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Fractionation, solid-phase immobilization and chemical degradation of long pectin oligogalacturonides. Initial steps towards sequencing of oligosaccharides

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Abstract—This work presents the optimized separation of pectin oligomers, their analysis by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), their subsequent immobilization to supports, and our initial steps towards solid-support assisted sequencing. The ambient pressure strong anion-exchange resin Source 15Q combined with ammonium formate buffer (AF) was used for the separation of unsaturated and saturated pectic oligogalacturonides (OGAs) derived from enzymatic digestion of pectin. Routinely, multi-milligram quantities of defined sizes OGAs with DPs from 5 to 19 were produced in excellent purity (>95%). Elution of OGAs followed by direct analysis of the peak fractions by MALDI-TOF MS. Purified OGAs (DP 5–7) were chemoselectively immobilized onto aminooxy-terminated polyethylene glycol polyacrylamide (PEGA) supports. Solid-phase anchoring took place at the reducing end of the oligosaccharide and resulted in the formation of an oxime linkage. The very high coupling yields confirmed the general suitability of aminooxy-PEGA resins for the immobilization of OGAs of different lengths. The OGA-functionalized PEGA supports were subsequently treated with aq TFA at 40 or 60 °C, and the chemical degradation products released from the support were analyzed by ESIMS. In all cases, the original OGA was degraded into smaller oligomers of various sizes down to the monomer. This work illustrates some of the basic principles underlying a strategy ultimately aimed at solid-support assisted sequencing of oligosaccharides.

Keywords: Preparative chromatography; Ion-exchange; Acid hydrolysis; Oxime; Mass spectrometry

1. Introduction

In recent years, there has been increasing focus on the structural analysis of proteins and nucleic acids through sequencing methodologies as a prerequisite to gaining a better understanding of their functions in complex biological processes. The development of automated sequencing techniques has very markedly contributed to the advancement of biophysical and biochemical studies. For instance, solid-phase methodologies routinely provide direct information on protein sequences. However, the other major biopolymers, such as polysaccharides, have not been equally studied, due in part to their vast heterogeneity and complicated structure.

Pectin belongs to the family of these complex heteropolysaccharides that are found in nature and more specifically in the middle lamella of higher plants and in the primary cell wall of all plants.[1,2] The main pectic

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polysaccharide is a homogalacturonan chain composed of a backbone of (1→4)-linked α-D-GalA residues. Some GalA moieties can be partially esterified at C-6 and/or O-acetylated at O-2 and O-3.[1] An important feature of pectin is its ability to form gels under defined conditions. Since the pioneering efforts of Goldthwaite (1909–1917), the manufacture of pectin food products has blossomed into a truly scientific and technological enterprise. From the 1950s onwards, the understanding of these polymers has evolved concurrently with advances in analytical techniques and instruments, such that now, pectin uses stretch far beyond its original application in jams and jellies.[1] Yet, despite growing interest in pectin in recent years, many fundamental questions concerning its roles in vivo, the pathways and mechanisms involved in its biosynthesis, the 'egg-box'-like interaction in pectin gels and structure–function interplay, remain unanswered.

The availability of large amounts of highly pure OGAs of defined sizes could permit many of these questions to be resolved, by for example, enabling the development of new tools for solid-phase sequencing of pectin. Indeed, the chemoselective immobilization of defined and pure OGAs onto solid supports may generate important and effective tools and provide sequence information not only on pectin, but also on polysaccharides in general. We reasoned that immobilized OGAs, with their simple homo-oligomers, should serve as ideal materials on which to perform partial degradation reactions under well-defined conditions—for instance with either chemicals or degradative enzymes, or a combination of the two—and in a controlled fashion. Such solid-phase degradation reactions would *partially* cleave the immobilized OGAs, releasing smaller hydrolysis products into solution. These could, in turn, be isolated simply by filtration and then analyzed. Sequential repetition of this procedure should afford a fractionation pattern for the OGA, and provide insight into its molecular structure.

Several procedures for fractionating mixtures of OGAs have already been reported.[3–5] Jin and West successfully used anion-exchange chromatography on a DEAE Sephadex A-25 column to separate GalA oligomers up to the undecamer.[6] Chromatography on polyacrylamide gel has also been employed to fractionate mixtures of OGAs, up to the nonamer.[7] However, none of these separation methods have been able to fully resolve pectin OGAs of a larger size, and moreover low amounts have usually been cited. Pectin is typically hydrolyzed with H_2SO_4 or HCl.[1] Harsh conditions, such as temperatures between 80 and 121 °C and reaction times up to 72 h,[8,9] are often applied due to the high stability of the glycosidic linkages between uronic acid residues. These procedures lead to *extensive* degradation of the pectin molecules and it is anticipated that they would be highly undesirable for achieving *partial* degradation (i.e., sequencing) of for instance immobilized OGAs. In this case, milder conditions are clearly required to limit the degradation of the solid support. TFA has found many applications in solid-phase peptide chemistry for the release of synthesized peptides from the support. It has been employed for the hydrolysis of pectin since the late 1960s.[8,10,11] We therefore envisaged that TFA might prove effective in the development of chemical methods for the partial degradation of pectin oligomers immobilized on solid supports.

Here we first describe rapid and simple protocols for the preparative separation of large quantities of defined OGAs (with DPs up to at least 19) centring on low pressure anion-exchange chromatography (AEC), using a high performance adsorbent designed for fast, high-resolution separation of other biomolecules, namely, proteins, peptides and oligonucleotides. We then extend the application of the solid-phase immobilization technique, developed in our laboratories and based on the formation of an oxime linkage between the reducing end of the OGA and the aminooxy-group installed on the support, to pure unsaturated OGAs of defined sizes (DP 5–7). Finally, we have demonstrated the use of OGA-functionalized resins as model substrates in solid-supported degradation studies with TFA. This work represents a preliminary step towards sequencing of pectin.

2. Results and discussion

2.1. Fractionation of pectin oligogalacturonides

2.1.1. Small-scale preparative experiments: purification of feedstock 1 containing unsaturated OGAs. In earlier work reported by Voragen and colleagues,[12,13] columns containing the high-resolution strong anion media Source 15Q were used to separate a mixture of partly methyl-esterified OGAs up to the 9-mer and demethylated OGAs with DP ranging from 2 to 8, respectively. This adsorbent also proved efficient in the purification of a 20-mer oligonucleotide synthesized on solid phase.[14] Based on these observations, we were encouraged to select Source 15Q for the preparative fractionation of pectin digests enriched in long OGAs with DP up to 22.

In initial scouting experiments, feedstock 1, which contained unsaturated demethylated OGAs was applied to 20 mL packed beds of Source 15Q (Fig. 1) and the resolving power of the matrix was examined judiciously by varying the steepness of linear gradients of AF. The latter was chosen as the eluent for the following reasons. First, its high volatility facilitates the removal of substantial amounts of buffer salts during lyophilization, thereby improving desalting performance by gel filtration. Second, organic ammonium salts have been reported to enhance signal intensities in MALDI-TOF

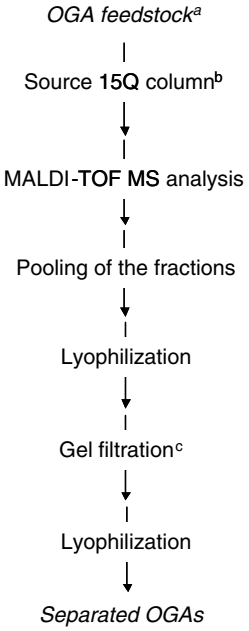


Figure 1. Preparative purification of OGA feedstocks by AEC: (a) feedstocks 1, 2 or 3; (b) the column dimensions and the AF gradient varied according to the amount of feed loaded (refer to individual separations depicted in Figures 3 and 4); (c) desalting was performed on a Sephadex G25 column using H_2O as eluent.

MS analysis.[15] Consequently, eluted fractions can be analyzed directly without the need for a cation-exchange step, which would otherwise be necessary when using sodium-containing buffers. Third, the UV absorption of AF buffer at 235 nm is limited, thus minimizing the need for and errors involved in baseline corrections. Finally, ammonium cations are believed to increase the solubility of OGAs,[3] and are superior to sodium and potassium ions for the quantitative elution of pectic polysaccharides.[16]

Figure 2 and Table 1 show the result of a small-scale chromatographic fractionation experiment conducted with OGA feedstock 1. The chromatogram (Fig. 2) was

Table 1. Summary of the data from the separation of OGA feedstock 1 enriched in unsaturated demethylated OGAs on a 20 mL Source 15Q column (see Fig. 2)

Peak fraction[a]	Main component		Purity[b] (%)	Minor components	
	Mass (Da)	Identity		Mass	Identity
I	527.0	U 3	n.d.		
II	704.0	U 4	n.d.		
III	879.2	U 5	n.d.		
IV	1055.5	U 6	n.d.		
V	1231.7	U 7	>99		
VI	1408.0	U 8	>99		
VII	1583.0	U 9	>99		
VIII	1759.9	U 10	>99		
IX	1936.1	U 11	>99		
X	2111.7	U 12	n.d.		
XI	2288.6	U 13	n.d.		
XII	2464.4	U 14	n.d.	2111.7	U 12
				2288.6	U 13
XIII	2639.9	U 15	n.d.	2288.1	U 13
				2463.9	U 14

n.d. not determined.
[a] See chromatogram in Figure 2.
[b] Based on HPAEC analysis.

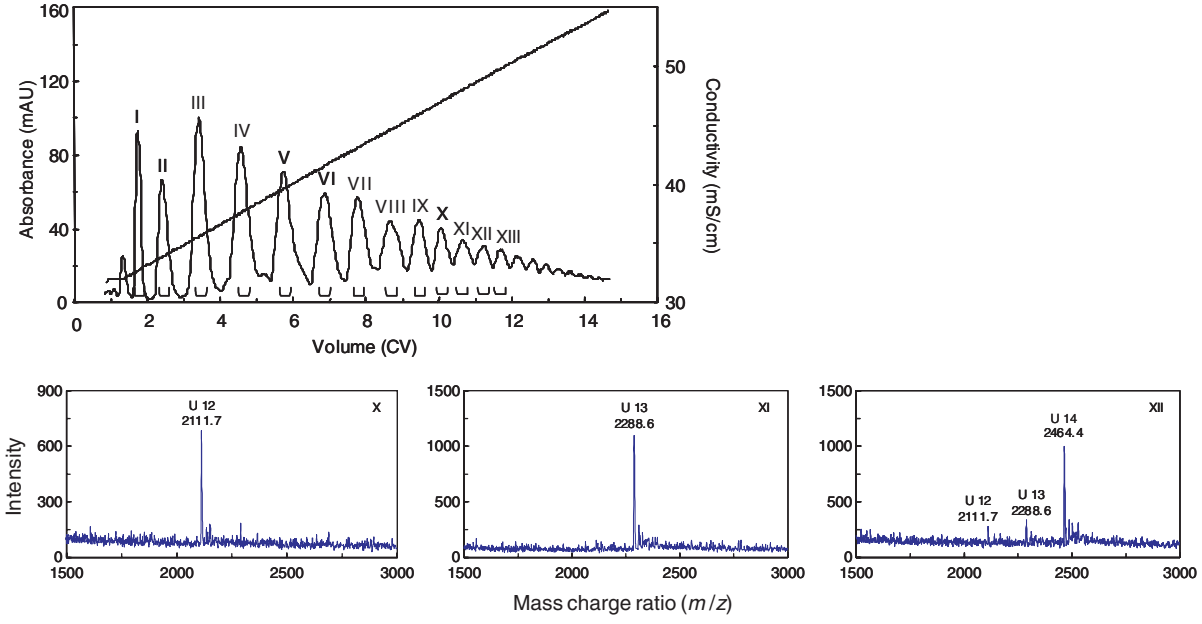


Figure 2. Chromatographic purification of OGA feedstock 1 on a Source 15Q high-resolution column at small scale. Load: 20 mg of feedstock 1; column dimensions: 20 mL, i.d. 16 × 100 mm; flow rate: 1.5 mL/min; gradient: from 0.3 to 0.55 M aq AF in 13.5 CV (corresponding to gradient changes per linear distance of fluid flow of 1.9×10^{-3} M/cm); detection: absorbance at 235 nm; the baseline was corrected for influence from the gradient; peak fractions (I–XIII) were collected ($V = 5$ mL) and analyzed by MALDI-TOF MS.

developed with a linear gradient from 0.3 to 0.55 M AF buffer (pH 6.5) applied over 13.5 column volumes (CV).

Each peak fraction contained one OGA as the major component (according to MALDI-TOF MS analysis), and the preparative purification afforded OGAs with a DP ranging from 6 to 15 (Table 1). MS spectra for fractions X, XI and XII shown below the chromatogram in Figure 2 confirm the suitability of the Source 15Q column to fractionate long OGAs in excellent purity. OGAs with a DP < 14 were purified to near homogeneity, whereas unsaturated $(GalA)_{14}$ (i.e., U 14) and $(GalA)_{15}$ (i.e., U 15) contained minor traces of shorter oligomers. The high purity (>99%) of U 7 through to U 11 was further confirmed by HPAEC analysis. Although we have not analyzed peak fractions beyond XIII under the applied conditions, the Source 15Q column nevertheless appeared to resolve peaks up to the 20-mer (Fig. 2).

2.1.2. Large-scale preparative experiments: fractionation of feedstock 1 containing unsaturated OGAs. Much of the challenge in the separation of OGAs lies in the production of large amounts of longer species (i.e., DP > 10), given that highly pure smaller oligomers can easily be produced in high quantities very quickly using steep gradients. The ability of Source 15Q to efficiently fractionate OGA mixtures was subsequently investigated on a much larger scale using feedstock 1. Half a gram of unsaturated OGA mixture was applied to a 0.65 L packed bed of Source 15Q and the column was then developed using a very steep linear gradient of AF (0.3–0.8 M) applied over 3.2 CV (results not shown). Even under such challenging and sub-optimal conditions, the column was nevertheless able to resolve distinct and regularly spaced peaks, presumably up to the 17-mer (not confirmed). Unsaturated 3-, 4-, 5-, 6- and 7-mer were obtained in excellent purity (92–100%) and in large amounts (29–55 mg), whereas the 8-, 9- and 10-mers were isolated in reasonable purity (72–85%).

2.1.3. Large-scale preparative experiments: fractionation of feedstock 2 containing unsaturated OGAs. In the previous large-scale chromatography experiment (2.1.2), the largest identified OGA separated was the 10-mer, which was produced by using highly unfavourable elution conditions. At this stage, it was deemed important to test the resolving power of the Source 15Q matrix under more optimal conditions in an effort to separate much longer OGAs. In order to do this, a new feedstock enriched in longer OGAs and depleted in shorter species was first produced (Supplementary data). This material (feedstock 2) was subsequently applied to a 0.65 L Source 15Q column and bound OGAs were then eluted in order of increasing size using a shallow linear gradient of AF (0.3–0.55 M) developed over 11 CV (Fig. 1). The resulting chromatogram and MS spectra of pooled peak fractions are presented in Figures 3 and 4, respectively, and the purities of each OGA are given in Table 2. The pooled fractions containing OGAs from the 7-mer through tentatively to the 22-mer were collected in excellent yields and very high purities (Table 2). It is noteworthy that although OGAs originating from partially-methylated pectin digests and with DP of up to 40 have been detected by MALDI-TOF MS,[17] OGAs of DP above 20 exhibit very poor ionization efficiency. In total, 221 mg of highly pure OGAs (6–27.2 mg individually with purities ranging from 88% to >99%) were generated, corresponding to a total OGA yield of 51% relative to that loaded onto the column. The procedures detailed herein represent a significant improvement over previously reported protocols. For example, although the best of these,[13] employing preparative HPLC on a preparative CarboPac PA-1 column managed to separate OGAs with DP from 7 to 20, however, yields and purities were lower than obtained in this work.

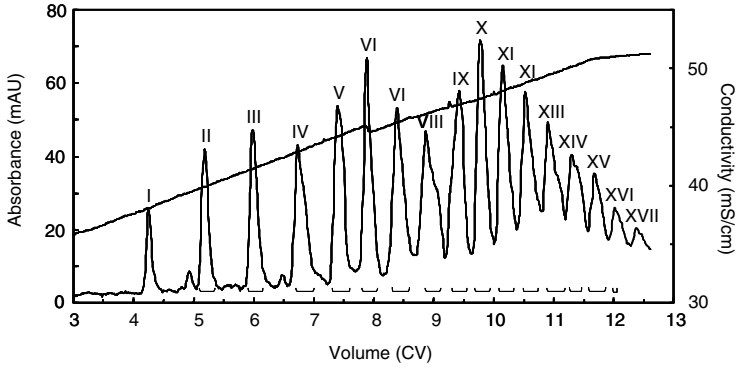


Figure 3. Chromatographic purification of feedstock 2 on a Source 15Q high-resolution column at large scale. Load: 430 mg of feedstock 2; column dimensions: 650 mL, i.d. 50 × 330 mm; flow rate: 7 mL/min; gradient: from 0.3 to 0.55 M aq AF in 11 CV (i.e., 6.9×10^{-4} M/cm); detection: absorbance at 235 nm; the baseline was corrected for influence from the gradient; peak fractions were collected, pooled (as indicated by the bars below the peaks and the roman numbers above them) and analyzed by MALDI-TOF MS.

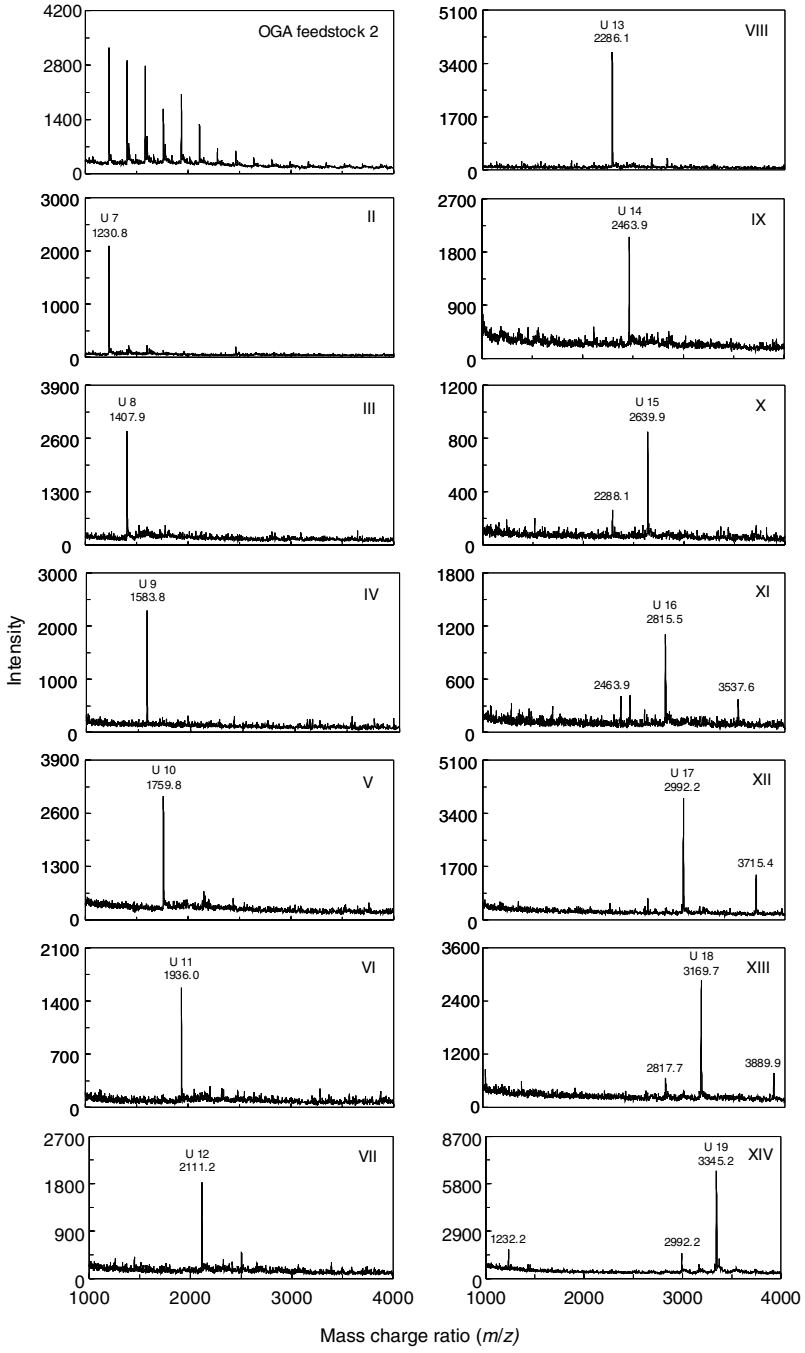


Figure 4. MS spectra of feedstock 2 and selected peak fractions containing OGA with a DP = 7–19. The collected fractions correspond to the separation depicted in Figure 4.

2.1.4. Large-scale preparative experiments: application to the purification of saturated OGAs from feedstock 3. The general procedure developed and detailed above was also successfully applied for fractionation of a digest (feedstock 3) containing saturated demethylated OGAs with DP from 4 to 18 (Fig. 1). Figure 5 represents the MS spectrum of a saturated OGA 17-mer (S 17), which was obtained in high yield and purity.

2.2. Solid-phase immobilization of defined long pectin oligogalacturonides followed by chemical degradation

2.2.1. Solid-phase immobilization of OGAs through the reducing end via oxime formation. Prior to immobilization of purified OGAs, amino-terminated PEGA support (0.2 mmol/g) was converted into aminooxy-terminated resin **A** (Scheme 1) by coupling of *tert*-butyloxycarbonyl-aminooxy acetic acid (Boc-Aoa-OH) under

Table 2. Summary of the data from the separation of OGA feedstock 2 enriched in unsaturated demethylated OGAs on a 650 mL Source 15Q column (see Fig. 3)

Pooled peak fraction[a]	Yield[b] (mg)	Main component[c]		Purity[d] (%)	Minor component(s)[c]	
		Mass (Da)	Identity		Mass	Identity
Feedstock 2						
I						
II	10.3	1230.8	U 7	>99		
III	12.1	1407.9	U 8	>99		
IV	11.0	1583.8	U 9	>99		
V	18.2	1759.8	U 10	>99		
VI	10.3	1936.0	U 11	80		
VII	18.8	2112.2	U 12	>99		
VIII	20.8	2286.1	U 13	>99		
IX	14.5	2463.9	U 14	97		
X	16.2	2639.9	U 15	94	2288.1	U 13
XI	27.2	2815.5	U 16	92	2463.9	U 14
					3537.6	S 20
XII	20.3	2992.2	U 17	88	3715.4	S 21
XIII	21.2	3169.7	U 18	n.d.	2817.7	U 16
					3889.9	S 22
XIV	15.0	3345.2	U 19	92	2992.2	U 17
XV	6.0	3521.5 (not shown)	U 20	90		
XVI			U 21	n.d.		
XVII			U 22	n.d.		

[a] See chromatogram in Figure 3.
[b] The mass was ascertained using the *m*-HDP assay.
[c] Based on MALDI-TOF MS analysis.
[d] Based on HPAEC analysis.

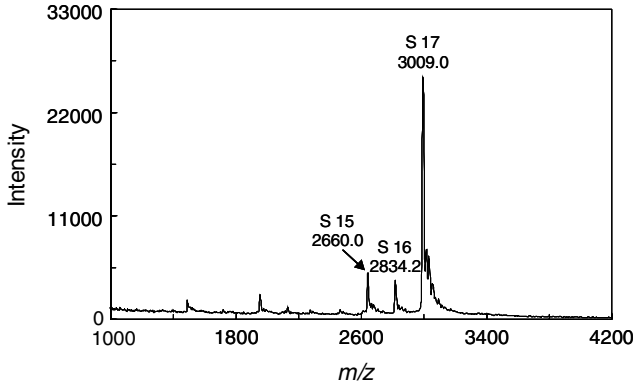
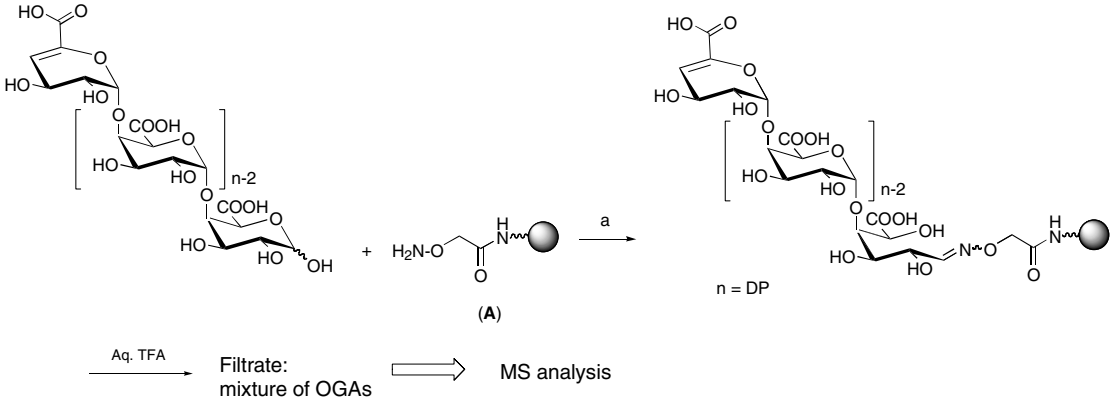


Figure 5. MALDI-TOF MS spectrum of a saturated OGA (DP 17) purified by AEC.

standard conditions, followed by acidic deprotection.[18] The extent of functionalization of resin **A** was quantified by the 'indirect' Trt quantitation method, which involves derivatization of the functionalized resin with trityl chloride followed by acidolytic release of Trt and quantification.[18] Unsaturated OGAs originating from preparative AEC, as described above, and with a DP ranging from 5 to 7 were subsequently immobilized onto resin **A** through the reducing end by formation of an oxime bond to produce resins **B**, **C** and **D**, respectively (Scheme 1).

The immobilization step was followed by on-resin quantitation with the anthrone assay.[18] At this stage, the resin was better handled in a dry state[19] and thus the same drying procedure reported earlier[18] was applied. In all cases, high coupling efficiencies ranging between 75 (±16)% and 107 (±23)% were obtained, although a trend of decreasing efficiency with increasing OGA size was observed (the value for the loading yield is obtained after subtraction of a 'blank' from acidolytic degradation of PEGA). Quantitative loading of an oligosaccharide is not required for the subsequent sequencing step. These findings nevertheless confirm that solid-phase immobilization onto aminooxy-terminated resins is efficient and applicable to pectin OGAs of different sizes.

2.2.2. Chemical degradation of immobilized OGAs. The development of tools for sequence analysis of polysaccharides is a complex and multi-step process. Here, initial efforts consisted of establishing efficient and simple methodologies for the chemoselective anchoring of OGAs of various sizes onto a solid matrix, as described above. We have further used the OGA-functionalized PEGA resins (**B**, **C** and **D**) to perform solid-supported chemical degradation reactions under mild conditions and involving treatment with aq TFA. Parameters such as TFA concentration, temperature and time were varied. The use of a solid-phase approach facilitated isolation of the supernatant by filtration, and the filtrate composition after chemical treatment was then analyzed by ESIMS (Scheme 1). Potential cleavage sites on an



Scheme 1. Solid-phase immobilization of OGAs ($n = 5$, 6 or 7) onto aminooxy-terminated PEGA supports A through the reducing end to form resins **B**, **C**, and **D**: (a) 1:1 DMF–water, pH 4.8, 40 °C, 24 h.

unsaturated OGA (e.g., 6-mer) are indicated in Figure 6. Both saturated ('S') and unsaturated ('U') fragments can be generated from cleavage of the glycosidic bonds between GalA residues. The S series contains the reducing end, while the U series contains the non-reducing end.

Mass spectra of collected fractions resulting from acidic treatment of immobilized U 5, U 6 and U 7 were acquired in the negative ion mode. Table 3 summarizes the composition of each sample. The results show that, in all cases, the initial immobilized OGA was degraded into smaller OGAs down to the monomer. Moreover, TFA was able to cleave *exo*-glycosidic and *endo*-glycosidic bonds on the OGA chain under the applied conditions, thereby generating both unsaturated and saturated OGAs from the non-reducing and reducing end, respectively. In nearly all of the samples, the 3-mer and the 4-mer were the most abundant fragments produced. The degradation pattern corresponding to

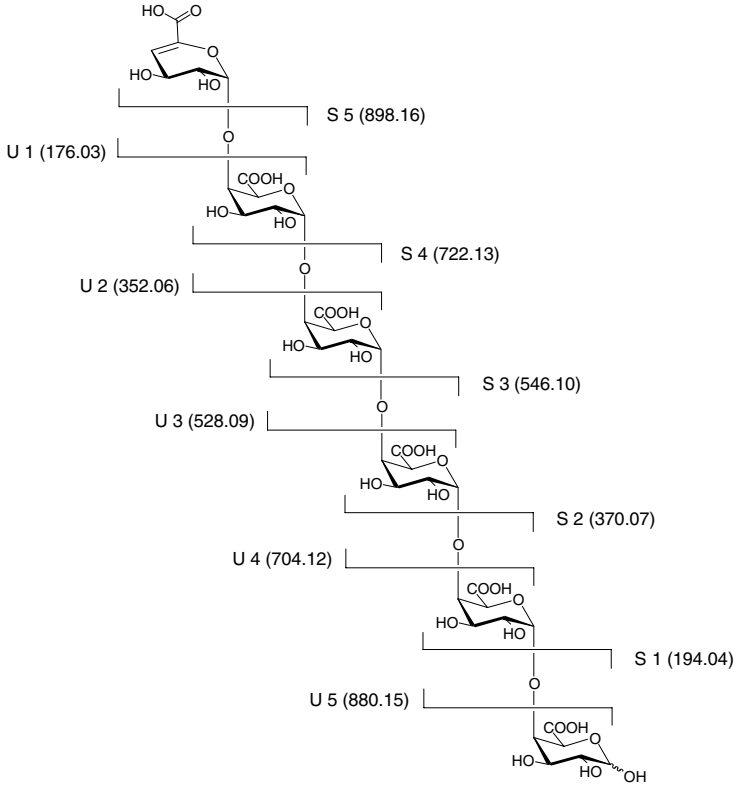


Figure 6. Fragmentation of an unsaturated 6-mer OGA. Saturated and unsaturated fragments are referred to as S and U, respectively. For example, U 4 corresponds to an unsaturated fragment containing 4 GalA residues. The calculated masses of the neutral fragments are indicated in parentheses.[20]

Table 3. ESIMS was used to determine the composition of samples resulting from chemical degradation of immobilized OGAs

Entry	Originally immobilized OGA	Degradation conditions	Major fragments	Minor fragments
1	U 5	2 M TFA, 40 °C, 1 h	S 1: 194.6 (100) U 3: 526.8 (71) U 4: 702.8 (67) U 5: 878.8 (63) S 3: 544.8 (60)	S 4: 720.8 (37) U 2: 350.7 (29) S 2: 368.7 (20) U 1: 174.7 (16)
2	U 5	2 M TFA, 40 °C, 20 h	U 5: 878.8 (100) U 4: 702.8 (90) U 3: 526.8 (88) U 2: 350.7 (48)	S 3: 544.7 (26) U 1: 174.7 (19) S 4: 720.8 (16) S 2: 368.7 (12) S 1: 194.7 (5)
3	U 6	5 M TFA, 40 °C, 1 h	U 4: 702.8 (100) U 3: 526.8 (88) U 6: 1054.8 (76) S 3: 544.8 (58) U 5: 878.8 (56) S 4: 720.8 (49) U 2: 350.7 (45) S 5: 896.8 (41)	S 2: 368.7 (21) U 1: 174.7 (12) S 1: 194.6 (9)
4	U 6	5 M TFA, 40 °C, 20 h	U 3: 526.8 (100) U 4: 702.8 (98) S 1: 194.6 (62) S 3: 544.8 (54) U 5: 878.8 (54) U 2: 350.7 (43) S 4: 720.8 (40)	U 6: 1054.8 (30) S 5: 896.8 (23) S 2: 368.7 (21) U 1: 174.8 (15)
5	U 7	2 M TFA, 60 °C, 1 h	U 4: 702.8 (100) S 3: 544.8 (90) U 3: 526.8 (80) U 7: 1230.7 (73) U 5: 878.8 (55) S 4: 720.9 (53) S 5: 896.8 (45) U 6: 1054.8 (44) S 6: 1072.7 (40)	U 2: 350.8 (35) S 2: 368.8 (19) U 1: 174.9 (18) S 1: 192.8 (15)
6	U 7	5 M TFA, 60 °C, 20 h	S 4: 720.8 (100) S 3: 544.7 (98) U 3: 526.8 (64) U 4: 702.8 (56) S 5: 1072.7 (48) S 6: 896.8 (46) S 1: 194.6 (41)	S 2: 368.7 (31) U 2: 350.7 (18) U 5: 878.8 (14) U 7: 1230.7 (11) U 6: 1054.8 (11) U 1: 174.7 (10)

The fragment ions are listed with their relative intensities shown as percentage of the most abundant fragment normalized to 100%. In each sample, the minor fragments correspond to the OGAs with an intensity below 40% of the maximum peak intensity.

entry 3 (Table 3) is 'bell-shaped' (Supplementary data), which suggests that the mild conditions used allow *partial* cleavage of the OGA chain. Increasing the reaction time and the TFA concentration resulted in larger amounts of degradation products, including some which could not be identified. In addition, the amount of saturated OGAs produced was higher in samples treated under harsher conditions (Table 3, entry 6). The reason for this currently remains unclear. Increasing the temperature from 40 to 60 °C did not have a significant effect on the degradation pattern (Table 3, compare entries 5 and 6 to 1 and 4, respectively). Finally, the PEGA support proved very resistant during acidic treatment under these conditions. It is noteworthy that the shortest saturated OGAs could also arise from the cleavage of two *endo*-glycosidic bonds on the OGA chain (Fig. 6). In addition, hydration of unsaturated OGAs at the reducing end or at the non-reducing end would lead to compounds with the same mass as the so-called 'saturated' OGAs. Further investigation would be required to identify the exact structure of the compounds referred to as 'saturated'. One possibility could be to label the fragments with ^{18}O at the reducing end in order to distinguish between the various isomers.[20]

We have also observed that each sample, collected by filtration after the reaction, contained the starting OGA,

which had initially been immobilized onto the solid matrix (Table 3). This observation indicates that the oxime linkage exhibited limited stability under the applied cleavage conditions. This could have been predicted, considering that the pH of the aq TFA solution was slightly below the limit of 2 reported for the oxime stability.[21] Important considerations arose from these observations, namely: did the production of smaller OGAs occur while the starting OGA remained attached on the solid support through the oxime linkage, or was the immobilized OGA released and then degraded in free solution? To test this, we performed the following control experiment.

2.2.3. Chemical degradation of OGAs in solution, control experiment. Unsaturated OGAs of two sizes (DP = 6 or 7) were treated with aq TFA in solution at 60 °C. ESIMS provided semi-quantitative information on the composition of each sample. Two typical examples are illustrated in Supplementary data.

Some general observations were similar to those made for degradation experiments on solid phase, that is: (i) all starting OGAs were degraded into smaller OGAs under the applied conditions; (ii) both unsaturated and 'saturated' oligomers were produced; (iii) the amount of 'saturated' OGAs was higher than the unsaturated ones when the sample was treated under harsher conditions; and (iv) degradation of unsaturated OGAs in solution was highly influenced by time. Indeed, prolonged treatment with TFA resulted in the near disappearance of U 6 and U 7, whereas a 1 h treatment produced only small amounts of shorter fragments (Supplementary data).

Interestingly, the amount of degradation products detected decreased in general with size. The fragmentation of U 7 appeared to proceed partially (i.e., not completely down to the monomer, but via the formation of a range of various smaller OGAs) even after a 20 h treatment, indicating that it may be possible to control the degradation by choosing much milder conditions.

Finally, to demonstrate that this control experiment could provide information on the degradation events occurring when using immobilized OGA substrates, we compared fragmentation patterns generated by the treatment of a 7-mer OGA with 2 M TFA at 60 °C and for 1 h in free solution and when immobilized. Treatment of the immobilized U 7 (Table 3, entry 5) yielded a 'bell-shaped' degradation pattern (spectrum not shown), in which U 3 and U 4 were the major compounds. In case of solution experiments, the starting U 7 was obtained as the major component and the degradation products were only present in very small amounts (Supplementary data). Collectively, these results confirm that, under the applied conditions, most of the immobilized OGA was degraded into shorter products whilst still anchored to the solid matrix. In other words, the control experiment showed that smaller OGAs resulted primarily from the chemical degradation of the *immobilized* OGA, and not once the latter substrate was released into solution as a result of instability of the oxime linkage. Finally, this control study highlights one advantage of the solid-phase approach, namely, the smaller OGAs can be analyzed without interference from the starting OGA.

The data presented illustrate the basic principles underlying a strategy we have begun to develop ultimately aimed at solid-support assisted sequencing of oligosaccharides. It is expected that further development of solid-supported partial chemical degradation methods such as those introduced here will provide important insight into structure-function relationships of pectin oligomers and other complex polysaccharides.

3. Experimental

3.1. Chemicals

Ammonium formate, 2,4,6-trihydroxyacetophenone (THAP), acetone, 2-propanol, methanol (all HPLC grade) and pectin (P9311, degree of methylesterification 28.5%) were purchased from Sigma (St. Louis, MO, USA). TFA was bought from Sigma–Aldrich Denmark A/S (Vallensbaek Strand, Denmark). *meta*-Hydroxydiphenyl (*m*-HDP, technical grade) and nitrocellulose (NC, Trans-blot transfer medium, 0.45 μm) were obtained from Fluka (Buchs, Switzerland) and BioRad (Hercules, CA, USA), respectively. NaOH (50% aqueous solution, HPLC grade) was supplied by Mallinckrodt Baker (Phillipsburg, NJ, USA) and NaOAc (anhyd, HPLC grade) was acquired from E. Merck (Darmstadt, Germany). Pectin lyase (E.C. nr. 4.2.2.10) was a gift from Danisco Cultor A/S (Copenhagen, Denmark). Amino-functionalized PEGA resins (0.2 mmol/g, 300–500 μm, 9.41 wt % in MeOH) were kindly supplied by Dr. Andrew Coffey of Polymer Laboratories, UK. The PEGA supports were weighed wet, unless indicated otherwise.

3.2. Preparation of oligogalacturonide feedstocks for chromatography on Source 15Q

Three different feedstocks were applied to packed beds of the high-resolution Source 15Q chromatographic matrix and their detailed preparation is illustrated schematically in Figure 1. Feedstocks 1 and 2 were made by controlled digestion of partially esterified pectins with the enzyme pectin lyase, at room temperature for different times (i.e., 24 h for feed 1 c.f. 1 h for feed 2). In the preparation of feedstock 1 (Supplementary data) OGAs of very high molecular weight were removed by ultrafiltration after enzymatic treatment. Following precipitation with cold propan-2-ol and centrifugal collection

of the pelleted material, the redissolved OGAs were saponified to yield a mixture of unsaturated demethylated OGAs with DP from 2 to ca. 20.

In order to produce a feedstock enriched in longer OGAs with DP ranging from 5 to 22 (Supplementary data), the pectin lyase digestion time was reduced to 1 h and the alkaline de-esterification step was performed immediately thereafter. The ultrafiltration step was eliminated and removal of very high molecular weight OGAs—produced by lowering the pH—was performed later in the purification sequence, using high-speed centrifugation. Unsaturated demethylated OGAs (1.2 g) isolated from the resulting pellet by dissolution, precipitation and recentrifugation, contained a substantial amount of small OGAs (DP 1–4). These, and species longer than DP = 22 were removed by chromatography on Q Sepharose Fast Flow to give 430 mg of OGAs with DP from 5 to 22, which constituted feedstock 2.

The third feedstock (3), a mixture of saturated demethylated OGAs (DP < 20), was produced using a modified version of the protocol described by Hotchkiss et al.,[22] involving chemical degradation of polygalacturonic acid under acidic conditions (Supplementary data).

3.3. Chromatography

The chromatography equipment, columns (XK16, XK26 and XK50) and media (Q Sepharose Fast Flow, Source 15Q and Sephadex G75) used in this work were from Amersham Biosciences (Uppsala, Sweden). All separations were performed at room temperature with an ÄKTA EXPLORER 100 workstation controlled by UNICORN software. Detailed information on the columns, packings, flow rates and gradients applied in different experiments is provided in the text and in legends to Figures 2 and 3.

OGA feedstocks (see above) were loaded on Source 15Q high-resolution columns equilibrated with AF buffer, as illustrated in Figure 1. Elution was performed using various gradients of AF. The purification of unsaturated OGAs (feedstocks 1 and 2) was monitored by UV detection at 235 nm. The collected fractions were then analyzed by MALDI-TOF MS and those containing pure OGAs were pooled and desalted by chromatography on a Sephadex G75 gel filtration column (XK25, 100 mL) to afford separated demethylated OGAs of defined sizes. The GalA content of each pooled fraction was determined using the specific *m*-HDP assay, as detailed below. The purity of the pooled materials was ascertained by high performance anion-exchange chromatography (HPAEC, see below).

3.4. Mass spectrometry analysis

MALDI-TOF MS analyses of GalA oligomers were carried out on a Voyager DE STR Biospectrometry workstation (PerSeptive Biosystems, Framingham, MA, USA). The instrument was operated in reflector mode with a delayed extraction time of 350 ns, a grid of 65% and accelerating voltage of 20 kV. An internal laser intensity of 2.6–2.9 kV was used and the low mass gate was set to 500 Da to avoid matrix ions saturating the detector. The spectrometer was externally calibrated using HP peptide standard mixture (Hewlett Packard, Denmark). All the spectra were acquired in the negative ion mode. THAP was dissolved in MeOH to a concentration of 200 mg/mL. NC was dissolved in acetone to a concentration of 30 mg/mL and then diluted with isopropanol to 15 mg/mL. The THAP and NC solutions were mixed in a 4:1 ratio, and a 0.2 μL volume of the THAP-NC matrix was applied in spots to the target plate. 0.2 μl of 20 mM diabasic ammonium citrate solution was added to the matrix spot. The analyte solutions were cation-exchanged using miniature Dowex 50WX8-columns[17] before applying 0.2 μL to the matrix spot. This step was performed to avoid the formation of sodium adducts during MS analysis. However, it could be omitted for routine screening of eluted fractions, in which Na^+ ions are removed by employing a large excess of AF.[12,20]

ESIMS spectra were acquired on an Esquire-LC quadrupole ion-trap mass spectrometer (Bruker Daltonic, Bremen). Analysis was done in the negative ion mode at a fundamental radio frequency of 781 kHz. A standard scan range of 50–2200 m/z was used at a scan speed of 13,000 $m/z\ s^{-1}$.

3.5. HPAEC analysis

Unsaturated OGAs were analyzed by HPAEC on a Dionex AI450 system (Dionex Corporation, Sunnyvale, CA, USA) using UV detection at 235 nm. NaOH (0.1 M aq, solution A) and 1 M aq NaOAc in 0.1 M aq NaOH (solution B) were used as eluents. Solutions A and B were degassed by sparging with Helium for 30 min and subsequently kept under He. Samples (20 μL) were loaded onto an analytical PA-100 strong anion-exchange Dionex column (internal diameter i.d. 4.9 × 250 mm) connected to a PA-100 guard Dionex column (i.d. 4.9 × 40 mm) and equilibrated with 90% A at a flow rate of 0.8 mL/min for 10 min. OGAs were eluted using a non-linear gradient of 10–62% B in 32 min followed by a shallower segment of 62–66% B in 18 min.

3.6. Determination of GalA content

The *m*-HDP assay originally developed by Blumenkrantz and Asboe-Hansen for the quantitation of uronic acids,[23] and later adapted for use in microtitre plates by van den Hoogen et al.,[24] was used in this study. The influence of the AF buffer on the assay response was

Table 4. Conditions for chemical degradation of immobilized unsaturated OGAs

Entry	Resin	Immobilized OGA[a]	Mass of resin (mg)	Aq TFA (concentration, M)	Temperature (°C)	Time (h)
1	**B**	U 5	13.9	2	40	1
2	**B**	U 5	11.0	2	40	20
3	**C**	U 6	11.3	5	40	1
4	**C**	U 6	11.8	5	40	20
5	**D**	U 7	7.1	2	60	1
6	**D**	U 7	11.1	5	60	20

[a] The OGAs were unsaturated ('U') at the non-reducing end. The number indicates the DP (e.g., U 5 = unsaturated $(GalA)_5$).

Table 5. Control experiment: conditions for the chemical degradation of unsaturated OGAs in solution

Entry	OGA	Mass of OGA (mg)	Aq TFA (concentration, M)	Temperature (°C)	Time (h)
1	U 6	2.3	5	60	20
2	U 7	2.5	5	60	20
3	U 6	3.0	5	60	1
4	U 7	2.7	2	60	1

studied in order so that accurate determination of the GalA content of fractions collected after purification on a Source 15Q column, could be made.

3.7. Preparation of aminooxy-terminated supports (A)

Aminooxy-terminated support **A** was prepared following the exact same procedure as that described by Guillaumie et al.[18] In this case, a low loading amino PEGA resin (5.31 g, 0.1 mmol) was used and the amount of reagents was adjusted accordingly. The loading was determined by the 'indirect' trityl (Trt) quantitation method.[18]

3.8. Immobilization of unsaturated OGAs onto aminooxy-terminated supports (resins B, C and D)

The procedure used was that described earlier by Guillaumie et al.[18] $(GalA)_5$ (3.5 mg, 4 μmol), $(GalA)_6$ (3.2 mg, 3 μmol) or $(GalA)_7$ (3.7 mg, 3 μmol) were immobilized onto dry resin **A** (40 mg, 2 μmol; 30 mg, 1.5 μmol; and 30 mg, 1.5 μmol, respectively), to give resins **B**, **C** and **D**, respectively. Following immobilization, coupling efficiencies were determined by the anthrone assay.[18]

3.9. Chemical degradation of immobilized OGAs

The different individual parameters for solid-phase reactions are listed in Table 4. The same basic procedure was applied to resins **B**, **C** and **D**. The dry resins were weighed into 2 mL syringes and washed with water (3×1 min, 1×10 min, 2×1 min). Portions (0.7 mL) of aq TFA were added and the syringes were placed in a sand bathes held at the appropriate temperatures. After the allotted time, the resins were filtered and washed with extra portions of H_2O (2×1 mL). Each filtrate was concentrated to dryness and redissolved in H_2O (100 μL) prior to ESIMS analyses.

3.10. Chemical degradation reaction in solution: control experiment

The DP (n) of the unsaturated OGAs and other reaction parameters are summarized in Table 5. 'U n' were weighed into 1 mL Eppendorf tubes, and following addition of aq TFA (0.5 mL), the vials were placed in a sand bath at 60 °C for 1 or 20 h. The samples were then freeze-dried and their composition was analyzed by ESIMS.

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Supplementary data

The supplementary data contains Figure 1. Preparation of OGA feedstocks and dimension of chromatographic columns; Figure 2. Negative ion mode ESIMS spectra of unsaturated OGA samples after chemical degradation. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2005.10.011.

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