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Okra pectin contains an unusual substitution of its rhamnosyl residues with acetyl and alpha-linked galactosyl groups

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ABSTRACT

The okra plant, *Abelmoschus esculentus* (L.) Moench, a native plant from Africa, is now cultivated in many other areas such as Asia, Africa, Middle East, and the southern states of the USA. Okra pods are used as vegetables and as traditional medicines. Sequential extraction showed that the Hot Buffer Soluble Solids (HBSS) extract of okra consists of highly branched rhamnogalacturonan (RG) I containing high levels of acetyl groups and short galactose side chains. In contrast, the CHelating agent Soluble Solids (CHSS) extract contained pectin with less RG I regions and slightly longer galactose side chains. Both pectic populations were incubated with homogeneous and well characterized rhamnogalacturonan hydrolase (RGH), endo-polygalacturonase (PG), and endo-galactanase (endo-Gal), monitoring both high and low molecular weight fragments. RGH is able to degrade saponified HBSS and, to some extent, also non-saponified HBSS, while PG and endo-Gal are hardly able to degrade either HBSS or saponified HBSS. In contrast, PG is successful in degrading CHSS, while RGH and endo-Gal are hardly able to degrade the CHSS structure. These results point to a much higher homogalacturonan (HG) ratio for CHSS when compared to HBSS. In addition, the CHSS contained slightly longer galactan side chains within its RG I region than HBSS. Matrix-assisted laser desorption ionization-time of flight mass spectrometry indicated the presence of acetylated RG oligomers in the HBSS and CHSS enzyme digests and electron spray ionization-ion trap-mass spectrum showed that not only galacturonosyl residues but also rhamnosyl residues in RG I oligomers were O-acetylated. NMR spectroscopy showed that all rhamnose residues in a 20 kDa HBSS population were O-acetylated at position O-3. Surprisingly, the NMR data also showed that terminal α-linked galactosyl groups were present as neutral side chain substituents. Taken together, these results demonstrate that okra contained RG I structures which have not been reported before for pectic RG I.

1. Introduction

The okra plant, *Abelmoschus esculentus* (L.) Moench, family Malvaceae, is cultivated for its immature pods. The immature pod contains a thick and slimy mucilage. The okra pod is used as a vegetable and as a thickening agent for soups and stews.[1] In addition, it is used in traditional medicine as a dietary meal in the treatment of gastric irritations[2] and dental diseases[3] due to its high content of polysaccharides. Physiological studies showed that the okra polysaccharides (OKPs) had hypoglycemic properties and lower plasma cholesterol levels in rats.[2] In food applications, the OKP was a suitable egg-white substitute[4] and a fat substitute in cookies and in chocolate frozen dairy dessert.[5,6]

The OKP was an acidic polysaccharide which consists of galactose, rhamnose, and galacturonic acid.[7] The OKP has been reported to have a backbone repeating units of -4-α-Gal*p*A-(1,2)-α-L-Rha*p*-1- dimers and, on average, dimeric side chains of β-Gal*p*-(1,4)-β-Gal*p*-1.[8] The acetyl content was about 5.5% w/w.[8] Sequential extraction of okra cell wall material showed that okra contained different types of polysaccharides, that is, pectins, xyloglucans, xylans, and celluloses.[9] The Hot Buffer Soluble Solids (HBSS) fraction was the main fraction which contained mainly rhamnogalacturonan (RG) I with short galactose-containing side chains. The CHelating agent Soluble Solids (CHSS) fraction mainly contained homogalacturonan (HG) and slightly longer galactose-containing side chains connected to the RG I segments. In addition, the degree of acetylation of the galacturonic acid moieties for HBSS was relatively high. About 58 moles of acetyl groups were present for every 100 galacturonic acid moieties. NMR studies of HBSS polymer after incubation with polygalacturonase (PG) and pectin methyl esterase

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(PME) showed that the majority of the acetyl groups were not linked to galacturonosyl residues and substitution to other sugars such as rhamnosyl residues had to be considered.[9] In this study, we provide further structural information of the HBSS and CHSS fractions by degradation studies using homogeneous and well-characterized enzymes. Furthermore, NMR spectroscopy and mass spectrometry were used to indicate the position of the acetyl groups and details concerning the galactose side chains in both samples.

2. Results and discussion

The okra AIS was sequentially extracted with hot buffer and chelating agent. The sugar composition (Table 1) showed that the HBSS contained mainly rhamnogalacturonan (RG) I (85%) with short galactose-containing side chains and hardly any homogalacturonan (HG). The CHSS contained mainly HG and some RG I (24%) with more galactose and arabinose present in side chains.[9] Moreover, the configuration of all sugar compositions present in HBSS and CHSS was in D-configuration except of rhamnose, which was in the L-configuration.[9] The degree of acetylation (DA) was quite high in HBSS (58%) which is in agreement with levels found, example for apple RG I.[10] However, NMR studies of HBSS polymer showed that no acetyl groups substitution was present on the galacturonosyl residues of RG I backbone which is normally the case.[9] The DA of CHSS was relatively low and NMR studies of CHSS polymer showed that acetyl may be linked to galacturonic acid as well as to some other sugar residues.

2.1. Enzymatic degradation of okra pectins

2.1.1. Polygalacturonase treatment

To characterize the HG segments within the extract sample, the samples were incubated with endo-polygalacturonase (PG) from *Aspergillus aculeatus*. This enzyme can cleave the α-1,4-D-galacturonosyl linkages of the HG-backbone by hydrolysis although PG action is hindered by the presence of methyl esters and acetyl groups.[11]

HPSEC of the PG digests of HBSS and saponified HBSS (sHBSS) (data not shown) showed a slight shift of the high Mw pectin population to lower Mw values. Some minor quantities of oligomers were released as well. Analyses by HPAEC showed the presence of monomers, dimers, and trimers of galacturonic acid (GalA) accounting only for about 0.1% (HBSS) and 3% (sHBSS) of all GalA residues present.

The HPSEC patterns of PG-treated CHSS (Fig. 1A) indicated that approximately 37% of CHSS remained as high Mw material, and 52% and 11% were found as intermediate and low Mw fragments, respectively. For sCHSS, about 50% of the polymer was present as low Mw fragments and no intermediate fragments were observed (Fig. 1B). The (limited) action of PG toward HBSS and CHSS confirms the relative abundance of HG segments in the samples and also reflects the methyl esterification in the CHSS sample.

2.1.2. Treatment with galactose releasing enzymes

To obtain more information about the length of the galactose-containing side chains, the samples were incubated with endo-Galactanase (endo-Gal) from *A. aculeatus*. This enzyme cleaves the 1,4 linkage between β-linked galactosyl residues within galactan chains and releases mono-galactose and galactose oligomers.[12]

The HPSEC patterns of HBSS and sHBSS after treatment with endo-Gal showed no shift of the polymer to low Mw material and no oligomers were released as indicated by HPAEC (results not shown). Only 5% of all galactose present was released by endo-Gal in HBSS and sHBSS. For CHSS and sCHSS, endo-Gal digest showed a minor shift to lower Mw values and HPAEC analysis showed a release of mono- and dimeric galactose representing about 10% (CHSS) and 15% (sCHSS) of all galactosyl residues present. These observations confirm our earlier indications for the presence of short galactan side chains within HBSS and CHSS,[9] mostly resistant against enzyme action.

Since endo-Gal showed only a limited action toward the HBSS fraction, we also studied the activity of α and β galactosidases for their ability to release galactose. Both enzymes were hardly able to remove galactosyl residues from the HBSS fraction.

2.1.3. Rhamnogalacturonan hydrolase (RGH) treatment

To gain information about the RG I structure, the samples were incubated with RGH from *A. aculeatus*. This enzyme acts exclusively on RG I[11] and is unable to split the RG I backbone in case of substitution with long galactose side chains and when acetyl groups are present in the backbone.[13]

The HPSEC patterns of the HBSS (Fig. 2) after incubation with RGH showed that about 20% of the HBSS polymer remained as high Mw material and 80% of the HBSS polymer was shifted to medium or low Mw material. The HPSEC pattern of sHBSS digests showed only 2 populations of which the <10 kDa Mw fraction was most dominant (66% of the sHBSS digest). The enzymatic degradation of sHBSS by RGH from sHBSS confirmed that the HBSS was indeed

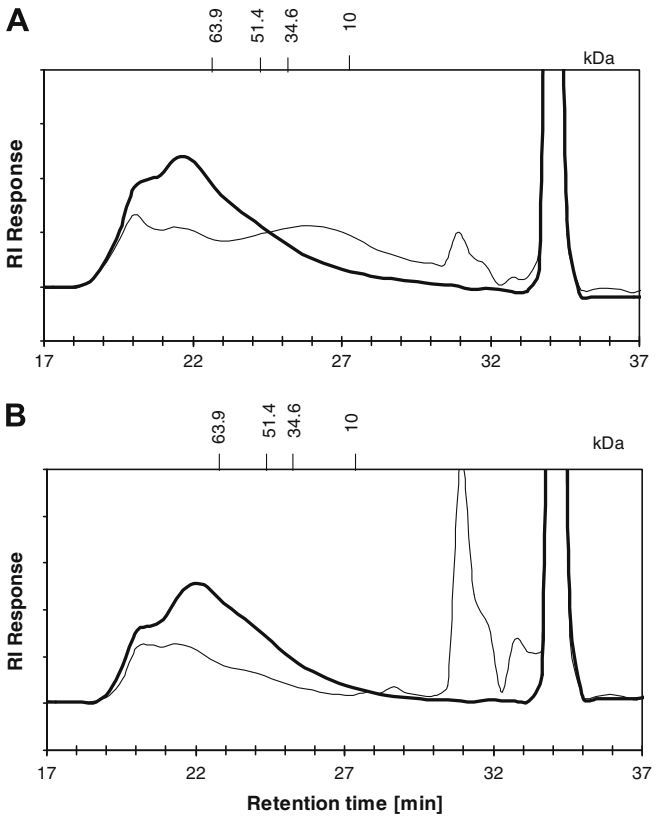

Figure 1. HPSEC elution patterns of CHSS (A) and sCHSS (B). Bold line: before incubation, thin line: after incubation with PG (the molecular weight indication is based on pectin standards).

Table 1
Sugar composition (mol %) of HBSS and CHSS fractions obtained from okra AIS[9]

	Rha	Ara	Gal	Glu	GalA	GlcA	DM[a] (%)	DA[a] (%)	Total sugar[b]
HBSS	26	0	34	1	35	3	24	58	90
CHSS	14	3	17	1	63	2	48	18	86

[a] Moles methanol or acetyl per 100 moles of galacturonic acid.
[b] Gram qualities per 100 g of fraction.

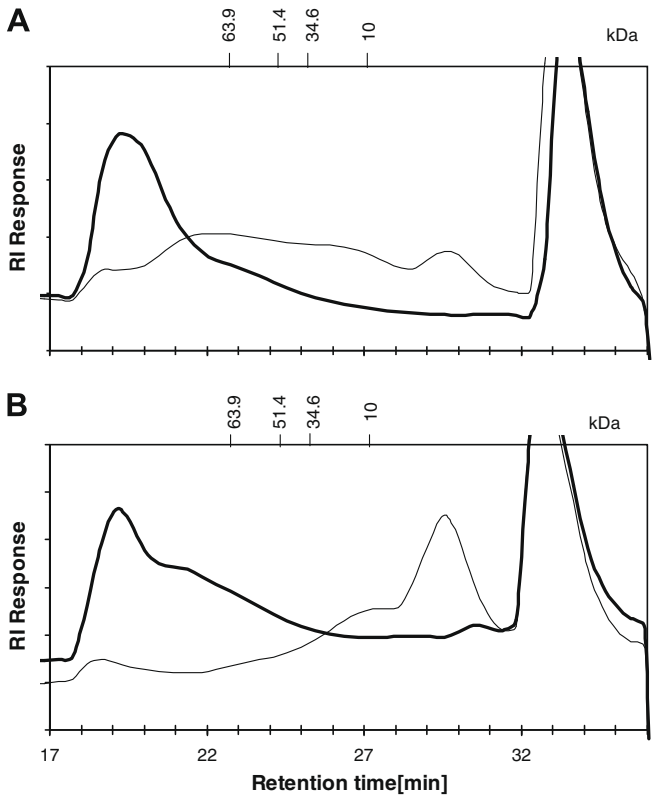

Figure 2. HPSEC elution patterns of HBSS (A) and saponified HBSS (B) before (bold line) and after (thin line) incubation with RGH (the molecular weight indication is based on pectin standards).

built up by RG I segments with rather short side chains and that acetyl groups were present partly inhibiting enzyme action toward HBSS.[14] This indicated that acetyl free regions were sufficiently present in RG backbones of HBSS to allow RGH to act. Another option we considered was that (some of) the acetyl groups may not be substituted to the galacturonosyl residues in the RG I backbone as indicated by the NMR results and so may not hinder the enzyme.[9]

Comparison of the HPAEC elution patterns of the HBSS RGH digest (Fig. 3) with those obtained for RGH-degraded RG I from apple[15] confirmed that HBSS digest indeed contained the typical RG oligomers. The RGH digest from HBSS differed slightly from the sHBSS digest in which less large oligomers were observed. The

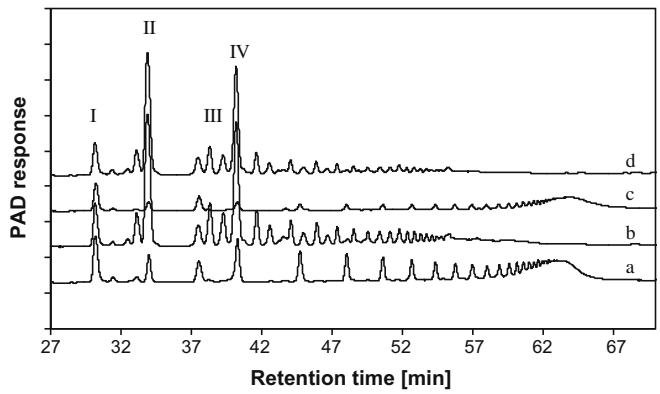

Figure 3. HPAEC elution patterns of sample after incubation with RGH: (a) HBSS, (b) saponified HBSS, (c) non-saponified CHSS, and (d) saponified CHSS (I: Rha_2GalA_2; II: $Rha_2GalA_2Gal_2$; III: Rha_3GalA_3; IV: $Rha_3GalA_3Gal_2$).

HPAEC pattern showed that RGH can release $Rha_2GalA_2Gal_2$ (34 min) and $Rha_3GalA_3Gal_2$ (40 min) and these oligomers represented 40% and 80% of released oligomers for the HBSS and sHBSS, respectively. These results demonstrate that sHBSS has much more RGH cleavable sites probably due to the hindering effect of acetyl groups in HBSS.

MALDI-TOF MS was employed to obtain mass and composition of the oligomers and established the presence and site of substitution of acetyl groups. The MALDI-TOF mass spectra of the HBSS and sHBSS digests are shown in Figure 4. The major RG oligomers released within the HBSS mass spectrum correspond to $(RhaGalA)_{2-3}$ and Rha_2GalA_3 oligomers, while the major fragments found in the sHBSS mass spectrum were $(RhaGalA)_2Gal_{2-3}$. The presence of Rha_2GalA_3 in RGH-HBSS digests was probably due to some contamination of RGH with RG-rhamnohydrolase which removed rhamnose from the non-reducing end of the RG segments.[15,16]

The mass spectrum of the HBSS digest showed clearly that the acetylated oligomer fragments $Rha_1GalA_1Gal_1Ac_3$, and $Rha_2GalA_2Ac_1$ were predominantly present while $Rha_2GalA_2Gal_2Ac_{1-2}$ and $Rha_2GalA_2Gal_3Ac_3$ were only minor products. So far it has never been reported that RGH is able to degrade acetylated RG I.[13] This information together with the NMR data of the HBSS polymeric fragments gave rise to doubt about the precise location of acetyl groups within okra RG I.[9]

The HPSEC patterns of RGH treated CHSS and sCHSS showed that about 54% of the sCHSS polymer shifted to low Mw fragments while in the non-alkali treated CHSS polymer only a small part of the molecules shifted to low Mw (data not shown). This finding revealed that RGH hardly degrades CHSS in contrast to the rather good degradation of sCHSS. Moreover, the HPAEC patterns of both samples (Fig. 3) showed that the released RG oligomers correspond to $Rha_2GalA_2Gal_2$ and $Rha_3GalA_3Gal_2$, although the level of oligomers released is quite different for CHSS and sCHSS. The MALDI-TOF mass spectra showed the presence of Rha_2GalA_2 and $Rha_2GalA_2Gal_2$ in CHSS and sCHSS digests, next to the minor fragments $Rha_2GalA_2Gal_4$ and $Rha_3GalA_3Gal_{4-5}$ in sCHSS (data not shown). In conclusion, also the RG I in sCHSS consists of the typical RhaGalA repeats. However, the preference of RGH to act on sCHSS rather than on CHSS pointed to the presence of acetylation on the galacturonic acid residues within RG I. The presence of small amounts of acetylated oligomers indicate that only a minor part of the acetyl groups may be located on other sugar residues as is the case in HBSS RG I.

2.2. The position of acetyl groups in the RG I backbone

The possible hindrance of RGH by acetyl groups was verified by using rhamnogalacturonan acetyl esterase (RGAE) known to remove the acetyl groups from RG I extracted from apple which contained similar amount of acetyl groups. RGAE was unable to release acetyl groups from HBSS (data not show) but was active toward CHSS confirming our conclusions stated above.

2.2.1. Electron spray ionization ion trap mass spectrometry

Electron spray ionization ion trap mass spectrometry (ESI-IT-MS) was performed to get an insight in the structure of RG oligomers obtained after treatment of sHBSS with RGH. The MS^2 mass spectrum of the ion with mass to charge ratio (m/z) of 1171, corresponding to a $Rha_2GalA_2Gal_3$ oligomer, is shown in Figure 5A. The parent ion peak $m/z = 1171$ was fragmented to $m/z = 863$ (releasing of Rha-Gal fragment) and 699 (releasing of Gal-Rha-GalA fragment), indicating that one galactosyl residue was attached to one rhamnosyl residue. In addition, the removal of Gal_2Rha_1 fragment ($m/z = 701$) and $Gal_2Rha_1GalA_1$ fragment ($m/z = 507$) in the MS^2 spectrum of $m/z = 1171$ pointed to two galactosyl residues linked to one rhamnosyl residue. The possible structure of oligomers with

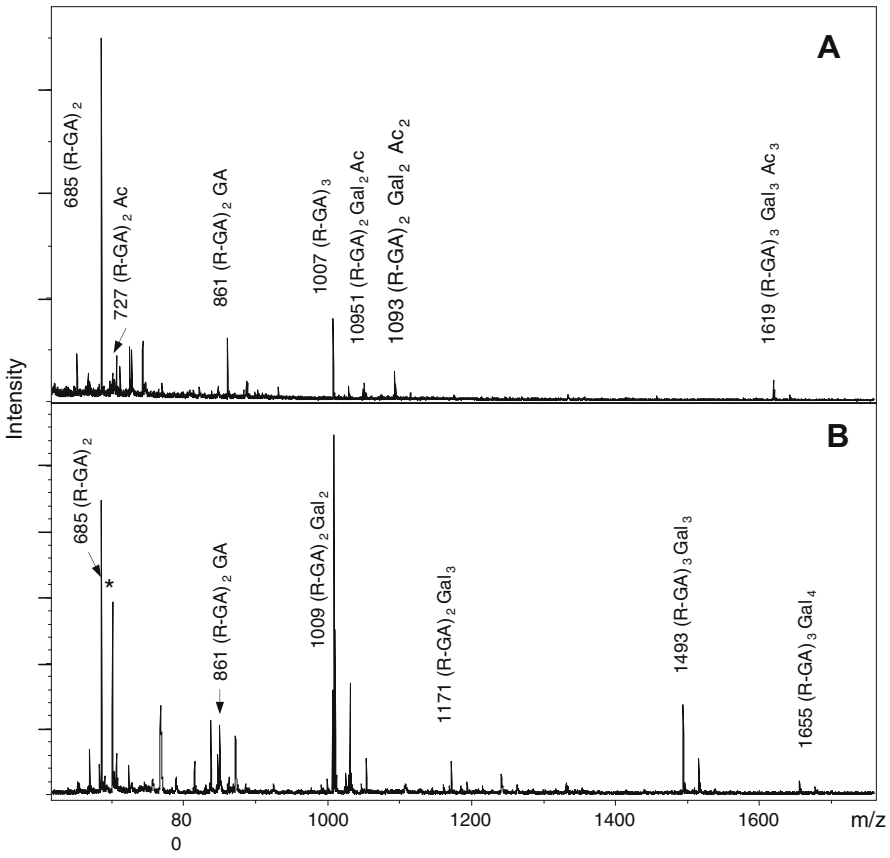

Figure 4. MALDI-TOF mass spectra of non-saponified HBSS (A) and saponified HBSS (B) after treatment with RGH (all ions are present as Na adduct except *: present as K adduct).

m/z = 1171 is shown in the right top corner of Figure 5A. However, it was not possible to make a difference between a side chain of two galactoses or two galactoses, both attached to the rhamnose at positions O-3 and O-4.

Also, the presence of fragment of m/z = 863 (leaving of a Gal_2Rha_1 fragment) and 699 (leaving of a $Gal_2Rha_1GalA_1$ fragment) in the MS^2 spectrum of m/z = 1333 representing $Rha_2GalA_2Gal_4$ (Fig. 5B) confirmed that two galactosyl residues are substituted to one single rhamnosyl residue. Fragments representing the removal of Rha_1Gal_1 (m/z 1025) and Rha_1Gal_3 (m/z 863) were only found as minor fragments in the MS^2 spectrum of m/z = 1333. The possible structure of main oligomers with m/z = 1133 is shown in the right top corner of Figure 5B. Therefore, the main RG oligomers released from sHBSS by RGH consist of a Rha-GalA repeat with galactose dimers linked to the rhamnose moiety, although rhamnoses without, with one or with three galactose units are also present in minor amounts. Linkage analysis already pointed out[9] that galactoses may be linked to each other.

To locate the acetyl groups within the HBSS RGH oligomers, ESI-IT-MS^n was performed. The ESI-IT-MS^2 spectrum of ions peak 729 (^{18}O-labeled $Rha_2GalA_2Ac_1$) is shown in Figure 6A and the most apparent fragments were m/z = 583 and 533 which were formed after removal of rhamnose and ^{18}O-labeled GalA, respectively. The MS^3 spectrum of m/z = 583 showed the leaving of $GalA_1Ac_1$ (m/z = 327 and 345) pointing out that an acetyl group was located on the galacturonosyl residue. Unexpectedly, the MS^3 spectrum of m/z = 533 ($Rha_2GalA_1Ac_1$) showed the release of Rha_1Ac_1 (m/z = 327 and 345) suggesting that an acetyl group was linked to a rhamnosyl residue. From this data, it is concluded that the oligomers with a m/z of 727/729 represent a mixture of different isomers with an acetyl group either on the GalA or Rha moieties. This finding was confirmed for ion peak m/z 1093 (^{18}O-labeled $Rha_2GalA_2Gal_2Ac_2$) also indicating that acetyl could be linked to either the GalA or Rha residues. Fragments with an additional loss of one water (−18) refer to different O-glycosyl fragmentation[17] while the loss of a second water molecule refers to a rearrangement of the galacturonosyl residues.

From the MS^2 and MS^3 spectra of HBSS RGH oligomers, it can be derived that rhamnosyl residues were O-acetylated. These findings might explain the activity of RGH and RGAE toward HBSS as well as the fact that the acetyl groups attached to the rhamnosyl residues cannot be removed by RGAE but do not hinder RGH either. Some acetyl groups were, however, located on galacturonosyl residues hindering RGH, explaining the different digestion of HBSS and sHBSS. However, MS is not a very reliable technique to quantify the acetylated oligomer since esters are known to have a positive effect on fragmentation efficiency.

2.2.2. Nuclear magnetic resonance of okra HBSS

To confirm the position of the acetyl groups within the HBSS on both the rhamnose and GalA moieties and to enable some quantitation, NMR was performed on a 20 kDa RGH HBSS fragment obtained by preparative SEC over Sephacryl 500 and Sepharcryl 300 (Fig. 7) after RGH digestion in order to overcome the high viscosity of the intact polymer.

Within the anomeric region of the ^{1}H NMR spectrum of recorded five major sugar moieties of a 20 kDa RGH HBSS fragment, 1,2,4-linked α-Rha*p* (A), 1,4-linked α-Gal*p*A (RG I) (B),[16,18–20] t-α-Gal*p* (C),[21,22] 1,4-linked Gal*p* (D) and t-β-Gal*p* (E)[18,23] could be observed (Table 2). In general, the spectra underline the regular structure of okra RG I being a highly branched rhamnogalacturonan with only one or two galactose units as side chains. A signal

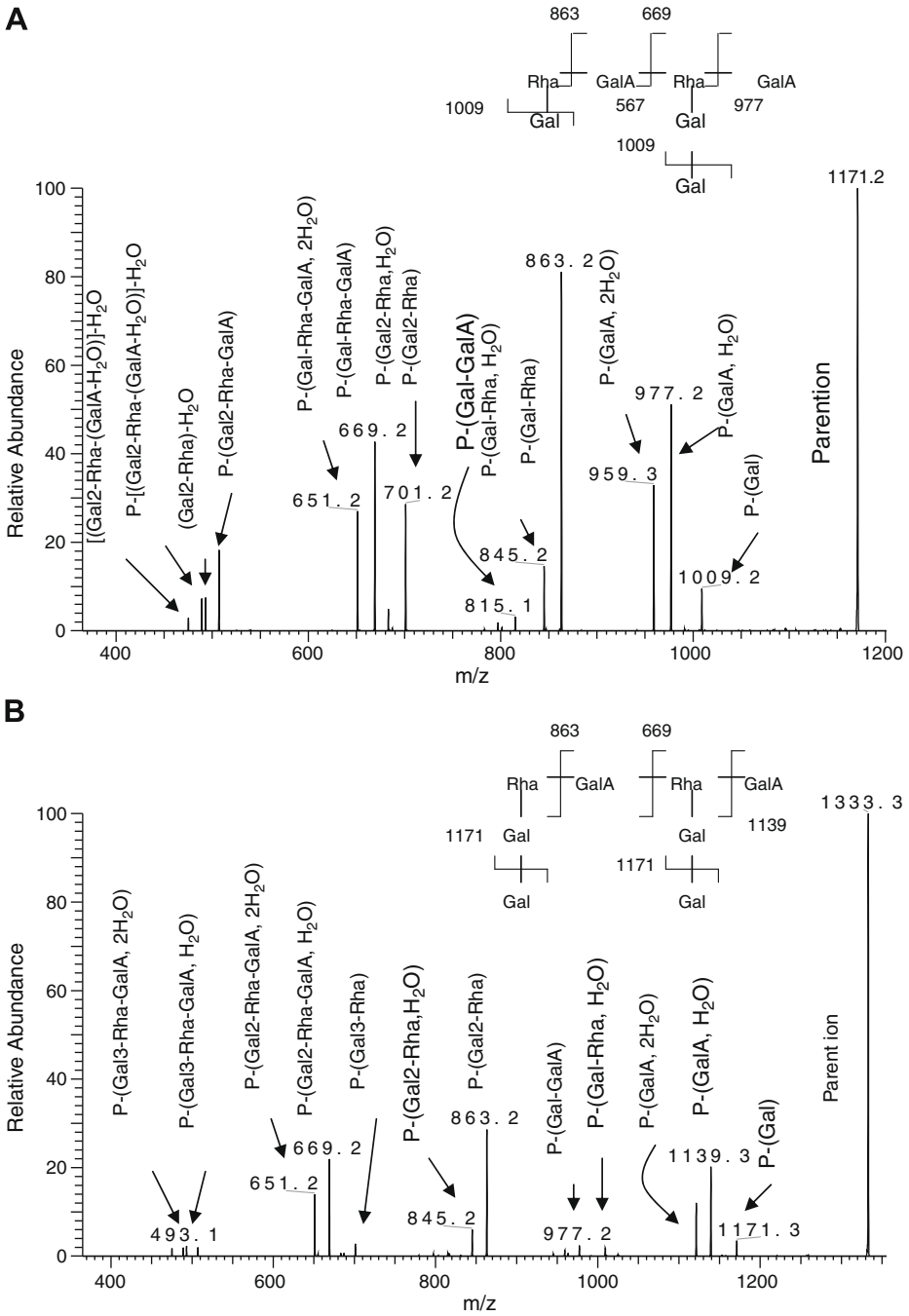

Figure 5. The MS^2 spectrum of $Rha_2GalA_2Gal_3$ (P: m/z = 1171) and $Rha_2GalA_2Gal_4$ (P: m/z = 1333) for sHBSS digest with RGH. All ions are present as Na adducts (possible structures of the oligomers, m/z = 1171 and 1333, are shown in the right top corner).

at 2.106 ppm typically belonging to an O-acetyl substituent was observed. In general, the O-acetyl units present on the RG I backbone are expected to be linked to O-3 and/or O-2 of the GalA units present in the backbone.[24–26] However, the spin systems found for the GalA units (3.95 and 4.11 ppm) did not show the expected significant downfield shift for proton 2 and/or 3 (5.10 and 5.40 ppm).[25,26] Surprisingly, the O-acetyl substituent was thus found to be attached to O-3 of the rhamnose moiety as indicated by the significant down field shift of H-2 and H-3, 4.226 and 5.254 ppm, respectively. Furthermore, no indications for methyl esterification or other O-acetylation sites were found. O-acetylation of rhamnose within RG I had never been reported before. The chemical shift of the O-acetyl group attached rhamnose seems to overlap with the chemical shift of an acetyl group at position 3 of a GalA in RG I, as observed around 2.10 ppm by Peronne et al.[27] in spinach pectin and by Lerouge et al.[25] in RG I from suspension-cultured sycamore cells.

Using a 2D homonuclear ROESY experiment the connectivity between the different residues observed could be established. The presence of a -2)-α-Rha-(1→4)-α-GalA-(1- dimeric repeat, representing the RG I backbone, could be established by the presence of ROESY cross-peaks between B H-1 and A H-1, B H-4 and A H-1, and A H-2 and B H-1 as indicated in Figure 8a and b. Furthermore, the integral of both anomeric signals, 1,2,4-linked rhamnose and 1,4-linked galacturonic acid showed a ratio of 1:1 again proving that all rhamnoses and galacturonic acid within this sample were derived from the RG I backbone. All rhamnose moieties within the samples were substituted at O-4 with either 1 or 2 galactosyl

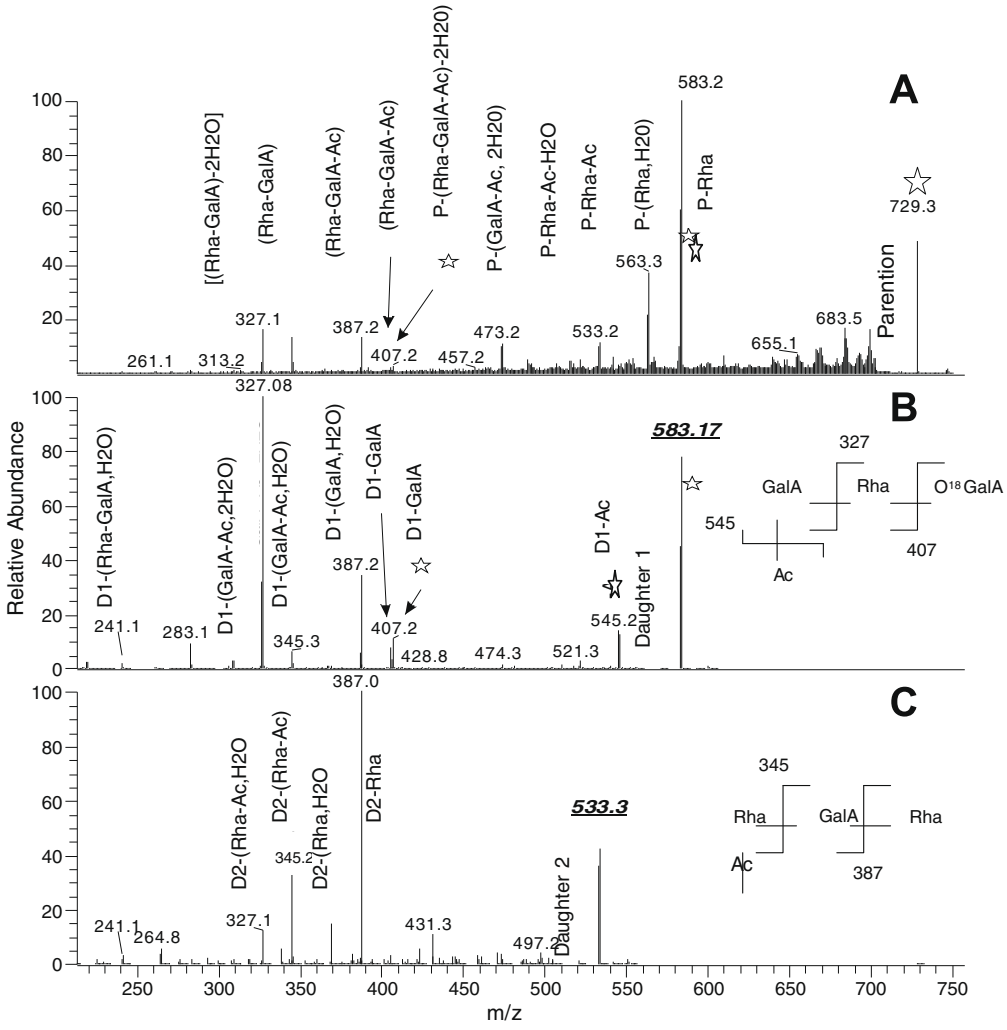

Figure 6. ESI MS^2 (A) of ^{18}O-labeled Rha_2GalA_2Ac (Parent, P: $m/z = 729$) and MS^3 of ion peak Daughter D_1: $m/z = 583$ (B) and D_2: $m/z = 533$ (C) obtained from the HBSS digest with RGH (the fragments having a star are ^{18}O-labeled). All ions are present as Na adducts (possible structures of the fragments, $m/z = 583$ and 533, are shown in the right top corner).

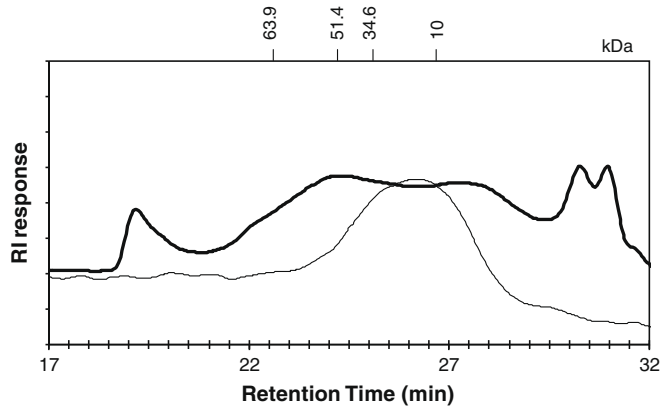

Figure 7. Molecular weight distributions of HBSS RGH digest (bold line) and a 20 kDa fragment obtained after fractionation of Sepharcryl S500 pool 3 over Sepharcryl S300 (thin line). (The molecular weight indications are based on pectin standards.)

residues as proven by the proton chemical shifts (Table 2), indicative for a 1,2,4-linked rhamnose[18,23,28] which is further substantiated by the substitution of acetyl at O-3 and by the chemical shift of H-6.

The anomeric signals D and E in the ROESY spectrum were β-galactosyl residues and could rather be well established using reference data from literature.[18,19,23,28] The chemical shift values of anomeric signal C, however, did not fit with any of the data observed for RG I or RG I fragments measured by proton NMR. However, according to the sugar composition of the RG I fragments measured, this anomeric signal C should be a galactose. Therefore, the proton chemical shift of anomeric signal C was inserted in the SweetDB database provided by the German Cancer Research Center Heidelberg, Central Spectroscopic Division, Germany (http://www.glycosciences.de/sweetdb/). The database search provided a close hit (ca. 98%) with a t-α-Gal linked to O-4 of a β-1,4-linked Gal.[21,22] The presence of an α-(1,4)-linked galactose dimer is proven by the D H-2, **C** H-5 ROESY cross-peak between the β(1,4)-linked galactosyl (D) and the t-α-galactosyl unit (C). The presence of a t-α-galactose at the terminus of RG I side chains had not been observed before in any other pectins. Moreover, the integral of t-α-galactose compared to 1,4-β-galactose was about 2:1 (Table 2).

To obtain additional proof for the linkage between and the sequence of the sugar moieties found, a 2D heteronuclear HMBC spectrum was obtained. A fully resolved spectrum seemed impossible due to the viscosity and heterogeneity of the samples. The O-Ac substituent could be used to determine the carbon chemical shifts of the more flexible moieties, for example, for rhamnose C-

Table 2
^{1}H chemical shifts in ppm of HBSS RGH, intact RG I HBSS and intact RG I CHSS PG/PME

HBSS RGH	H-1	H-2	H-3	H-4	H-5	H-6	Ratio[b]
(A) 1,2,4-α-L-Rha[a]	5.238	4.227	5.249	3.903 (78.11)	3.965 (69.33)	1.332 (18.19)	1
(B) 1,4-α-D-GalA(RGI)	4.984	3.947	4.109	4.441	4.555		0.92
(C) t-α-D-Gal	4.952 (101.57)[c]	3.808 (70.55)	3.923 (70.29)	4.039	4.399 (72.06)	3.697 (61.84)	1
(D) 1,4-β-D-Gal	4.603	3.477	3.743	4.025	–[d]	–	0.66
(E) t-β-D-Gal	4.492 (104.98)	3.428	3.647	3.891	–	–	0.69
CH_3 (*O*-Acetyl)	2.106 (22.09)						0.98
COO	174.91						
RG I HBSS							
1,2-α-L-Rha	5.257	4.12	3.889	3.415	3.765	1.252	0.55
1,2,4-α-L-Rha	5.234	4.224	5.251	3.905	3.951	1.333	0.87
t-α-L-Rha	5.229	4.059	3.797	3.353	–	1.231	
1,4-α-D-GalA (RGI)	5.012	3.909	4.119	4.417	–		1.00
t-α-D-Gal	4.955	3.802	3.935	4.035	4.404	3.696	0.97
1,4-β-D-Gal	4.602	3.491	3.756	–	–		0.84
t-β-D-Gal	4.492	3.425	3.65	3.888	–		1.11
Unknown	4.556	3.563	3.836	4.334	4.563		
CH_3	2.099						1.18
RG I CHSS							
1,2-α-L-Rha	5.243	4.116	3.879	3.409	3.745	1.245	0.15
1,2,4-α-L-Rha (OAc)	5.199	4.221	5.243	–	3.911	1.328	0.38
1,2,4-α-L-Rha	5.163	4.137	3.966	–	3.811	1.313	0.26
1,4-α-D-GalA (RGI)	5.007	3.905	4.114	4.427	–		1.00
	4.907	3.719	4.003	4.456	–		
	4.965	3.719	4.003	4.456	–		
	4.951	3.798	3.924	–	–		
t-α-D-Gal	4.951	3.789	3.923	4.027	4.392	3.96	0.53
1,4-β-D-Gal	4.6	3.482	3.76	3.956	–	–	0.36
t-β-D-Gal	4.483	3.421	3.557	3.882	–	–	0.34
1,4-α-D-GalA (O-Acetylated)							
	5.093	4.135	–	–	–		1.66
	5.165	3.976	–	–	–		
	5.216	3.988	–	–	–		
	5.309	4.002	4.416	–	–		
CH_3	2.094						0.31
CH_3	2.071						0.21
CH_3	2.181						0.03
1,4-α-D-GalA(HG)							
E	5.105	3.731	4.014	4.466	–		1.01
F	5.143	3.748	4.044	_	–		

[a] Arabic letters correspond to annotation in Figure 8.
[b] Ratio was calculated from the integrals of individual proton signals.
[c] ^{13}C chemical shift between brackets.
[d] – = not determined; values are expressed against internal acetone at 2.225 and 31.55 ppm.

6. These chemical shifts are represented between brackets in Table 2. From the data that could be obtained, the presence of a t-α-galactose could also be confirmed based on the carbon chemical shifts. Furthermore, the only inter residual scalar coupling observed was between rhamnose C-4 and H-1 of the t-α-galactose proving the direct linkage between this moiety and the RG I backbone.

By using 20 kDa HBSS RGH population as a reference, the structure of intact RG I from HBSS could be revealed. A COSY, TOCSY, ROESY, and HMBC spectra of intact samples were recorded. Figure 9 shows the ^{1}H NMR spectrum of intact RG I in comparison to the 20 kDa RGH HBSS fragment. Obviously, intact structures showed the same structural elements as found within the 20 kDa RGH HBSS fragment. From the data obtained for intact RG I HBSS, it is obvious that the sample resembles the 20 kDa RGH HBSS fragment except for the presence of unbranched α-1,2-linked rhamnose in the intact RG I HBSS. The unbranched rhamnose has been removed from the 20 kDa RGH HBSS fragment by the treatment with RG hydrolase as already indicated by MALDI-TOF MS. Furthermore, the chemical shifts found for the non-substituted rhamnose correlated well with the chemical shifts found in literature.[20,21,28,29] This indicates that the unbranched rhamnose is not acetylated at position O-3 like the branched rhamnosyl residues. The relative amount of the different residues observed was calculated from the integrated signals present within the anomeric region of both the acetyl groups and rhamnosyl substituents with galatosyl units (Table 2). The ratios indicate that within HBSS, 62% of the rhamnosyl units were substituted. The remaining rhamnose moieties were not substituted. According to the integral of GalA (0.92) that was lesser than integral of Rha (1.0), it could be suggested that part of the galacturonosyl units are O-acetylated resulting in a downfield shift of the anomeric signals which are not fully recognized in the spectrum. From these NMR data, it also becomes clear that RGH is able to cleave the linkage between acetylated rhamnosyl and galacturonosyl moieties within RG I backbone, although the enzyme is much more active against non-acetylated structures. In addition, RGH is also tolerant for both α and β galactosyl residues present as short side chains positioned at rhamnose O-3.

2.2.3. Nuclear magnetic resonance of okra CHSS

Converse to HBSS, RGAE is able to remove the acetyl groups from CHSS. To obtain more structural information of RG I within CHSS compared to RG I in HBSS, the structure of intact RG I from CHSS was revealed by using the 20 kDa RGH HBSS fragment. The ^{1}H NMR spectrum of CHSS intact RG I in comparison to the 20 kDa RGH HBSS fragment is shown in Figure 9. Since the PG treatment could not completely remove the HG part, possible acetylation of HG next to acetylation of the RG could not be ruled out.

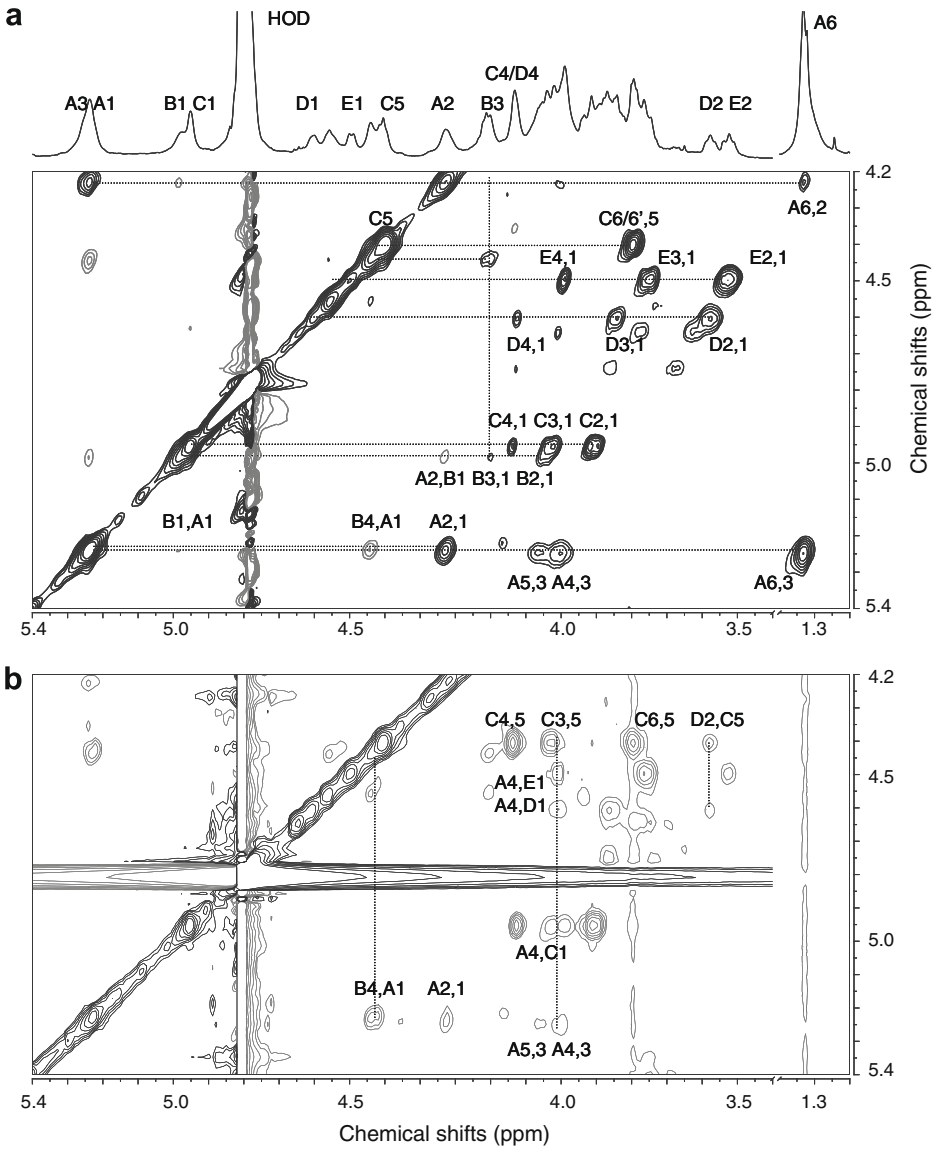

Figure 8. (a) Segment of 2D homonuclear TOCSY NMR spectrum with annotated cross-peaks; A1 = H-1 residue A, B3,1 = cross-peak between B H-1 and B H-3, etc. Gray cross-peaks are ROESY peaks appearing negative in the spectrum they are annotated A4, B1 = inter residual coupling between A H-4 and B H-1, etc. (b) Segment of 2D homonuclear ROESY NMR spectrum with annotated cross-peaks; A1 = H-1 residue A, B3,1 = cross-peak between B H-1 and B H-3, etc., and A4, B1 = inter residual coupling between A H-4 and B H-1, etc. (A: 1,2,4-linked α-Rha*p*, B: 1,4-linked α-Gal*p*A (RG I), C: t-α-Gal*p*, D: 1,4-linked Gal*p* and E: t-β-Gal*p*).

Therefore, the assignment of the different spin systems present is quite complex and the chemical shifts of the different galacturonosyl residues present could only be partially resolved. Similar to the intact RG I HBSS sample, intact RG I CHSS contained the t-α-galactosyl residues and O-acetylated rhamnosyl residues as observed in 20 kDa RGH HBSS fragment. Either 1,2-linked- and 1,2,4-linked rhamnosyl units, O-acetylated at O-3, could be found within the sample. The GalA interconnecting the 3-O-acetylated rhamnosyl units could be assigned completely except for the proton 5 and had similar chemical shifts as the 20 kDa RGH HBSS fragment. Furthermore, three non-acetylated GalA residue signals (RG I) could be observed as proven by the chemical shifts.[18,28,29] Moreover, two anomeric signals of GalA residues, with proton 2 chemical shift of 3.731 and 3.748 ppm, respectively, suggest that these signals are non-acetylated and belong to HG. These chemical shifts of GalA belonging to HG have been shown to appear at lower field than GalA belonging to RG I.[27,30,31] The other galacturonosyl moieties detected are probably derived from O-acetylated GalA since none of the residues showed a COSY cross-peak from the anomeric region to a proton with a chemical shift between 3.75 and 3.80 ppm typical for a non-substituted GalA. This reasoning is further substantiated by finding three significant signals at 2.094, 2.071, and 2.181 ppm, all belonging to an O-acetyl group attached to different positions.

Also for intact RG I CHSS, an estimate of the abundance of the different sugar moieties was made by integrating the proton signals (Table 2). However, due to extreme overlap of the signals sometimes, the values should only be seen as a rough estimate and can rather be under- or over-estimated.

In summary, HBSS contains predominantly RG I structures with monomeric and dimeric galactan side chains, although HG is part of the same molecule to a small extent. The methyl esters present in HBSS seem to be present on the HG part of the molecule. The acetyl groups are predominantly located at position O-3 of the rhamnose moiety. Another novelty of Okra RG-I as found in the HBSS fraction is the presence of α-galactose substitution at O-4 of the backbone rhamnose residue. In contrast to HBSS RG-I, the pectic material extracted by chelating agent and being homoge-

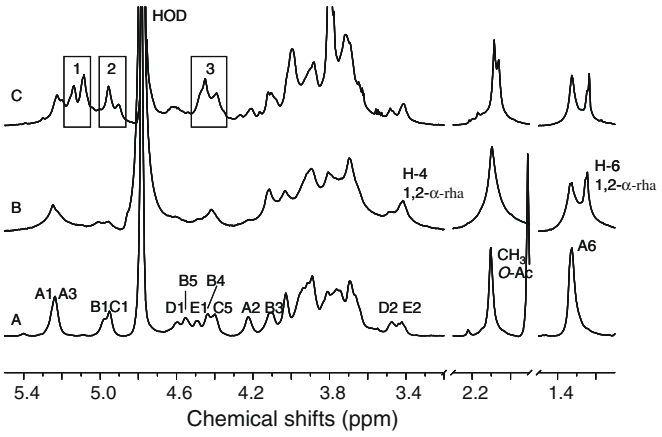

Figure 9. ^{1}H proton NMR spectra of A: 20 kDa HBSS RGH population; B: Intact RG I HBSS; C: Intact RG I CHSS, A = 3-O-acetylated-1,2,4-α-L-rha; B: 1,4-α-D-galA; C: t-α-D-gal; D: 1,4-β-D-gal; E: t- β-D-gal; 1: galA derived from HG or O-acetylated GalA 2: galA derived from RGI and non substituted; 3: galA H-4.

neous with respect to size and charge contains much more HG which is relatively highly methyl esterified. Minor amounts of the rhamnose-acetylated RG-I containing α-galactose substitutions are found. In addition, also more common RG-I structural elements are present consisting of a galacturonic acid-acetylated backbone, without α-linked galactose residues and with longer arabinose- and galactose-containing side chains.

3. Methods

3.1. Sequential extraction of okra AIS

Soft and mature okra pods (5–10 cm in length) were collected at local market in June 2005, Thailand. Okra AIS was prepared and then extracted with 0.05 M sodium acetate buffer (Hot Buffer Soluble Solids, HBSS), followed by 0.05 M EDTA and 0.05 M sodium oxalate in 0.05 M sodium acetate (CHelating agent Soluble Solids, CHSS) according to Sengkhamparn et al.[9]

Intact RG I from HBSS and CHSS were prepared by incubation with endo-polygalacturonase (PG) and pectin methyl esterase (PME) according to Sengkhamparn et al.[9]

3.2. Analytical methods

3.2.1. Total neutral sugar content and uronic acid content

The total neutral sugar and uronic acid contents were determined by the automated colorimetric orcinol/sulfuric acid method[32] and by the automated colorimetric *m*-hydroxydiphenyl method,[33–35] respectively. Galactose and galacturonic acid were used as a standard.

3.2.2. Enzyme degradation

A saponification step was performed for removing methyl ester and acetyl groups before incubation with enzymes. The HBSS and CHSS (3–4 mg) were dissolved in 300 μl millipore water and were saponified by adding 300 μl of 0.1 N NaOH. After storage overnight at 4 °C, the solutions were neutralized with 300 μl of 0.1 M acetic acid and the volume was adjusted to 1 ml with 0.2 M NaOAc buffer of pH 5.

The non-saponified (final concentration of 3–4 mg/ml in 50 mM NaOAc buffer of pH 5) and saponified solutions of HBSS and CHSS were incubated with the following enzymes individually: 0.547 μg protein/mg substrate of rhamnogalacturonan hydrolase (RGH) from *A. aculeatus*, 0.016 units of endo-polygalacturonase (PG) from *A. niger*,[36] 2.5 units of endo-galactanase(endo-Gal) from *Aspergillus niger*,[37] 0.04 units of β-galactosidase from *Kluyveromyces lactis*, 0.04 units of α-galactosidase from Guar seed (Megazyme) and *A. niger*. The incubations were performed at 40 °C for 24 h and were stopped by heating at 100 °C for 5 min for inactivating enzymes. The decrease in molecular weight (Mw) and the release of oligomeric products were analyzed by high performance size exclusion chromatography (HPSEC) and high performance anion-exchange chromatography (HPAEC), respectively.

3.2.3. High performance size exclusion chromatography (HPSEC)

The high performance size-exclusion chromatography (Thermo Separation Products, USA) equipped with three TosoH Biosep-TSK-Gel G columns in series (4000PW_{XL}-3000PW_{XL}-2500PW_{XL}) in combination with a guard PW_{XL} column (TosoH, Japan) was used to observe the change in Mw distribution of samples. The samples were eluted with 0.2 M sodium nitrate at 30 °C and with a flow rate of 0.8 ml/min.[38] The eluent was monitored using RI detector (Shodex SE-61, Showa Denko K.K., Japan).

3.2.4. High performace anion-exchange chromatography (HPAEC)

High performance anion-exchange chromatography was performed on a Dionex ISO 3000 system with PAD detector system (USA). For the analysis of enzyme digests, a (2 × 250 mm) CarboPac PA 1 column (Dionex, USA) was equilibrated with 100 mM NaOH. Gradients of NaOH and NaOAc were used simultaneously to elute the oligomers with a flow of 0.3 mL/min. The results are in the following gradients of NaOH: 0.0–15.0 min, 0–16 mM; 15.0–15.1 min, 16–100 mM NaOH; 15.1–85.0 min, 100 mM. The simultaneous gradient of NaOAc was 0.0–15.0 min, 0 mM NaOAc; 15.0–15.1, 0–100 mM NaOAc; 15.1–55.0 min, 100–400 mM NaOAc; 55.0–80.0 min, 400–700 mM NaOAc; 80.0–85.0 min, 700–1000 mM NaOAc. The column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH and equilibrated for 15 min with 16 mM NaOH.

3.2.5. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

A small spatula of AG 50W-X8 Resin (H^+ form; Bio-Rad, USA) was added to 10 μl of digest sample for desalting, followed by mixing and centrifugation for 5 min at 13,000*g*. One microliter of sample solution was placed on a MALDI-TOF plate together with 1 μl of matrix solution, 10 mg/mL of 2,5-dihydroxybenzoic acid (Bruker Daltonic's, Germany) in water, and dried under a constant flow of warm air. The oligomers were determined by using MALDI-TOF MS (Ultra flex instrument, Bruker Daltonics, Germany) with a lowest laser energy (35%) of Nitrogen 337 nm laser beam to correcting data from average of 200 shots. A mixture of maltodextrins, mass range of 300–3000 Da, was used for calibration.

3.2.6. Electron spray ionization ion trap mass spectrometry (ESI-IT-MS)

For ESI-IT-MS^n analysis, HBSS was dissolved in water and incubated with 0.547 μg/mg substrate of RGH at 40 °C for 24 h. The digest sample obtained after freeze drying was dissolved in 50% acetonitril/49.9% water/0.1% formic acid in H_2O (0.5 mg/ml). For labeling the reducing end with ^{18}O, the sample was dissolved in 50% acetonitril/49.9% water/0.1% formic acid in $H_2{}^{18}O$ (0.5 mg/mL) and incubated for 72 h at 40 °C. After centrifugation (15 min, 24.000*g* at 20 °C) to remove solid impurities, the samples were applied on a LTQ Ion Trap Mass Spectrometer (Thermo Electron, USA) using direct infusion (3 μl/min) with the use of electrospray ionization and detection in positive mode. The capillary voltage was between 4 and 4.9 kV, and the capillary temperature was 225 °C.

Before use, the instrument was tuned with the peak at *m/z* 1009 that is present in HBSS-Sap-RGH sample. The instrument was controlled by XCalibur 2.2 software (Thermo Electron, USA). The scan range was set on *m/z* 300–2000. MS^2 – MS^n was performed with a mass window of *m/z* 2 and the collision energy was optimized for every compound. Mass Frontier 5.0 (Thermo Electron, USA) was used for identifying unknown fragments.

3.2.7. Size exclusion chromatography (SEC)

In order to perform NMR analysis of the rather viscous HBSS sample, the viscosity was lowered by treating with RGH. HBSS (600 mg in 100 mL water) was incubated with RGH from *A. aculeatus* (0.547 Katal) for 24 h at 40 °C and the enzyme was then inactivated at 100 °C for 5 min. An Akta explorer system (Amersham Biosciences, Sweden) was used for fractionation of the digest on preparative scale. The preparative SEC was performed on Sephacryl S500 column (560 × 160 mm ID.; Amersham Biosciences, Sweden) and Sephacryl S300 column (900 × 50 mm ID., Amersham Biosciences, Sweden). The sample was loaded and eluted with 20 mL/min of 0.1 M ammonium acetate buffer of pH 5 on the Sephacryl S500 column. Fractions of 250 mL were collected (28 fractions) and determined for the uronic acid and neutral sugar contents as described above. After pooling appropriate fractions, the pool with the smallest Mw fragments from the Sephacryl S500 was fractionated using a Sephacryl S300 column. The sample was eluted with 4 mL/min 0.1 M of ammonium acetate buffer of pH 5. Fractions of 12.5 ml were collected (54 fractions) and analyzed for uronic acid and neutral sugar contents as described above. After pooling, all pools were desalted by dialysis through 0.1 kDa membrane and were freeze dried.

3.2.8. ^{13}C and ^{1}H Nuclear magnetic resonance (NMR)

Prior to NMR analyses, the sample was dissolved in 0.5 mM NaOAc buffer of pH 5, freeze dried, and then exchanged twice with 99.96% D_2O (Cambridge Isotope Laboratories). A Bruker AV-600 cryoprobe NMR spectrometer located at Biqualys, Wageningen, was used to record NMR spectra at a probe temperature of 25 °C. The 1D ^{1}H proton was recorded at 600.13 MHz as described by Sengkhamparn et al.[9] The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. 2D TOCSY spectra were acquired using standard Bruker pulse sequences with 110 ms mixing time. For all homonuclear 2D spectra, 512 experiments of 2048 data points were recorded using 16–64 scans per increment. Chemical shifts were expressed in part per million relative to internal acetone: $\delta = 2.225$ ppm for ^{1}H and $\delta = 31.55$ ppm for ^{13}C.

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