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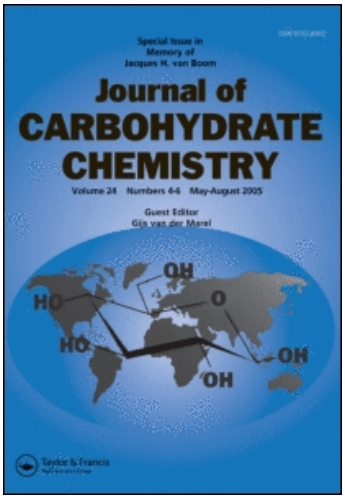

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J. W. Timmermans[a]; M. G. J. W. Bitter[a]; D. de Wit[a]; J. F. G. Vliegenthart[b]
[a] ATO-DLO, Wageningen, The Netherlands [b] Department of Bio-organic Chemistry, Bijvoet Center, Utrecht University, Utrecht, The Netherlands

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THE INTERACTION OF INULIN OLIGOSACCHARIDES WITH Ba^{2+} STUDIED BY ^1H NMR SPECTROSCOPY.

J.W. Timmermans,* M.G.J.W. Bitter,* D. de Wit,* and J.F.G. Vliegenthart+

*ATO-DLO, P.O.Box 17, 6700 AA, Wageningen, The Netherlands
+Department of Bio-organic Chemistry, Bijvoet Center, Utrecht University, P.O.Box 80.075, 3508 TB, Utrecht, The Netherlands

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ABSTRACT

A High Performance Anion Exchange Chromatography method with gradient elution in combination with a refractive index detector has been developed. This method enables a convenient way to determine Pulsed Amperometric Detector (PAD) responses using mixtures without prior separation. In addition, large quantities of salts could be separated from small oligomers (Degree of Polymerisation (DP) 5-12) using RP-18 chromatography. The interaction between Ba^{2+} ions and inulin oligomers with DP 2-12 was studied with ^1H NMR spectroscopy and was shown to be strongly dependent on the degree of polymerisation. The interaction appeared to be more pronounced for DP 4-5 than for both smaller and larger molecules, which suggests a conformational change around this DP.

INTRODUCTION

Commercially available inulin (from Chicory root) consists of oligosaccharides built up from (2->1)-linked ß-D-fructofuranosyl residues, and mostly terminated by an α-D-glucopyranosyl unit in a (1<->2)-linkage (Fig. 1). In the search for new applications for inulin, structure function relationships are frequently studied. Based on X-ray data combined with computer modelling[1] and ^1H-relaxation studies[2] it has been suggested that inulin adopts a five fold helical structure. Computer modelling has

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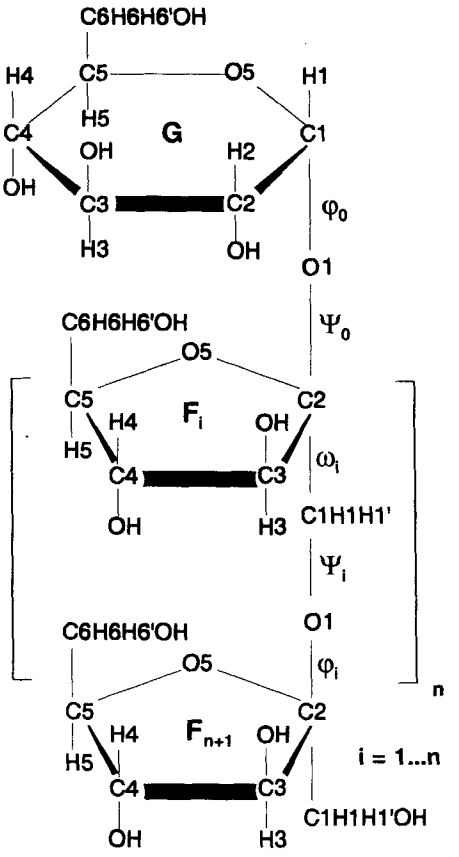

Fig. 1. Primary structure of oligosaccharides present in inulin.

shown that inulin can form many types of helices but these calculations do not indicate a preferred structure.[3] From variations in ^{13}C chemical shift values it has been concluded that a simple regular helix is not the predominant conformation of inulin oligomers in aqueous solution.[4]

Although crystal structures of 1-kestose[5] and nystose[6,7] have been published, for inulin only low resolution X-ray data are available.[1] Recently, it has been shown that the indexation of the older X-ray data,[1] from which the five fold helical structure was concluded, has to be revised.[8] Combination of this indexation with new electron diffraction data and computer modelling resulted in a significantly different unit cell and a sixfold helical structure.[9]

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The complexation of neutral carbohydrates with metal ions has been studied extensively.[10] For inulin, however, such data are not available. Cycloinulo-hexaose has shown a large selectivity with respect to the complexation with different metal ions.[11,12,13] Ba^{2+} was found to form the most stable complex. A comparable selectivity has been observed for permethylated cycloinulo-hexaose.[14,15] Suitable arranged combinations of two or three hydroxyl groups will result in significant complex-formation, but single hydroxyl groups cannot compete with the solvent.[10] This means that the complexation will be dependent on the conformation of the carbohydrate. Therefore, in this report the interaction between Ba^{2+} ions and isolated inulin oligomers with degree of polymerisation 2 - 12 was studied in search of a possible dependency of the conformational behaviour in relation to the Degree of Polymerisation (DP).

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For NMR analysis, inulin oligomers were separated using High Performance Anion Exchange Chromatography (HPAEC). Analysis with HPAEC and Pulsed Amperometric Detection (PAD) is very sensitive and shows a large resolution. However, quantitative analysis is difficult because the detector response can vary much depending upon the nature of the analyte, which often makes the isolation of standards necessary. Therefore, a gradient elution has been developed which enables the use of a refractive index (RI) detector.

RESULTS AND DISCUSSION

The selective precipitation of Chicory inulin with methanol yielded a filtrate which contained oligomers with a DP up to about 30 (Fig 2a), whereas the starting Chicory inulin contained oligomers with DP up to about 60. Dilution with one volume of water gives a stable (towards precipitation and microbial growth) clear solution which is suitable for automated fractionation on at least a gram scale using gel filtration on a P2 column. This fractionation resulted in several fractions with different $\overline{DP}$ and a smaller molecular mass distribution (Fig 2a).

These fractions were very suitable for separation into individual oligomers (Fig 2b) on High Performance Anion Exchange Chromatography (HPAEC) columns because of a more efficient use of the small capacity of these columns. Another

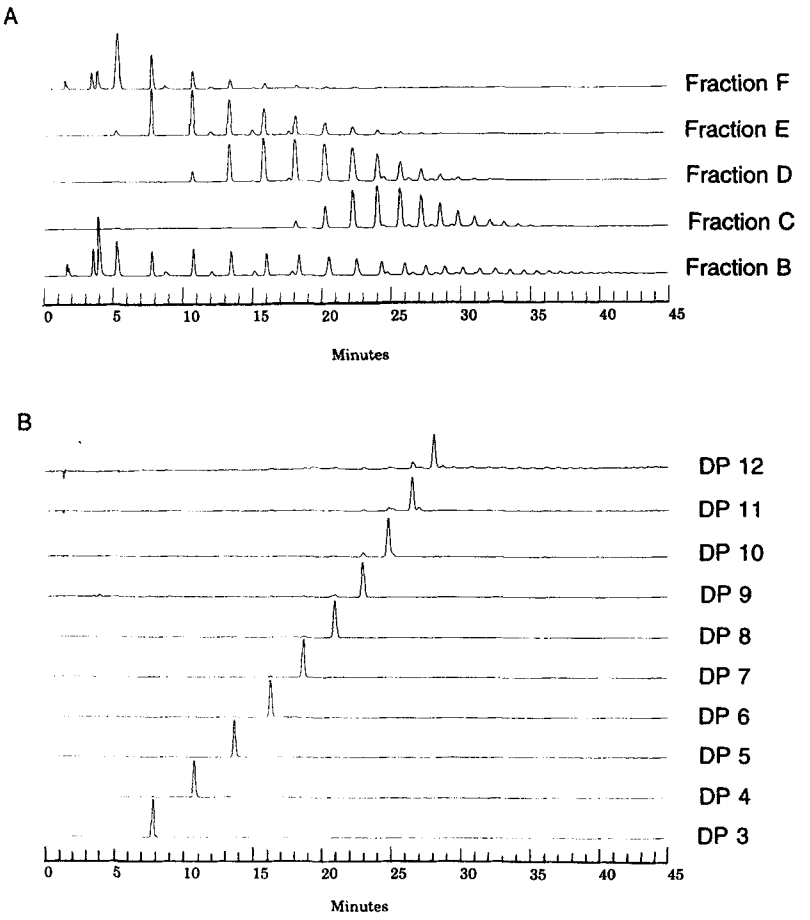

Fig. 2. HPAEC-PAD chromatograms of inulin fractions (A) and oligomers (B). Gel filtration (P-2 Bio Gel) of fraction B (liquid fraction, obtained after selective precipitation followed by filtration, of Chicory inulin) resulted in fractions C-F. HPAEC chromatography of fraction D yielded isolated oligomers with DP 5-12, and RP-18 HPLC chromatography of 'NEOSUGAR' yielded isolated oligomers with DP 3-4.

advantage of these fractions with smaller DP distributions is the possibility to apply a short gradient of eluents, allowing the isolation on HPAEC columns of up to 26 mg of isolated oligomers in less than one day.

Although HPAEC has the advantage of a high chromatographic resolution, it has the disadvantage of the necessity of a gradient which results in fractions with a large salt / carbohydrate ratio. Routinely, gel filtration is used after chromatography for

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desalting of carbohydrate oligomers with a DP higher than about 8. The application of RP-18 HPLC enabled fast desalting of small oligomers. Elution with water / 4% methanol allowed for the isolation of inulin oligomers with DP 4-12 from fractions with salt / carbohydrate ratios of up to 75. Because the carbohydrates are baseline separated from salts it can also be expected that much larger amounts of salt can be removed. For smaller oligomers the methanol content should be reduced and for much larger oligomers the methanol content should be increased.

Inulin oligomers with a DP of 5-12 were separated on a semipreparative HPAEC column equipped with a RI detector. Up to now only a PAD detector was used, because of the large acetate gradients and the high sensitivity. For making RI detection possible an acetate gradient was developed with a constant refractive index.

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In general, for HPAEC-PAD analysis an increasing sodium acetate gradient is applied in combination with a constant sodium hydroxide concentration. The sodium acetate concentration determines mostly the retention times of the oligomers and has no large influence on the detection. Sodium hydroxide is necessary for elution and detection. Because the sodium hydroxide concentration is not very critical, a gradient with constant refractive index could be developed which allows both RI and PAD detection. Therefore, the sodium acetate concentration should be increased in order to obtain the desired fractionation, and the sodium hydroxide concentration should be decreased in order to keep the refractive index constant. Application of this gradient enables comparison of RI- and PAD-responses of the compounds which are analyzed using HPAEC. Because the response of the RI detector depends mostly on the mass concentration of the analyte and not on the nature of the analyte, the PAD-response can be estimated for the detectable compounds. This led to a fast and convenient method for quantitative analysis of mixtures of oligomers which can be separated on HPAEC columns and detected using a PAD detector, without isolation of these oligomers (Timmermans *et al.*, unpublished results).

The combined use of RP-18 and HPAEC chromatography afforded the isolation of inulin oligomers with DP 3-12 (Fig. 2b) which were pure according to ^1H NMR spectroscopic results. For inulin oligomers with DP 2-12 ^1H NMR spectra were recorded. To these solutions increasing amounts of Ba^{2+} were added stepwise. After each step a ^1H NMR spectrum was measured. The chemical shift values of the

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Table 1. ^{1}H chemical shift values of inulin oligomers with DP 2-12. The values which are omitted could not be determined because of severe spectral overlap. The terminal fructosyl residue is designated as t and the fructosyl residues in the middle of the oligomer, which have not been assigned sequentially, are referred to as i or j.

a) DP 2: [Ba2+]	G-H2 G-H1	G-H3	G-H4	G-H5	G-H6,6′	F-H1,1′	F-H3	F-H4	F-H5	F-H6′	F-H6
0	5.410	3.553	3.757	3.465	3.841	3.813	3.673	4.210	4.047	3.885	3.820	3.804
0.0070	5.410	3.554	3.757	3.465	3.841	3.813	3.673	4.210	4.047	3.885	3.819	3.804
0.0237	5.410	3.554	3.758	3.465	3.841	3.813	3.673	4.210	4.047	3.885	3.819	3.803
0.0533	5.410	3.555	3.759	3.465	3.841	3.813	3.674	4.210	4.047	3.886	3.819	3.803
0.101	5.411	3.557	3.760	3.466	3.842	3.813	3.675	4.209	4.047	3.887	3.818	3.802
0.168	5.411	3.559	3.762	3.466	3.842	3.813	3.676	4.209	4.047	3.888	3.817	3.800
0.288	5.413	3.564	3.766	3.468	3.843	3.813	3.679	4.209	4.046	3.890	3.817	3.797
0.476	5.417	3.571	3.773	3.471	3.845	3.813	3.684	4.208	4.047	3.893	3.817	3.793
0.729	5.422	3.581	3.781	3.474	3.848	3.813	3.691	4.209	4.047	3.898	3.818	3.786

b) DP 3: [Ba2+]	G-H2 G-H1	G-H4	G-H5	F1-H1	F1-H1′	F1-H3	F1-H4	F1-H5	Ft-H1′	Ft-H1	Ft-H3	Ft-H4	Ft-H5
0	5.429	3.539	3.467	3.840	3.825	3.713	4.273	4.042	3.868	3.738	3.675	4.187	4.079	3.864
0.0070	5.429	3.539	3.466	3.840	3.825	3.714	4.273	4.042	3.868	3.738	3.675	4.187	4.079	3.864
0.0237	5.429	3.540	3.466	3.840	3.826	3.714	4.273	4.041	3.868	3.738	3.676	4.187	4.079	3.864
0.0533	5.430	3.540	3.467	3.841	3.826	3.714	4.272	4.041	3.869	3.738	3.677	4.187	4.079	3.865
0.101	5.431	3.542	3.467	3.841	3.826	3.715	4.272	4.041	3.870	3.738	3.678	4.187	4.079	3.865
0.168	5.433	3.544	3.467	3.841	3.827	3.716	4.271	4.041	3.872	3.739	3.680	4.187	4.080	-
0.288	5.437	3.549	3.468	3.843	3.830	3.718	4.270	4.041	3.876	3.739	3.685	4.188	4.081	3.870
0.476	5.445	3.558	3.469	3.845	-	3.722	4.267	4.042	3.883	3.740	3.692	4.191	4.082	-
0.729	5.458	3.571	3.471	3.847	3.834	3.730	4.265	4.043	3.886	3.743	3.702	4.196	4.084	3.875

c) DP 4: [Ba2+]	G-H2 G-H1	G-H4	F1-H1	F1-H3	F1-H4	F1-H5	F1-H6	Ft-H1′	Ft-H1	Ft-H3	Ft-H4	F2-H1	F2-H3	F2-H4
0	5.433	3.533	3.469	3.737	4.271	4.044	3.893	3.782	3.757	3.680	4.185	4.105	3.720	4.224	4.074
0.0060	5.434	3.534	3.468	3.737	4.271	4.044	3.893	3.782	3.757	3.680	4.185	4.105	3.721	4.224	4.074
0.0135	5.435	3.534	3.468	3.737	4.271	4.045	3.894	3.783	3.757	3.681	4.185	4.106	3.721	4.224	4.074
0.0372	5.438	3.535	3.467	3.739	4.269	4.044	3.895	3.784	3.757	3.683	4.186	4.106	3.723	4.225	4.075
0.123	5.454	3.541	3.463	3.746	4.263	4.047	3.903	3.787	3.756	3.693	4.190	4.110	3.733	4.229	4.078
0.186	5.464	3.544	3.460	3.751	4.258	4.047	3.906	3.789	3.756	3.699	4.193	4.112	3.740	4.231	4.079

d) DP 5: [Ba2+]	G-H2 G-H1	G-H4	F1-H3	F1-H4	Ft-H1	Ft-H3	Ft-H4	F2-H1′	F2-H3	F2-H4	F(t-1)-H1′	F(t-1)-H1	F(t-1)-H3	F(t-1)-H4
0	5.432	3.536	3.469	4.275	4.044	3.682	4.189	4.101	3.882	4.219	4.076	3.885	3.721	4.232	4.100
0.101	5.439	3.542	3.469	4.273	4.045	3.692	4.193	4.104	3.890	4.216	4.076	-	3.728	4.236	4.100
0.260	5.452	3.552	3.470	4.269	4.048	3.709	4.200	4.108	3.903	4.215	4.078	3.894	3.742	4.245	4.103
0.471	5.471	3.568	3.473	4.264	4.052	3.731	4.211	4.115	3.923	4.215	4.080	-	-	4.257	4.105
0.698	5.492	3.585	3.476	4.260	4.056	3.754	4.223	4.120	3.942	4.216	4.083	-	-	4.271	4.108
0.893	5.511	3.600	3.480	4.258	4.061	-	4.234	4.125	3.958	4.218	4.087	-	-	4.282	4.109

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e) DP 6:

[Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi-H3	F1-H4	Ft-H4	F2-H4	F(t-1)-H4	Fi-H4
0	5.431	3.539	3.470	3.687	4.281	4.189	4.224	4.239	4.231	4.045	4.104	4.075	4.099	4.096
0.101	5.436	3.542	3.469	3.694	4.278	4.193	4.225	4.242	4.231	4.045	4.105	4.077	4.100	4.097
0.259	5.446	3.550	3.469	3.707	4.274	4.201	4.228	4.250	4.232	4.046	4.109	4.080	4.102	4.100
0.471	5.464	3.564	3.469	3.723	4.269	4.213	4.232	4.261	4.235	4.049	4.114	4.085	4.106	4.106
0.698	5.481	3.577	3.470	-	4.265	4.224	4.237	4.271	4.239	4.052	4.119	4.090	4.109	4.109
0.893	5.500	3.590	3.471	-	4.263	4.235	4.242	4.280	4.244	4.056	4.122	4.096	4.111	4.113

f) DP 7:

[Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj2-H4	Fj3-H4
0	5.434	3.540	3.470	3.686	4.280	4.189	4.230	4.240	4.239	4.239	4.045	4.101	4.077	4.098	4.098	4.097
0.101	5.437	3.543	3.469	3.694	4.277	4.193	4.229	4.244	4.238	4.238	4.044	4.103	4.077	4.099	4.099	4.096
0.259	5.446	3.551	3.469	3.706	4.273	4.200	4.230	4.252	4.240	4.240	4.045	4.107	4.078	4.102	4.102	4.098
0.471	5.461	3.564	3.470	3.722	4.269	4.212	4.233	4.265	4.245	4.245	4.048	4.113	4.082	4.107	4.107	4.101
0.698	5.479	3.578	3.471	3.743	4.265	4.224	4.238	4.277	4.246	4.250	4.051	4.118	4.087	4.111	4.111	4.104
0.893	5.495	3.590	3.472	3.758	4.263	4.234	4.242	4.286	4.250	4.255	4.054	4.122	4.091	4.115	4.112	4.106

g) DP 8:

[Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	Fi3-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj2-H4 F(j3→j4)-H4
0	5.434	3.541	3.471	3.686	4.280	4.189	4.230	4.242	4.247	4.242	4.242	4.044	4.101	4.076	4.097	4.097 4.097
0.101	5.438	3.544	3.470	3.694	4.277	4.193	4.230	4.243	4.248	4.243	4.243	4.045	4.103	4.077	4.098	4.098 4.098
0.259	5.445	3.551	3.470	3.706	4.273	4.200	4.231	4.252	4.252	4.243	4.243	4.045	4.107	4.079	4.100	4.100 4.100
0.471	5.458	3.563	3.470	3.722	4.269	4.211	4.234	4.263	4.255	4.247	4.247	4.047	4.112	4.082	4.103	4.103 4.103
0.698	5.475	3.576	3.471	3.738	4.266	4.222	4.239	4.274	4.259	4.253	4.253	4.050	4.117	4.086	4.107	4.107 4.107
0.893	5.490	3.588	3.471	3.754	4.263	4.231	4.243	4.283	4.263	4.258	4.253	4.053	4.121	4.089	4.110	4.106 4.110

h) DP 9:

[Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	Fi3-H3	Fi4-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj2-H4 F(j3→j5)-H4
0	5.435	3.541	3.471	3.686	4.279	4.189	4.232	4.240	4.253	4.248	4.240	4.244	4.045	4.097	4.076	4.097	4.097 4.097
0.101	5.438	3.544	3.470	3.694	4.277	4.193	4.231	4.244	4.253	4.250	4.241	4.244	4.045	4.103	4.077	4.097	4.097 4.097
0.259	5.445	3.551	3.470	3.706	4.273	4.200	4.232	4.253	4.253	4.253	4.243	4.246	4.045	4.107	4.078	4.099	4.099 4.099
0.471	5.458	3.562	3.471	3.722	4.269	4.211	4.236	4.263	4.258	4.258	4.247	4.247	4.249	4.047	4.113	4.081	4.104 4.101 4.104
0.698	5.482	3.580	3.471	3.738	4.265	4.226	4.243	4.278	4.265	4.265	4.251	4.257	4.052	4.119	4.088	4.109	4.104 4.109
0.893	5.498	3.594	3.472	3.753	4.263	4.234	4.246	4.285	4.268	4.268	4.254	4.263	4.055	4.122	4.091	4.111	4.106 4.111

i) DP 10:

[Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	Fi3-H3	F(i4→i5)-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj3-H4 F(j3→j6)-H4
0	5.434	3.542	3.472	3.686	4.279	4.189	4.232	4.245	4.254	4.248	4.240	4.240	4.045	4.102	4.076	4.097	4.097 4.097
0.101	5.437	3.545	3.471	3.694	4.276	4.193	4.232	4.249	4.254	4.249	4.241	4.244	4.045	4.104	4.077	4.097	4.097 4.097
0.259	5.445	3.551	3.471	3.706	4.273	4.200	4.234	4.256	4.256	4.252	4.243	4.247	4.046	4.107	4.078	4.099	4.099 4.099
0.471	5.458	3.562	3.471	3.721	4.269	4.211	4.236	4.262	4.257	4.257	4.247	4.250	4.047	4.112	4.082	4.102	4.102 4.102
0.698	5.476	3.576	3.472	3.740	4.266	4.222	4.241	4.274	4.262	4.262	4.251	4.256	4.050	4.117	4.086	4.106	4.103 4.106
0.893	5.491	3.588	3.473	3.755	4.261	4.231	4.245	4.282	4.266	4.266	4.252	4.261	4.054	4.121	4.090	4.110	4.106 4.110

(continued)

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Table 1. Continued.

j) DP 11: [Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	Fi3-H3	F(i4⋯i6)-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj2-H4	F(j3⋯j7)-H4
0	5.435	3.542	3.472	3.687	4.279	4.190	4.233	4.245	4.255	4.248	4.240	4.240	4.046	4.097	4.077	4.097	4.097	4.097
0.100	5.438	3.545	3.472	3.694	4.277	4.194	4.233	4.251	4.255	4.250	4.242	4.244	4.046	4.104	4.077	4.098	4.098	4.098
0.259	5.445	3.551	3.471	3.706	4.274	4.200	4.232	4.255	4.257	4.253	4.243	4.247	4.046	4.107	4.078	4.099	4.099	4.099
0.471	5.458	3.563	3.471	3.722	4.270	4.211	4.236	4.262	4.262	4.258	4.247	4.250	4.048	4.112	4.081	4.102	4.102	4.102
0.698	5.475	3.577	3.472	-	4.266	4.222	4.242	4.274	4.266	4.263	4.250	4.256	4.051	4.117	4.086	4.106	4.104	4.106
0.893	5.491	3.589	3.472	-	4.263	4.230	4.245	4.282	4.270	4.266	4.252	4.261	4.054	4.121	4.090	4.110	4.106	4.110

k) DP 12: [Ba2+]	G-H1	G-H2	G-H4	Ft-H1	F1-H3	Ft-H3	F2-H3	F(t-1)-H3	Fi1-H3	Fi2-H3	Fi3-H3	F(i4⋯i7)-H3	F1-H4	Ft-H4	F2-H4	Fj1-H4	Fj2-H4	F(j3⋯j8)-H4
0	5.435	3.542	3.472	3.687	4.279	4.190	4.233	4.245	4.255	4.248	4.240	4.240	4.046	4.102	4.076	4.097	4.097	4.097
0.101	5.438	3.545	3.471	3.694	4.277	4.193	4.232	4.250	4.255	4.250	4.242	4.244	4.045	4.104	4.077	4.097	4.097	4.097
0.259	5.445	3.552	3.471	3.708	4.273	4.201	4.233	4.257	4.257	4.253	4.243	4.247	4.046	4.108	4.078	4.099	4.099	4.099
0.471	5.459	3.564	3.471	3.723	4.270	4.212	4.237	4.263	4.259	4.259	4.248	4.252	4.048	4.112	4.081	4.102	4.102	4.102
0.698	5.476	3.576	3.471	-	4.267	4.223	4.242	4.274	4.264	4.264	4.252	4.257	4.051	4.117	4.088	4.109	4.105	4.109

glucosyl H-1,2,4 and the fructosyl H-3,4 signals and some other signals were determined for each $BaCl_2$ concentration (Table 1). A plot of the chemical shift of a particular proton of a certain oligomer as a function of the $BaCl_2$ concentration, which will be referred to as titration curve, is depicted in Fig. 3.

For interpretation of the ^1H NMR signals of the various oligomers assignments available from literature were used.[2,16,17,18] In comparison with cycloinulo-hexaose[11,13] the chemical shifts of the protons of the inulin oligomers did not change very much upon increasing the Ba^{2+} concentration. The largest absolute value of the slope of the titration curves of the H3 protons was 40 times smaller than the initial slope of the titration curve of the H3 protons of cycloinulo-hexaose. This implied that the interaction between Ba^{2+} and inulin oligomers was much weaker than with cycloinulo-hexaose. The chemical shift of the protons of inulin oligomers with DP 2-12 was found to be linear with the Ba^{2+} concentration. This can be explained by assuming that one Ba^{2+} ion formed a complex with one inulin oligomer as was found for cycloinulo-hexaose, but the complexes are much weaker because no saturation was observed. The small changes in chemical shift suggested a much higher value for the dissociation constant than the 36 mmol/L for the cycloinulo-hexaose-Ba^{2+} complex. Linear regression of the titration curves for some selected protons resulted in slopes as depicted in Fig. 4 and Fig 5 (Table 2).

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The addition of Ba^{2+} caused a change in chemical shift but did not introduce new signals, indicating that the interaction between Ba^{2+} and the inulin oligomers is fast on the NMR time scale. The coupling constants of the protons did not change significantly upon addition of Ba^{2+}. Consequently, it can be concluded that the ring conformations did not change by complexation with Ba^{2+}.

In Fig. 3 the titration curves of the H3 protons of the different fructosyl residues of the oligomers with DP 2-12 are depicted. From these curves it can be concluded that, in the case of oligomers with a DP larger than 5, the change in chemical shift depended more on the localisation of the fructosyl residue in the oligomer than on the DP of the oligomer. Because all fructosyl rings have the same $^4T_3$ conformation[18] this suggested that a complex between Ba^{2+} and the fructosyl residues alone did not play a major role. Apparently, the conformation of the backbone, formed by the glycosidic linkages, was important for the interaction with Ba^{2+}.

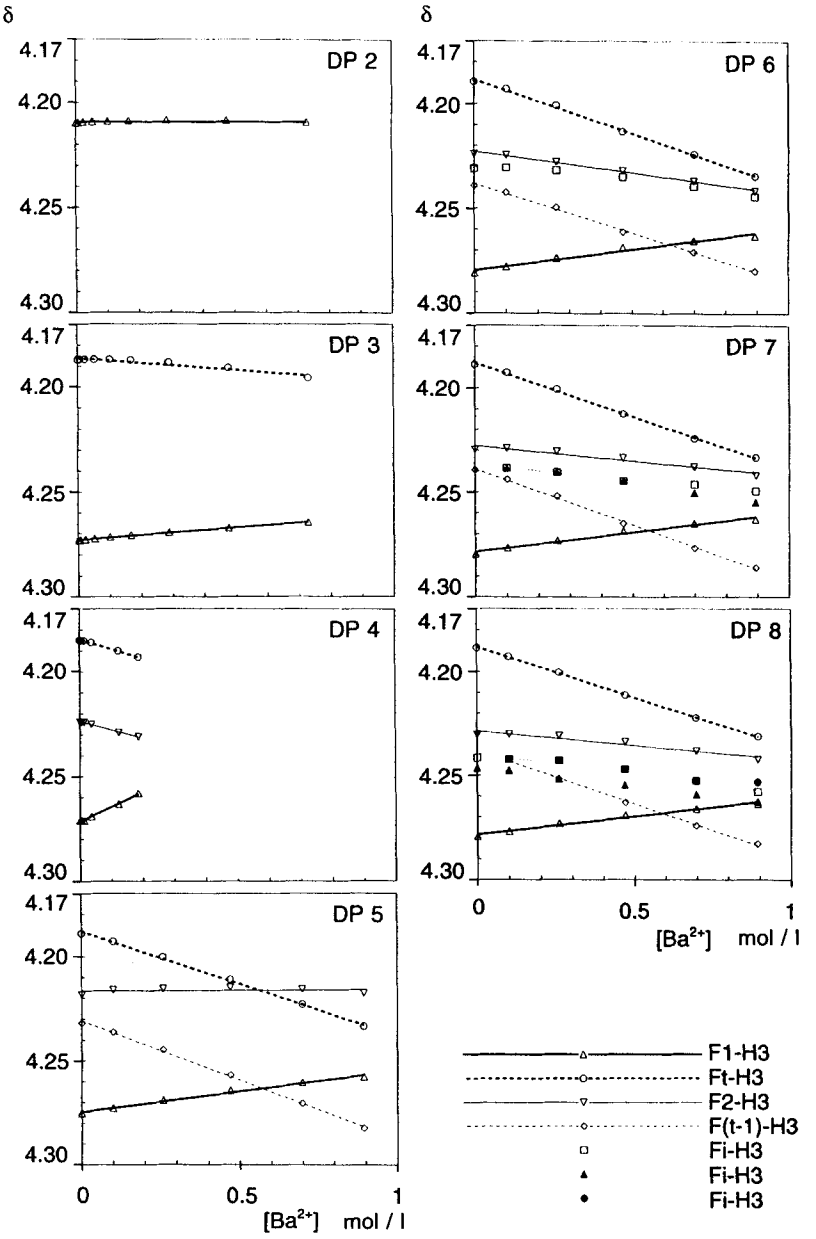

Fig. 3. Chemical shift dependency on the Ba^{2+} concentration (titration curves) of the fructosyl H-3 atoms of the inulin oligomers DP 2 - DP 8. Ft is the terminal fructosyl residue, F1 is the fructosyl moiety adjacent to the glucosyl residue, and the Fi residues are located in the middle of the oligomer chain.

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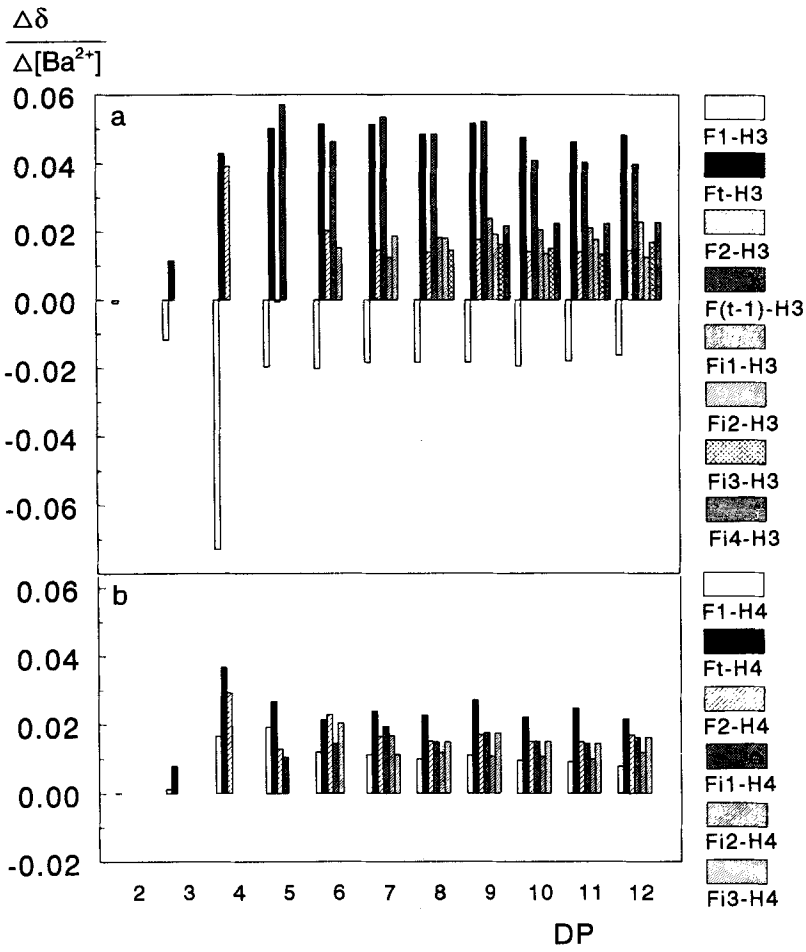

Fig. 4. Slope of the titration curves of the fructosyl H-3,4 ^1H NMR signals of inulin oligomers with DP 2-12.

In the case of the fructosyl H3 atoms the largest dependency of the chemical shift on the Ba^{2+} concentration (Fig. 4a) was found for the F1-H3 signal of DP 4, which was much larger than for the other oligomers. The slopes of the titration curves of the other fructosyl H3 signals showed the largest values for DP 4 or DP 5, and were approximately constant for larger oligomers. Furthermore, the slopes of the titration curves of the fructosyl rings in the middle of the oligomers (Fi) were much lower than for the terminal fructosyl rings and had opposite signs compared to the fructosyl residue adjacent to the glucosyl moiety (F1).

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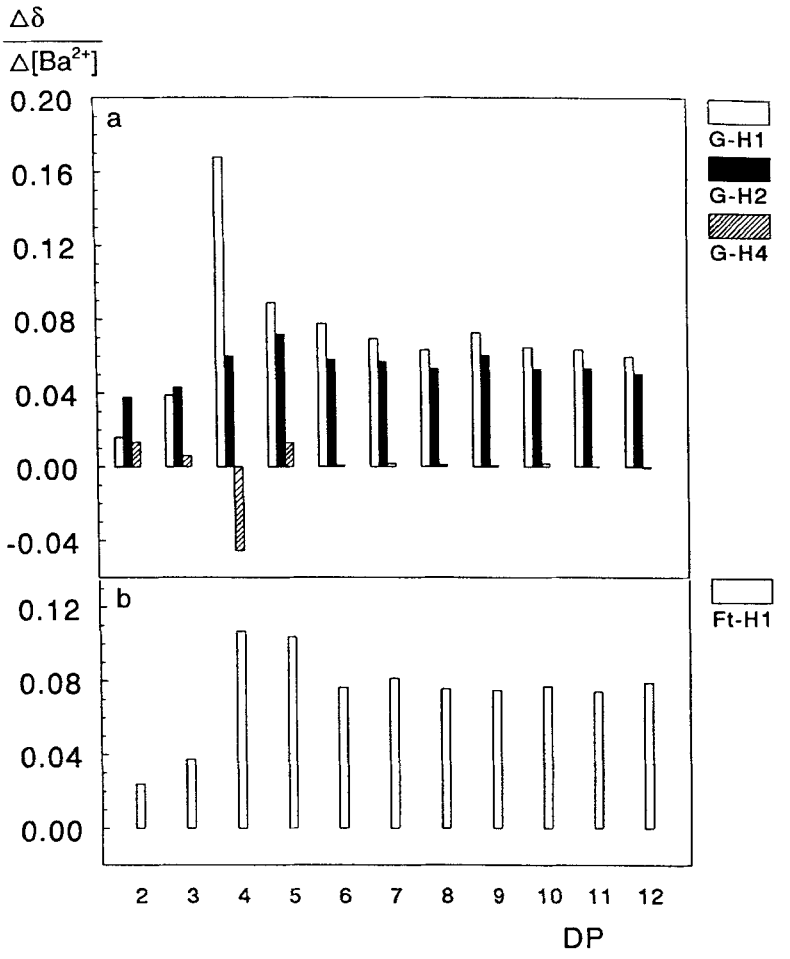

Fig. 5. Slope of the titration curves of the glucosyl H-1,2,4 and terminal fructosyl H-1 ^{1}H NMR signals of inulin oligomers with DP 2-12.

The chemical shift values of the fructosyl H4 signals had an increasing dependency on the Ba^{2+} concentration (Fig. 4b) upon increasing the DP from 2 to 4 or 5. For longer oligomers the Ba^{2+} dependency of the chemical shift decreased.

The slope of the titration curves is depicted for the glucosyl H1, H2 and H4 atoms in Fig. 5a, and for the titration curve of Ft-H1 in Fig. 5b. Clearly, the relation between the chemical shift of a certain proton and the Ba^{2+} concentration was strongly dependent on the DP, and the slopes of the titration curves had the largest value for DP 4 or DP 5.

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The strongly increasing dependency of the proton chemical shift values of inulin oligosaccharides on the Ba^{2+} concentration, when the DP increased from 2 to about 4 to 5, suggests that oligomers with a DP up to 5 can adopt structures resembling the conformation of cycloinulo-hexaose. For larger oligomers the chemical shift alterations were significantly smaller and approximately constant. The decreased interaction with Ba^{2+} can be explained by a change in conformational behaviour for longer oligomers.

This change around DP 5 is in agreement with a model based on molecular mechanics calculations for DP 5 combined with ^{13}C NMR measurements of DP 4 - 9[4] and molecular dynamics calculations on DP 4.[18] Small oligomers are flexible and can adopt (part of) a helical structure with a small pitch. Contrarily, oligomers with DP 6 and higher cannot adopt this preferred conformation for Ba^{2+} complexation, because of severe steric interaction between the ends of the oligomers which would result in a higher pitch. Presumably, these oligomers form helical structures which become more rigid on enlargement of the DP, due to the larger interaction between the fructosyl residues of subsequent turns. This might explain a conformational change around DP 7 to 8 into rigid structures, which was suggested on the basis of chromatographic behaviour[19] and measurements of rotation of the polarisation plane or ^{1}H relaxation.[2] For inulin oligomers with DP up to at least DP 8 the described structures do not necessarily represent the predominant conformation for inulin oligomers, as was concluded for oligomers up to at least DP 9 based on variations in ^{13}C chemical shift values.[4]

The weak interaction of the small inulin oligomers with Ba^{2+} in comparison with cycloinulo-hexaose may be a direct result of the higher flexibility of these structures. However, it can not be excluded that only a small fraction of these oligomers adopt flat bended conformations which behave like cycloinulo-hexaose. Because the interaction between Ba^{2+} and inulin oligomers is very weak it is reasonable to assume that the conformation of these oligomers does not change significantly upon complexation.

CONCLUSIONS

A method was developed to relate PAD responses to Refractive Index (RI) responses which allows quantitative HPAEC-PAD analysis without isolation of

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Table 2. ^{1}H chemical shift dependency on Ba^{2+}, in 10^{-3} ppm.l/mol, for inulin oligomers with DP 2-12.

DP 2: G-H1	G-H2	G-H3	G-H4	G-H5	G-H6,6′	F-H1,1′	F-H3	F-H4	F-H5	F-H6′	F-H6		
16	38	33	13	10	-1	24	-1	0	18	-3	-24		
DP 3: G-H1	G-H2	G-H4	G-H5	F1-H1′	F1-H1	F1-H3	F1-H4	F1-H5	Ft-H1′	Ft-H1	Ft-H3	Ft-H4	Ft-H5
39	43	6	10	12	22	-12	2	27	7	37	12	8	016
DP 4: G-H1	G-H2	G-H4	F1-H1	F1-H3	F1-H4	F1-H5	F1-H6	Ft-H1′	Ft-H1	Ft-H3	Ft-H4	F2-H1	F2-H3	F2-H4
168	60	-45	76	-73	17	73	39	-9	107	43	37	106	39	29
DP 5: G-H5	G-H2	G-H4	F1-H3	F1-H4	Ft-H1	Ft-H3	Ft-H4	F2-H1′	F2-H3	F2-H4	F(t-1)-H1′	F(t-1)-H1	F(t-1)-H3	F(t-1)-H4
89	72	13	-20	19	104	50	27	86	-1	13	35	83	57	11

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DP 6:	G-H2		F4-H1		Ft-H3		F(t-1)-H3	F1-H4		F2-H4		Fi-H4				
G-H1		G-H4		F1-H3		F2-H3		Fi-H3		Ft-H4		F(t-1)H4				
78	58	1	76	-20	51	20	46	15	12	21	23	14	20			
DP 7:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		Ft-H4		Fj1-H4		Fj3-H4		
G-H1		G-H3		F1-H3		F2-H3		Fi1-H3		F1-H4		F2-H4		Fj2-H4		
69	57	2	81	-18	51	15	53	12	19	11	24	17	19	17	11	
DP 8:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		F1-H4		F2-H4		Fj2-H4		
G-H1		G-H4		F1-H3		F2-H3		Fi1-H3		Fi3-H3		Ft-H4		Fj1-H4		F(j3→j4)-H4
63	53	1	76	-18	48	14	49	18	18	15	10	23	15	15	12	15
DP 9:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		Fi4-H3		Ft-H4		Fj1-H4		F(j3→j5)-H4
G-H1		G-H4		F1-H3		F2-H3		Fi1-H3		Fi3-H3		F1-H4		F2-H4		Fj2-H4
73	60	1	75	-18	52	18	52	19	24	16	22	11	27	17	17	11	17
DP 10:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		F(i4→i5)-H3		Ft-H4		Fj1-H4		F(j3→j6)-H4
G-H1		G-H4		F1-H3		F2-H3		Fi1-H3		Fi3-H3		F1-H4		F2-H4		Fj2-H4
65	53	2	77	-19	48	14	41	14	21	15	22	10	22	15	15	11	15
DP 11:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		F(i4→i6)-H3		Ft-H4		Fj1-H4		F(j3→j7)-H4
G-H1		G-H4		F1-H3		F2-H3		Fi1-H3		Fi3-H3		F1-H4		F2-H4		Fj2-H4
63	53	0	74	-18	46	14	40	18	21	13	22	9	25	15	14	10	14
DP 12:	G-H2		Ft-H1		Ft-H3		F(t-1)-H3	Fi2-H3		F(i4→i7)-H3		Ft-H4		Fj1-H4		F(j3→j8)-H4
G-H1		G-H4		F1-H3		F2-H3		Fi1-H3		Fi3-H3		F1-H4		F2-H4		Fj2-H4
59	50	-1	79	-16	48	15	40	12	23	17	23	8	22	17	16	12	16

standards. Using reversed phase (RP-18) chromatography enabled fast isolation of inulin oligomers with DP 4-12 from fractions with large salt / carbohydrate ratios.

Comparison of the ^1H NMR chemical shift changes, induced by the addition of Ba^{2+}, of DP 2-12 with each other and with cycloinulo-hexaose suggested that cyclic conformations which resemble that of cycloinulo-hexaose may play a role for inulin oligomers with DP smaller than 6. For larger oligomers this conformation is probably perturbed via interactions between the beginning and the end of the oligomer resulting in a weaker interaction with Ba^{2+}.

EXPERIMENTAL

Fractionation of inulin. Inulin from Chicory was fractionated by selective precipitation, by keeping a solution of 50 g inulin and 50 mL methanol in 250 mL water at room temperature (about 20 °C) for 18 h. The precipitate (Fraction A: 29.1 g, $\overline{DP}$=16.4 and dispersion (D) =1.47) was removed by filtration and the filtrate (Fraction B: $\overline{DP}$=4.9/D=1.94) was diluted with one volume of water and was fractionated on a P-2 Bio Gel column (300 x 50 mm) using water as eluent and a flow rate of 7 mL/min at room temperature. For each run 2 g oligomers were injected, and continuous automated fractionation was achieved by the use of a gradient controller for injection of the diluted filtrate and a fraction collector. This resulted in four fractions with a smaller DP distribution: $\overline{DP}$=10.9/D=1.05, $\overline{DP}$=7.7/D=1.08, $\overline{DP}$=4.9/D=1.17, and $\overline{DP}$=2.8/D=1.35 (Fraction C, D, E, and F, respectively).

Isolation of inulin oligosaccharides. A mixture of inulin oligosaccharides with degree of polymerisation (DP) 3, 4, and 5 ('NEOSUGAR' obtained from Meiji Seika Ltd.) was fractionated,[20] at room temperature, using a RP-18 HPLC column of 300 x 50 mm (prep Nova-Pak, 6 μm particles) and water / 1% methanol as eluent. During the total run time of 36 min the flow rate was kept at 50 mL/min. For each run 0.4 g was injected (using a 10% m/v solution) and fractions of 100 mL, and 175 mL were collected for DP 3, and DP 4, respectively. Lyophilisation yielded the isolated oligomers which had a purity of 99 %, and 96 % according to HPAEC-PAD[19] for DP 3, and DP 4, respectively.

The separation of individual oligomers with DP 5-12 was performed using High Performance Anion Exchange Chromatography (HPAEC). For each run 27 mg inulin

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fraction D (with $\overline{DP}$=7.7) was injected on a Carbopac PA1 column (9 x 250 mm) equipped with a Waters 410 differential refractometer. A gradient with increasing sodium acetate concentration and decreasing sodium hydroxide concentration was applied in order to keep the refractive index constant. The gradient is running from solution A to solution B in 30 min, followed by elution with solution B for 10 min. To solution A (2.30 mol NaOH in 5 L water) water was added (462 mL) until it had exactly the same refractive index as solution B (0.494 mol NaOH / 1.50 mol NaAc in 5 L water). The fractions (2-11 mL) of six runs were combined and neutralised with 10% acetic acid. The salt/carbohydrate ratio varied from 38 to 75. Each combined fraction was desalted on a RP-18 column (300 x 50 mm) by elution with water / 4 % methanol (50 mL/min), in less than 20 min for each sample, and analyzed using HPAEC-PAD. Lyophilisation yielded 10.6 mg DP 5 (purity: 99 %), 17.2 mg DP 6 (purity: 98 %), 21.5 mg DP 7 (purity: 96 %), 26.4 mg DP 8 (purity: 98 %), 16.7 mg DP 9 (purity: 92 %), 10.6 mg DP 10 (purity: 90 %), 5.5 mg DP 11 (purity: 79 %), and 2.5 mg DP 12 (purity: 79 %).

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NMR spectroscopy. ^{1}H NMR spectra were recorded on a BRUKER AMX-400-WB (ATO-DLO/RIKILT-DLO, Wageningen) spectrometer at 300 K. For each spectrum 16 scans of 32 K data points were accumulated with a spectral width of 2000 Hz. Chemical shifts (δ) are expressed in ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured relative to internal acetone (2.225 ppm). Solutions of 5.1 mg DP 2, 4.8 mg DP 3, 5.5 mg DP 4, 6.3 mg DP 5, 10.3 mg DP 6, 13.0 mg DP 7, 15.9 mg DP 8, 10.1 mg DP 9, 6.4 mg DP 10, 3.3 mg DP 11, and 1.5 mg DP 12 in D_2O (99.9 atom %D) were lyophilised and each residue was dissolved in 0.55 mL D_2O (99.96 atom %D). A solution of $BaCl_2$ in D_2O (with known concentration) was added stepwise to these solutions. After each step a ^{1}H NMR spectrum was recorded. For DP 2 and DP 3 $BaCl_2$ concentrations of 0, 0.0237, 0.0533, 0.101, 0.168, 0.288, 0.476, and 0.729 mol/L were used, and for DP 4 the $BaCl_2$ concentrations were 0, 0.0060, 0.0135, 0.0372, 0.123, or 0.186 mol/l. Concentrations of 0, 0.101, 0.259, 0.471, 0.698, and 0.893 mol/L $BaCl_2$ were used for DP 5-12. The chemical shifts of some selected protons were determined for each $BaCl_2$ concentration.

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