabinose while this sugar was not found in the HBSS fraction. No xylose was found in the HBSS, neither in the CHSS fractions providing evidence that no xylose-containing pectic polymer like xylogalacturonan was present in both fractions. In addition, the HBSS and CHSS fractions contained low levels of glucuronic acid (3 and 2 mol %, respectively) which was lower than the levels of glucuronic acid found in water-extracted okra polysaccharides (8.8 mol %) as described by Lengsfeld et al.[9]

The HBSS fraction had a higher ratio of rhamnose to galacturonic acid (0.7) than the CHSS fraction (0.2). Generally, rhamnogalacturonan I (RG I) consists of alternating rhamnose and galacturonic acid residues as a backbone.[13] The ratio of rhamnose:galacturonic acid within a RG I backbone is 1:1. Consequently, the HBSS fraction was found to contain mainly RG I segments (85%) and much less homogalacturonan (HG) segments. The CHSS contained mainly HG segments (74%) and less RG I segments. The high amounts of RG I segments in the HBSS fraction is a bit uncommon since no water extract from other sources showed such a high amount of RG I segments. For example, water soluble soybean polysaccharide contained 43% of RG I segments within the polysaccharides,[14] water extracts from sugar beet pulp contained 22% of RG I segments within the polysaccharides.[15] The ratio of neutral sugars to rhamnose roughly indicates the length of the side chains. The ratio of (galactose and arabinose) to rhamnose was 1.3 and 1.4 for the HBSS and CHSS fractions, respectively. This suggests that the CHSS fraction contained slightly longer side chains than the HBSS fraction.

The absolute configuration of sugar moieties in the HBSS and the CHSS fractions were determined by using GC-FID after methan-

Table 1
Relative amount of the different parts of fresh okra pods

Parts of okra pods	Fresh weight[a]	Dry weight[a]
Calyx	14.6	1.4
Pulp	71.9	7.4
Seed	9.1	1.3

[a] Gram qualities per 100 g of fresh okra pods.

Table 2
Yield, composition and degree of acetylation (DA) and methyl esterification (DM) of okra AIS and okra polysaccharide extracts and residues

	Yield (g/100 g AIS)	Rha	Ara	Fuc	Xyl	Man (mol %)	Gal	Glc	GalA	GlcA	DA[a] (%)	DM[a] (%)	Carbohydrate content[b]	Protein content[b]
AIS		3(2)[c]	5(2)	0	5(2)	3(2)	17(10)	44(25)	16(14)	7(1.7)	40	59	57.5	15.8
HBSS	11.2	26(2.5)	0	0	0	0	34(3.2)	1(0.1)	35(4.0)	3(0.3)	58	24	90.0	3.5
CHSS	4.8	14(0.5)	3(0.1)	0	0	0	17(0.6)	1(0)	63(3.0)	2(0.1)	18	48	86.2	10.5
DASS	13.2	13(1.2)	13(1.0)	0	0	0	19(1.8)	4(0.4)	48(5.5)	2(0.2)	—[d]	—[d]	76.9	16.6
CASS	4.1	1(0.05)	3(0.1)	2(0.1)	27(0.8)	3(0.1)	8(0.3)	52(2.0)	3(0.1)	1(0.1)	—[d]	—[d]	86.8	13.2
RES	26.5	3(0.5)	5(0.8)	0	5(0.9)	tr(0)	7(1.3)	78(15.3)	0	1(0.2)	—[d]	—[d]	73.7	16.1

tr: Trace amounts.
[a] Moles acetyl or methanol per 100 mol of galacturonic acid.
[b] Gram qualities per 100 g of fraction.
[c] Values in brackets give g/100 g of AIS for the individual sugars.
[d] Not determined.

olysis and conversion to their corresponding butylglycosides. The results showed that all sugars as present in the HBSS and CHSS fractions were in D-configuration except for the rhamnosyl residues which were in the L-configuration. These results are in contrast with those reported by Agarwal et al.[10] who stated that the okra gum contain L-galacturonic acid. The HBSS, okra rhamnogalacturonan I is also different from rhamnogalacturonan I of flax seed mucilage which contains L galactose as a neutral sugar.[16]

The DASS fraction contained high amounts of galactose and galacturonic acid. The ratio of rhamnose to galacturonic acid (0.3) was higher than that found for the CHSS fraction (0.2). It can be calculated that the DASS fraction contained 43% of RG I segments and 57% of HG segments. Furthermore, the DASS fraction was relatively enriched in arabinose and galactose. The ratio of arabinose to rhamnose and the ratio of (arabinose and galactose) to rhamnose of the DASS fraction were 1.0 and 2.5, respectively, which was higher than that found for the CHSS fraction (0.2 and 1.4, respectively).

The CASS fraction contained glucose (52 mol %) and xylose (27 mol %) as the main neutral sugars, and only low amounts of glucuronic acid were present which were quite similar to other fractions. The presence of glucose xylose and glucuronic acid residues may indicate that next to xyloglucans, acidic xylans were also part of this fraction.

In the extraction residue, the main sugar was glucose (78 mol %) representing approximately 50% of all glucose present in AIS. This glucose originates from cellulose and hemicellulose. The presence of xylose (5 mol %) and galactose (7 mol %) residues indicated that xyloglucan partly remained in the residues. This xyloglucan is either strongly embedded in the structure of the cellulose fibrils or so extensively hydrogen bonded to the cellulose fibrils that it resisted extraction by 6 M NaOH.

The residue fraction showed that the solvents used to extract the okra AIS were able to solubilise pectic material. However, only one-third of polysaccharides in the AIS could be extracted while 26% was recovered in the residue. This results in a recovery of 60%. In addition, respectively, 26%, 32% and 15% of all glucose, galactose and xylose residues in AIS were not covered by the analysis of all fractions. They most probably belong to the hemicellulosic material and were lost during the extraction step with concentrated alkali.

2.3. Degree of methyl esterification and acetylation

The degree of methyl esterification (DM) of the HBSS pectins (24%) was surprisingly low and much lower than the DM of the CHSS. The DASS fraction was not included in the analysis due to the removal of methyl esters and acetyl groups during the dilute alkaline extraction. According to the sugar composition (Table 2), 75% of all galacturonic acid present in the HBSS pectins originates from RG I for which there is no evidence that galacturonic acid residues in RG I segments are methyl esterified.[17] Assuming that the methyl ester is only present within HG segments of HBSS pectin, the DM of this HG could be as high as 96%. The DM of the CHSS pectins (48%) was quite low since chelating agents are expected to extract calcium-sensitive pectins with low DM[18] being present in the form of calcium pectate gels.[12]

The degree of acetylation (DA) was much higher in the HBSS fraction (58%) compared to the CHSS fraction (18%). So far there is only evidence for the presence of O-acetyl groups on O-2 and/or O-3 of galacturonic acid residues in HG segments and RG I segments.[17,19] In general, the DA is high in the RG I segments (hairy regions) of pectin as illustrated by the DA of 60% found for modified hairy regions from apple.[20] Therefore the HBSS fraction represents pectins having a unique structure which differs from other pectins for instance apple, sugar beet and soya pectin. Moreover, a pure RG I with high DA in water extraction has not frequently been reported for other plants, although recently an arabidopsis seed mucilage was described by Deng et al.,[21] showing a water-extractable linear rhamnogalacturonan I.

2.4. Glycosidic linkage composition

To obtain more information about the different cell wall polysaccharides present in the different extracts, the samples were subjected to linkage analysis by permethylation. In general the data obtained were more qualitative than quantitative. First of all the uronic acids were not reduced completely to their neutral sugar analogues and therefore they were not measured. Secondly an underestimation of the terminal pentose and 1,4-linked galactosyl residues could occur due to the evaporation of terminal pentose and complex formation of 1,4-linked galactosyl residues with borate during the acetylation procedure,[22] respectively.

Table 3
Sugar linkage composition of okra polysaccharide fractions

Glycosyl residue	HBSS	CHSS (mol %)	DASS	CASS
t-ara*f*	0.5	4.2	7.5	0.3
1,2-ara*f*		0.3	0.2	0.6
1,5-ara*f*	0.8	29.4	22.4	
1,3-ara*f*	0.6	0.2	0.6	
1,3,5-ara*f*		6.8	4.5	0.2
1,2,5-ara*f*		1	1.7	0.2
1,2,3,5-ara*f*	0.4			0.2
Total ara	2.3	41.9	36.9	1.5
t-xyl*p*	0.1	0.3	0.1	0.8
1,4-xyl*p*	0.9		2.6	11
1,2-xyl*p*				1.7
1,3-xyl*p*	0.5			1.3
1,3,4-xyl*p*	0.4	0.2	0.4	
1,2,4-xyl*p*		1.7	1.8	0.7
1,2,3,4-xyl*p*		0.4		
Total xyl	1.9	2.6	4.9	15.5
t-rha*p*	0.3			
1,2-rha*p*	3.3	1.4	2.7	
1,3-rha*p*	0.1	0.3		
1,4-rha*p*	0.5	1.1	0.2	
1,2,4-rha*p*	34	5.5	5	0.1
Total rha	38.1	8.3	7.9	0.1
t-fuc*p*			0.03	1.2
Total fuc			0.03	1.2
t-glc*p*	1	1.8	2.2	2.7
1,4-glc*p*			25.4	52.1
1,2,4-glc*p*				0.1
1,4,6-glc*p*	0.6	0.3	0.3	16.5
1,3,4-glc*p*	0.7	0.1	0.3	
1,2,3,4,6-glc*p*	0.8	0.3		
Total glc	3.1	2.5	28.2	71.4
1,4-man*p*	0.7	2.1	0.4	2.2
1,2,3,4,6-man*p*	0.8	0.6	0.2	0.01
Total man	1.5	2.7	0.6	2.2
t-gal*p*	34.5	9.4	6.1	5.3
1,6-gal*p*	1.9	3.5	2.3	0.1
1,4-gal*p*	12.2	12.5	4.8	0.6
1,2,6-gal*p*		0.6		0.2
1,2,4-gal*p*	0.4	0.5	0.3	0.8
1,4,6-gal*p*	1.5	3.7	2.2	0.7
1,3,4-gal*p*	0.2	0.7	0.2	
1,3,6-gal*p*	1.6	9	4.6	0.2
1,3,4,6-gal*p*		0.6		
1,2,3,4,6-gal*p*	0.8	1.4	1	0.2
Total gal[a]	53	42	21.5	8.1
Ratio *t*/*b*	0.8	0.4	0.7	0.4

[a] Uronic acid was not reduced prior to methylation.

For the HBSS fraction, the sugar linkage composition results (Table 3) indicate the presence of highly branched RG I structures, since the majority (89%) of all 1,2-linked rhamnosyl residues were O-4 substituted. These rhamnosyl residues were substituted with short galactan side chains containing 1 or 2 galactosyl residues since 65% of all galactose was present as terminal residues and 23% as 1,4-linked units.

The CHSS fraction was found to represent a slightly less branched RG I structure as shown by lower levels of O-4 substituted rhamnosyl residues (66% of all rhamnose). Only 22% of all galactosyl residues were present terminally linked revealing the fact that slightly longer galactan side chains were present in the CHSS compared to the HBSS. Summarising, it can be stated that the HBSS fraction contained RG I backbones with monomeric or dimeric galactan side chain, while the CHSS fraction contained RG I with slightly longer galactan side chains. This observation was also reported by Tomada et al.[11] for water-extracted okra polysaccharides. Apart from the 1,4-linked galactosyl residues found in rather high amounts, some 1,6-linked and 1,3,6-linked galactosyl residues were found to be present indicating the presence of arabinogalactan type II as side chain. The amounts of arabinose estimated in the linkage analysis procedure for the CHSS fraction were higher than found in the sugar composition analysis as reported in Table 1. About 42% of all arabinose were present as 1,5-linked which indicated the presence of linear arabinan side chains.

Compared to HBSS and CHSS, the DASS fraction contained less branched RG I as shown by the low levels of 1,4- and 1,2,4-linked rhamnosyl moieties. The majority of all arabinose present in the CHSS and DASS were present as 1,5-; 1,3,5- and 1,2,5-linked arabinosyl residues in the furanose form, which indicate the presence of 1,2 and 1,3 branched α-(1,5)-arabinans. The DASS fraction is relatively rich in branched arabinans in which the number of terminally linked arabinosyl residues fitted rather well with the number of branching points. Besides arabinan side chains, the DASS fraction contains a mixture of AG I and AG II structures as shown by the presence of both 1,4-linked and 1,3,6-linked galactosyl residues.

The CASS fraction is obviously rich in hemicelluloses such as xylan, mannan and glucan present as long linear 1,4-linked chains. Besides, xylose, galactose and fucose were also present as terminal residues and in combination with the presence of 1,4,6-linked glucosyl residues, this points to the presence of a xyloglucan.[23]

2.5. Molecular weight distribution

The Mw distribution of the polysaccharides in the different fractions obtained from okra AIS is shown in Figure 1. The Mw distribution pattern of HBSS showed only one population having a rather high Mw, while CHSS shows a much broader Mw distribution representing populations with molecular weights higher and lower than HBSS. Moreover, the Mw distribution pattern of the DASS is similar to the Mw distribution pattern of the CHSS. The CASS fraction that represented predominantly containing hemicellulosic polysaccharides showed one broad Mw distribution representing populations with lower Mw values than the other fractions. These trends are also reported for olives,[24] blue berries and black currents.[25] It should be mentioned that the ultrafiltration step of the extract as was performed using a 30 kDa membrane did remove possibly present low Mw material.

2.6. Pectin-rich fractions

The sugar (linkage) composition of the HBSS fraction indicates that the HBSS mainly contains RG I segments next to some HG with high acetyl levels representing uncommon structure for water-extracted polysaccharides. Therefore, anion-exchange chromatography was performed to obtain information about the homogeneity of HBSS. The structural features of the sub-fractions were determined and compared to pectin structures present in the CHSS fraction.

2.6.1. Anion-exchange chromatography

The homogeneity with respect to charge of the HBSS and CHSS fractions was determined using anion-exchange chromatography. The sample solution was applied to a DEAE Sepharose Fast Flow column, and the material which bound to the resin was eluted with a linear gradient of NaOAc buffer of pH 5. Figure 2 shows the DEAE anion-exchange chromatography patterns of the HBSS and CHSS samples. Almost all material eluted in one major peak at about 0.8 and 0.6 M NaOAc buffer for the HBSS and CHSS, respectively. The sugar composition of this major peak was determined after pooling the fraction and was found to be comparable to the starting material. The co-elution of all sugars in one single peak having the same sugar composition as the starting material indicateed that only 1 pectin population is present in both the HBSS and CHSS fractions.

2.6.2. Nuclear magnetic resonance

The pectin fractions were found to consist mainly of two structural elements, HG and RG I. The sugar composition and degree of acetylation analysis exhibited that the HBSS fraction contained mainly RG I and a high substitution with acetyl groups while the CHSS fraction contained mainly HG and less acetyl groups. To reduce the viscosity of the pectins to improve the quality of the NMR spectra, HBSS was digested with polygalacturonase and pec-

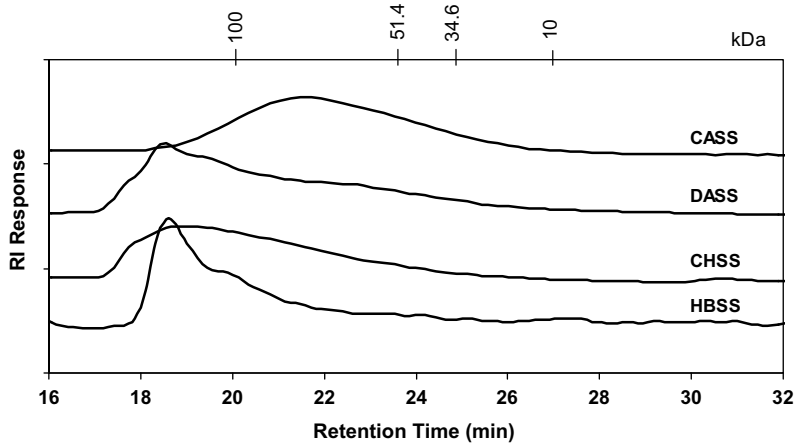

Figure 1. Molecular weight distributions of okra polysaccharide fractions (the molecular weight indications are based on pectin standards).

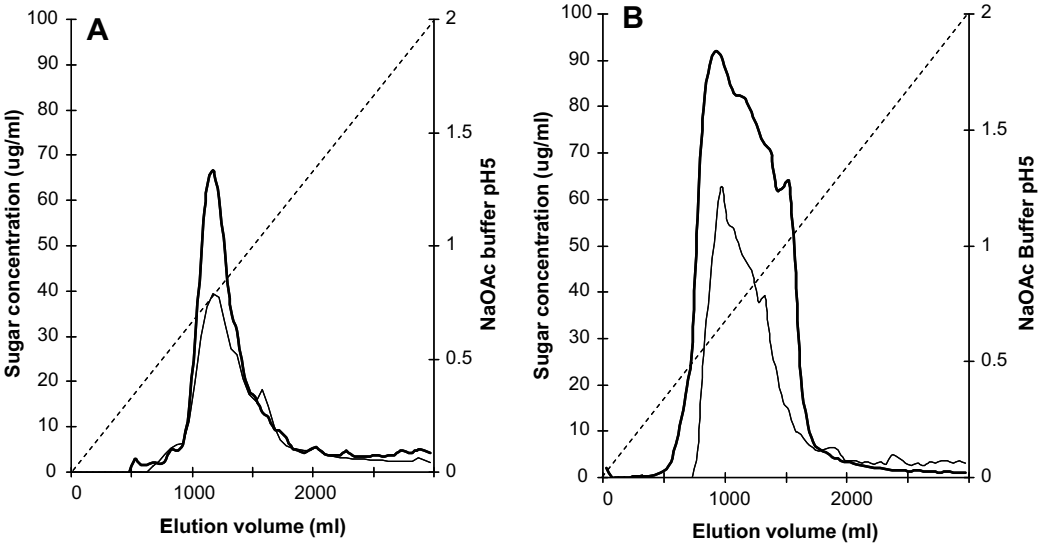

Figure 2. DEAE anion-exchange chromatography patterns of HBSS (A) and CHSS (B). Bold line: uronic acid, thin line: neutral sugars, dotted line: NaOAc gradient.

tin methyl esterase and CHSS was digested with polygalacturonase only. The digests were then dialysed, freeze-dried, dissolved in D_2O and then proton NMR spectra were recorded.

The spectra are shown in Figure 3. From the data obtained for HBSS PG/PME, it is obvious that the sample contains both unbranched α-1,2-linked rhamnose (1.25 ppm) and branched α-1,2,4-linked rhamnose (1.33 ppm); the chemical shifts typically found for H-6 of these rhamnosyl moieties and 1 or 2 anomeric signals between 5.22 and 5.25 ppm were assigned to rhamnosyl residues. The anomeric signals at 5.01 and 4.96 ppm in combination with signal at 4.42 ppm were assigned to H-1 and H-4 of galacturonic acid.[26–30]

Similar to the HBSS sample, also for PG treated CHSS 1,2-linked-, 1,2,4-linked-rhamnosyl units were found. In addition, several signals (signal in boxes 1, 2 and 3 in Fig. 3) belonging to galacturonic acid were found that these were derived from HG and RG I, and suggests the presence of substitution of acetyl group on both structural entities.[28–34]

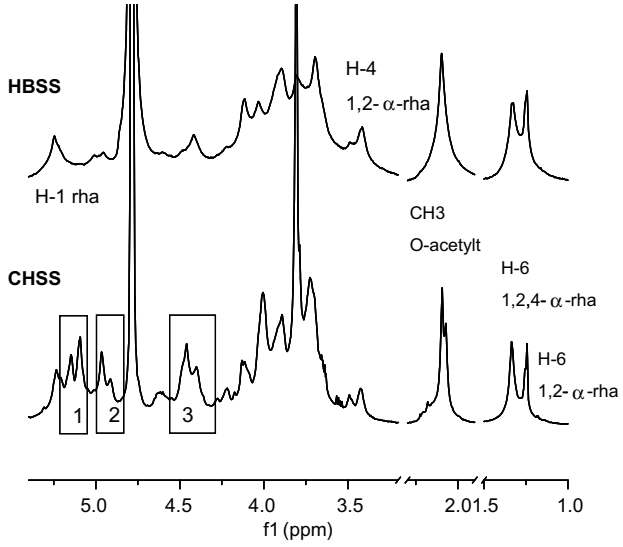

Figure 3. ^{1}H NMR spectra of ChSS PG and HBSS PG/PME; 1: galacturonic acid derived from HG or O-acetylated galacturonic acid; 2: galacturonic acid derived from RG I and non substituted; 3: galacturonic acid H-4.

Furthermore, the presence of several anomeric signals in region between 4.45 and 4.65 ppm of both samples indicated the presence of terminal galactose and 1,4-linked galactose within samples. These indicate that both samples contain short galactan side chains and are in agreement with the sugar composition and linkage analysis. Both samples also showed a signal between 2.10 and 2.20 ppm typically for an O-acetyl substituent. Lerouge et al.[30] found that when an *O*-acetyl group is attached to galacturonic acid at position O-2 or O-3, two extra signals appear within the anomeric region at 5.1 and 5.4 ppm. These signals are not present in the HBSS spectrum. The CHSS NMR spectra showed different galacturonic acid residues in combination with two major signals for *O*-acetyl at 2.10 and 2.07 ppm. This indicates that acetyl groups are linked to different positions of the galacturonic acid present in HG and RG I. Most reasonably for HBSS the acetyl should be linked to either a rhamnose or a galactose building block. However, it cannot be ruled out that also within CHSS other sugars are acetylated.

2.7. Hemicellulose-rich fractions

The sugar linkage composition demonstrated that the CASS fraction obviously consists of hemicelluloses. Generally, the hemicelluloses in plant cell wall are xyloglucans, xylans, mannans and/or arabinogalactans.[17] Therefore, to obtain more information about the structure of hemicellulose, the okra CASS fraction was incubated with specific enzyme and the digest subsequently analysed for the oligomers released.

2.7.1. Xyloglucan-specific *endo*-glucanase (XEG) degradation of okra CASS fraction

Xyloglucans represent a major hemicellulosic polysaccharide in the primary cell wall of many plants. In general, xyloglucan can be classified into three types, XXXG-type, XXGG-type and XXXX-type, which can be distinguished by the variation in the backbone substitution with xylose residues.[35,36] The high amounts of glucose and xylose found in okra CASS indicate the presence of xyloglucan. To obtain more information about this xyloglucan, this fraction was incubated with a xyloglucan-specific glucanase, and fragments released were analysed. This specific xyloglucanase[38] cleaves xyloglucans between the xylose-substituted glucose residues and the unsubstituted glucose residues releasing oligosaccharide building blocks from the xyloglucan.[35] Oligomeric building blocks were

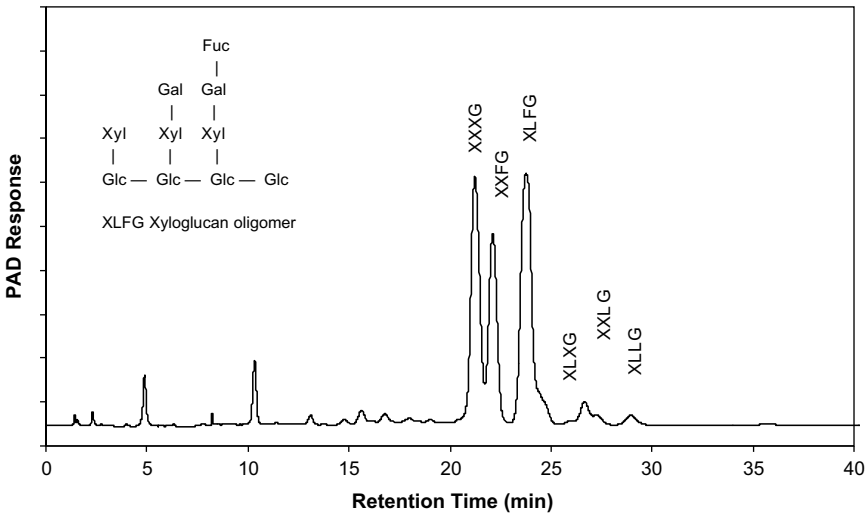

Figure 4. HPAEC pattern of okra CASS fraction after incubation with xyloglucan-specific *endo*-glucanase.

identified using xyloglucanase digests from potato and tamarind seeds,[37] and confirmed by MALDI-TOF mass spectrometry (results not shown).The HPAEC pattern (Fig. 4) of the digest shows the release of three main (XXXG, XXFG and XLFG) and three minor (XLXG, XXLG and XLLG) xyloglucan oligomers. These patterns are rather similar to the xyloglucan oligomers obtained from many plant cell wall,[39] and were also recently found for black currents.[40] These results indicated that the okra CASS fraction contained poly-XXXG type xyloglucan carrying both galactose and fucose substitutions which is generally found in dicotyledonous plants.[40]

2.7.2. *endo*-Xylanase degradation of okra CASS fraction

Besides xyloglucan, xylose could be presented as a constituent of another hemicellulosic polysaccharide existing in cell walls, for example, xylan. Xylans are present in monocotyledonous plants that are usually of the arabinoxylan type, while in the dicotyledonous plants often an acidic xylan almost without any arabinose substitution is found.[41,42] The okra CASS fraction was incubated with *endo*-Xylanase from *Aspergillus awamori* CMI 142717 (Xyl III belonging to GH family 11)[36] to obtain more information about the xylan structure present.

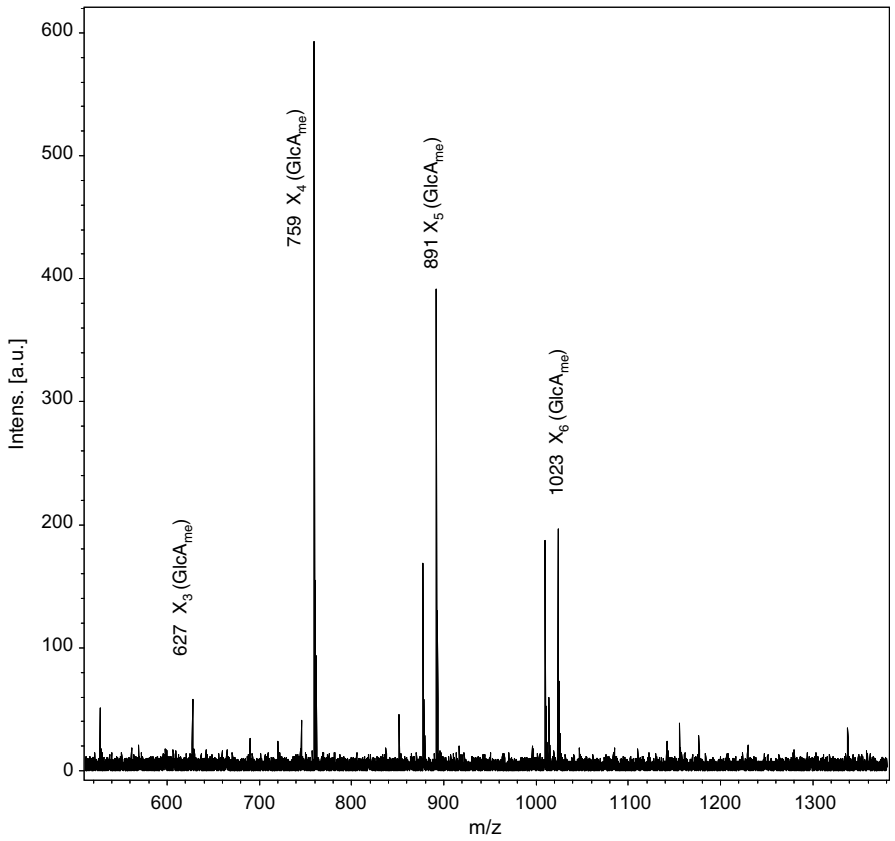

Figure 5. MALDI-TOF mass spectrum of the okra CASS fraction digest with Xyl III.

After 24 h incubation of the okra CASS fraction with Xyl III, the products released were analysed by HPAEC (data not shown), and compared with data found before for, for example, olive xylans.[36] From the HPAEC pattern, mainly xylose, xylobiose and xylotriose were found to be present, while arabinose-substituted xylan oligomers were only found as minor product in addition to some compounds which were retained longer by the anion-exchange. For additional information the digests were also analysed with MALDI-TOF MS. The spectrum (Fig. 5) shows the presence of series of xylo-oligomers substituted with one 4-*O*-methylglucuronic acid [$X_{3\text{-}7}(GlcA_{me})$]. The major MS signals were m/z of 759 and 891 corresponding to $X_4(GlcA_{me})$ and $X_5(GlcA_{me})$, respectively. The formation of these oligomers can be explained by the substrate specificity of the enzyme used since Xyl III is hindered by substitution of the xylan backbone, and can only cleave glycosidic linkages between two unsubstituted xylose residues which are not adjacent to singly or doubly substituted xylose residue.[24,43] Therefore, the xylan presented in okra CASS fraction is a 4-*O*-methylgluconoxylan which is generally found in the dicotyledonous plants such as olive[24] and also in hard wood such as eucalyptus wood.[44] Furthermore, the 4-*O*-methyl glucuronic acid groups seem to be distributed rather randomly over the xylan backbone.

In summary, sequential extraction of okra AIS yielded different polysaccharide populations. The sugar (linkage) composition showed that the galactose-rich pectic molecules are found in the three different extracts (HBSS, CHSS and DASS) which have different chemical structures. The HBSS fraction is a homogenous charged polymer and contains a regular structure which consists of mainly highly branched RG I with very short galactan side chains (1-2 galactose residues on average). The HBSS further contains high level of acetyl groups which, unexpectedly, as NMR analysis revealed that the acetyl groups are not attached to galacturonic acid units.

The CHSS fraction is also a homogeneously charged polymer and contains mainly HG and some RG I with galactan side chains consisting of on average 2 to 3 galactose residues. Also more heterogeneous side chains of arabinan and AG II are present. Both HBSS and CHSS showed one population on anion-exchange chromatography indicating different ratios of homogalacturonan and rhamnogalacturonan in HBSS and CHSS. The NMR studies of PG-treated HBSS and CHSS showed the presence of acetyl groups that are linked to other sugar residues present like rhamnose and/or galactose than galacturonic acid. Moreover, the DASS fraction contains even more complex pectins with arabinans, AG I and AG II, as neutral side chains.

Besides pectic material, the okra AIS also contains hemicellulosic polysaccharides which were mainly present in the CASS fraction. The hemicellulosic material in this fraction was found to consist of mainly XXXG-type xyloglucan and 4-methylglucuronoxylan.

To obtain more information about the position of the acetyl groups and the length of galactan side chains, more enzyme degradation studies of the polymer and NMR and MS characterisations of oligomers will be performed.

3. Material and methods

3.1. Material

The soft and mature okra pods, *A. esculentus* (L.) Moench (5–10 cm in length), were grown and collected at the local market in June 2005, Thailand.

3.2. Isolation of alcohol-insoluble solid

After removal of the seeds, the okra pods were sliced and homogenised two times with 70% (v/v) aqueous ethanol at room temperature. After filtration, the insoluble residues were combined and washed with two volumes of chloroform/methanol (1/1, v/v) with gentle stirring for 30 min to remove low molecular weight (coloured) compounds.[45] After filtration, the filtrates were washed with acetone and air dried (alcohol-insoluble solids, AIS).

3.3. Sequential extraction of okra AIS

Okra AIS (20 g) were sequentially extracted according to Vierhuis et al.[24] with 600 mL of the following extractants; 0.05 M sodium acetate buffer at pH 5.2 and 70 °C (hot buffer soluble solids, HBSS), 0.05 M EDTA and 0.05 M sodium acetate in 0.05 M sodium oxalate at pH 5.2 and 70 ° C (chelating agent soluble solids, CHSS), 0.05 M sodium hydroxide at 0 °C and 20 mM $NaBH_4$ (diluted alkali soluble solids, DASS), and 6 M sodium hydroxide at 0 °C and 20 mM $NaBH_4$ (concentrated alkali soluble solids, CASS). The extraction was continually performed until the total sugar content of the last supernatant was lower than 40 μg/mL, which was determined by phenol–sulfuric acid assay.[46] After each extraction, solubilised polymer was separated from the insoluble residue by centrifugation (19,000*g* for 25 min). The supernatants were ultra filtrated through a 30 kDa membrane (A/G Technology Corporation) and freeze-dried. The final residues were dialysed and freeze-dried.

3.4. Analytical methods

3.4.1. Total neutral sugar and uronic acid content

The total neutral sugar content and uronic acid content were determined colorimetrically by automated orcinol/sulfuric acid assay[47] and *m*-hydroxydiphenyl assay,[48–50] respectively, using an auto-analyser (Skalar Analytical BV, Breda, The Netherlands). Galactose and galacturonic acid were used as a standard. Corrections were made for the interference of uronic acid in the samples. Distinction between galacturonic acid and glucuronic acid was made by high performance anion-exchange chromatography (HPAEC, Dionex, USA) after methanolysis according to De Ruiter et al.[51]

3.4.2. Sugar composition

The neutral sugar composition of okra AIS was determined by gas chromatography[52] using inositol as internal standard. The samples were submitted to a prehydrolysis treatment with 72% w/w sulfuric acid at 30 °C for 1 h followed by a hydrolyses step using 1 M sulfuric acid at 100 °C for 3 h. The sugars were converted to their alditol acetates and analysed by GC according to Hilz et al.[25]

For the obtained fractions, after drying at 40 °C under vacuum over P_2O_5, the samples were hydrolysed with 2 M HCl in dry methanol at 80 °C for 16 h and followed by 2 M TFA at 121 °C for 1 h. The monomers were analysed by using high performance anion-exchange chromatography (HPAEC, Dionex, USA) equipped with the (2 × 250 mm) CarboPac PA 1 column (Dionex, USA) and post column (Dionex, USA) addition. Millipore water, 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH with a flow of 0.3 mL/min at 20 °C were used as eluent, and 0.5 M NaOH with a flow of 0.1 mL/min at 20 °C was added to post column for allowance of the PAD detection.

The following gradient was applied using a flow of 0.3 mL/min at 20 °C of NaOH: 0–30.0 min, 0 mM; 30.0–30.1 min, 0–100 mM; 30.1–50.0 min, 100 mM. The simultaneous gradient of NaOAc was 0.0–30.1 min, 0 mM; 30.1–45.0 min min, 0–400 mM; 45.1–50.0, 1000 mM. Millipore water was used from 0.0 to 30.0 min. For the post column, 0.5 M NaOH with a flow of 0.1 mL/min was used during 0.0–30.1 min. After each run the column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, for 8 min with 0.1 M NaOH and subsequently equilibrated with water for 15 min.[53]

3.4.3. Absolute configuration

The configuration of all sugars present was analysed by gas chromatography, and methyl-α-D-galactopyranoside was used as

internal standard. The sample was methanolysed with 1 M HCl in dry methanol. After conversion to their corresponding (−)-2-butyl glycosides and trimethylsilation, the trimethylsilated (−)-2-butyl glycosides were analysed by GC-FID.[54]

3.4.4. Degree of acetylation and methyl esterification

The degree of acetylation and methyl esterification of samples were determined after saponification with 0.4 M sodium hydroxide in isopropanol/water (50/50 v/v) by using Thermo Finnigan (USA) high performance liquid chromatography equipped with Aminex HPX 87H column (Bio-Rad, USA).[55] The elution took place at 40 °C with 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min. The degree of acetylation and methyl esterification were calculated as moles of acetic acids and methanol per 100 moles of galacturonic acid, respectively.

3.4.5. Protein content

Protein content was determined by the combustion method[56] with a Thermo Quest NA 2100 Nitrogen and Protein Analyser (Interscience, The Netherlands). D-Methionine was used as external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

3.4.6. High performance size exclusion chromatography (HPSEC)

High performance size exclusion chromatography (HPSEC) was performed on a Thermo Separation Products (USA) HPLC, equipped with three TosoH Biosep-TSK-Gel G columns (Methyacrylate resin) in series (7.8 mm × 30 cm, 4000PW_{XL}–3000PW_{XL}–2500PW_{XL}) in combination with a PW_{XL}-guard column (TosoH, Japan). Samples (5 mg/mL; except for HBSS: 1 mg/mL) were eluted at 30 °C with 0.2 M sodium nitrate at a flow rate of 0.8 mL/min.[57] The column effluent was monitored using a reflective index detector (Shodex SE-61, Showa Denko K.K., Japan). Calibration was done using pectins having known molecular weight.[20]

3.4.7. Glycosidic linkage analysis

The glycosidic linkage composition of each sample was analysed as described by Hakomori[58] and modified by Verhoef et al.[59] The partially methylated alditol acetates were analysed by GC-FID and GC–MS according to Verhoef et al.[59]

3.4.8. Enzymatic degradation

3.4.8.1. Pectin degradation. The HBSS fraction was dissolved (3 mg/ml) in 50 mM NaOAc buffer, at pH 5, and incubated with 0.016 units of polygalacturonase from *A. aculeatus* and 1.08 units of pectin methyl esterase from *A. niger*.[20] The CHSS fraction (4 mg/mL) was incubated with 0.024 units of polygalacturonase. The incubations were performed at 40 °C for 24 h, and the digests were subsequently heated for 5 min at 100 °C to inactivate the enzyme. The digests were dialysed by Centricon centrifugal filter devises with 3 kDa cut off at 1500*g* for 30 min and then, freeze-dried.

3.4.8.2. Xyloglucan-specific *endo*-glucanase (XEG) degradation. The CASS fraction was dissolved (5 mg/mL) in 50 mM NaOAc buffer, at pH 5, and incubated with 2.3 units of xyloglucan-specific *endo*-glucanase from *A. aculeatus*[38] at 40 °C for 16 h. The digest was heated at 100 °C for 5 min to inactivate the enzyme.

3.4.8.3. *endo*-Xylanase III degradation. The CASS fraction was dissolved (10 mg/mL) in 150 mM NaOAc buffer, at pH 5, and incubated at 40 °C for 24 h with 0.09 units of *endo*-xylanase III from *A. awamori*.[36] The enzyme was inactivated by heating the digest for 5 min at 100 °C.

3.4.9. HPAEC of oligosaccharides

For the determination of xyloglucan oligomers, enzyme digests were analysed by using a 4 × 250mm CarboPac PA 100 column (Dionex, USA) with pulsed amperometric detection. Gradients of NaOH and NaOAc with a flow of 1 mL/min were used to elute the oligomer according to Vincken et al.[37]

For the determination of digest from *endo*-xylanase III, the (2 × 250 mm) CarboPac PA 1 column (Dionex, USA) with pulse amperometric detection was equilibrated with 100 mM NaOH. Gradients of NaOH and NaOAc were used simultaneously to elute the oligomers according to Vierhuis et al.[36]

3.4.10. Matrix–assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) analysis

MALDI-TOF MS analysis was performed on Ultra flex instrument (Bruker Daltonics, Germany) with a Nitrogen 337 nm laser beam. The data were collected from average of 200 shots with the lowest laser energy (35%). The digest sample was desalted with resin (AG 50W-X8 Resin, H^+ form; Bio-Rad, USA). A small spatula of Dowex resin was added to 10 μL of sample solution, mixed and centrifuged for 5 min at 13,000*g*. One microliter of matrix solution, 10 mg/mL of 2,5-dihydroxybenzoic acid in millipore water (Milli-Q Gradient A-10, USA) was placed on a MALDI-TOF plate together with 1 μl of sample solution and dried under a constant warm air.

3.4.11. Anion-exchange chromatography

Anion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column (50 × 2.6 cm, Amersham Bioscience, Sweden) using an akta explorer system (Amersham Biosciences, Sweden). The sample (280 ml; 0.5 mg/mL) was loaded to the column (10 mL/min). After loading with sample, the column was washed with water for 1 column volume (250 mL, 50 mL/min) and eluted successively with a linear gradient of 0–2 M of NaOAc buffer, pH 5, within 10 column volumes. Finally the column was washed with 2 column volumes of 1 M NaOH. During elution with NaOAc buffer, fractions of 50 mL were collected as well as the column washed and analysed for neutral sugar and uronic acid content as described. The alkaline fractions were neutralised directly by adding acetic acid. After pooling appropriate fractions, pools were dialysed and freeze-dried.

3.4.12. ^{13}C and ^{1}H nuclear magnetic resonance (NMR)

Prior to NMR analyses, the sample was dissolved in 5 mM NaOAc, pH 5, to set the pD = pH and freeze-dried. Then the sample was dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved again in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 25 °C on a Bruker AV-600 spectrometer equipped with a cryoprobe located at Biqualys, Wageningen. Chemical shifts were expressed in ppm relative to internal acetone: $\delta = 2.225$ ppm for ^{1}H. The 1D ^{1}H proton spectra were recorded at 600.13 MHz using 64 scans of 8192 data points and a sweep width of 3000 Hz.

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