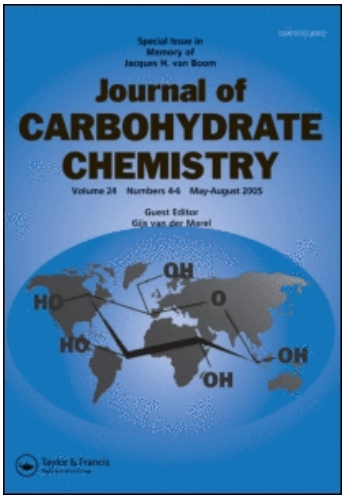

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Liu, Jianhua , Waterhouse, Andrew L. and Chatterton, N. Jerry(1994) 'Do Inulin Oligomers Adopt a Regular Helical Form in Solution?', Journal of Carbohydrate Chemistry, 13: 6, 859 — 872
To link to this Article: DOI: 10.1080/07328309408011686
URL: http://dx.doi.org/10.1080/07328309408011686

J. CARBOHYDRATE CHEMISTRY, 13(6), 859-872 (1994)

DO INULIN OLIGOMERS ADOPT A REGULAR HELICAL FORM IN SOLUTION?[1]

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Received July 28, 1993 - Final Form May 5, 1994

ABSTRACT

The ^{13}C NMR spectra of inulin oligomers in D_2O with degree of polymerization (DP) of 3 through 9, along with two other inulin oligomer mixtures of average DP = 17 and DP = 31 were recorded. Significant variations in the chemical shift of some fructofuranose carbon signals indicates that unlike glucans, simple helical structures are not the predominant conformation for inulin oligomers—at least up to DP = 9. Models of the DP = 5 oligomer show that it should prefer a single helical conformation which however, would not be accessible to longer DP oligomers due to severe steric interactions.

INTRODUCTION

Inulin (Figure 1) is in a class of compounds named fructans with the structural feature of (2→1) linked β-D-fructofuranosyl units usually terminated by a (2↔1) linked α-D-glucopyranoside unit. It occurs in numerous plants, and apparently acts as a storage carbohydrate.[2] It has a structural feature that may be unique among carbohydrates—the subunit (fructose) rings are not part of the polymer backbone, and this has important implications in its conformational structure. A better understanding of the inulin

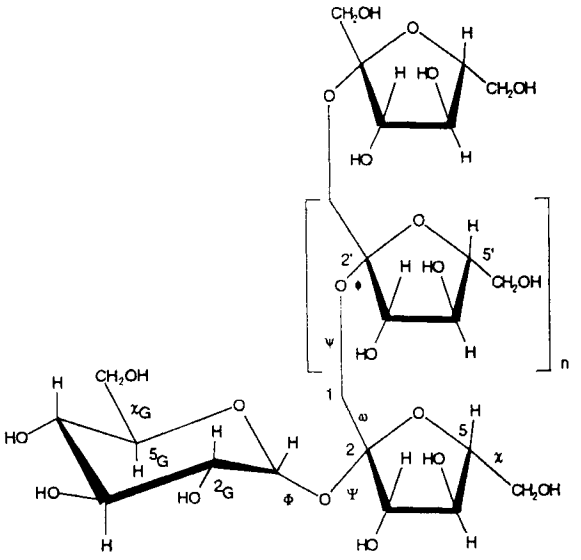

Figure 1. Structure of Inulin Oligomers. Nystose, n = 1; 1,1,1-kestopentaose (DP = 5), n = 2; 1,1,1,1-kestohexaose (DP = 6), n = 3; 1,1,1,1,1-kestoheptaose (DP = 7), n = 4; 1,1,1,1,1,1-kestooctaose (DP = 8), n = 5; 1,1,1,1,1,1,1-kestononaose (DP = 9), n = 6. Φ = H-C1$_G$-O2-C2, Ψ = C1$_G$-O2-C2-O5, ϕ = C1-O2′-C2′-O5′, ω = O5-C2-C1-O2′, χ = O5-C5-C6-O6

oligomer/polymer conformation may help to explore the biological role of this class of compounds in plants as well as in clinical uses since inulin is almost universally used in measuring the glomerular filtration rate in renal physiological examinations.[3]

Although NMR spectroscopy has long been used to identify plant fructans, complete ^{1}H and ^{13}C NMR assignments of inulin oligomers are rare. ^{13}C NMR assignments of 1-kestose, nystose and inulin polymer were reported some time ago,[4,5] with limited accuracy and completion. It was not until recent years that the unambiguous and complete ^{1}H and ^{13}C NMR chemical shift assignments of 1-kestose,[6] nystose[7,8] and 1,1,1-kestopentaose,[7,9] (nystose with one more (2→1) linked β-D-fructofuranosyl unit) have been reported. ^{13}C NMR spectra of inulin oligomers of DP = 2 to 5, along with that of the DP = 6 & 7 mixture, were reported.[10] Other available data include recent X-ray diffraction studies of nystose[11,12] and a study of crystalline inulin by X-ray and electron

diffraction.[13] There is a recent report on studies on the conformational aspects of inulin oligomers by ^{1}H NMR.[14]

In contrast to the few reports on inulin oligomers, there is much previous work on the structural features of glucose homooligomers.[15,16,17,18]

Carbohydrates have been extensively studied by various modeling techniques, but studies on larger oligomers are rare due to limitations of both the available software and computing power. Molecular mechanics modeling results of inulin oligomers are available for inulobiose,[19] 1-kestose[20] and nystose.[9,21]

RESULTS

1. ^{13}C NMR spectra analysis

The ^{13}C NMR spectra of the inulin oligomers (taken in D_2O) with DP of 4 through 9, along with mixtures that have higher DP averages, the cold water soluble and insoluble inulin samples from Jerusalem Artichoke, are presented in Figure 2. The chemical shift assignments for nystose (DP = 4) indicated in Figure 2 are obtained from previous work.[7]

It is evident that all the signals from the glucopyranoside unit are essentially unchanged with the increase in DP. This enables us to identify one previously missed signal, the C-6 of the glucopyranoside unit, in the earlier study by Jarrell *et al.*[4] Importantly, this also suggests that the inter-residue interactions between the glucopyranoside unit and the fructofuranosyl units remain unchanged as the oligomer/polymer chain increases in length.

If the inulin oligomer or polymer forms a helical structure, then the internal repeating units should possess the same conformation and the internal residues should experience the same chemical environment. Consequently, the ^{13}C signals of the same carbon in the repeating units should coalesce. This is true for α- and β-(1→4)-D-glucose homooligomers,[15] in which signal coalescence is observed at DP = 5. For maltooligomers,[22] the ^{13}C NMR chemical shifts do not vary with the degree of polymerization in the range of DP = 4 through 13 and are identical with those measured for monodisperse amyloses of DP = 35 and 4000. This is not the case for inulin oligomers.

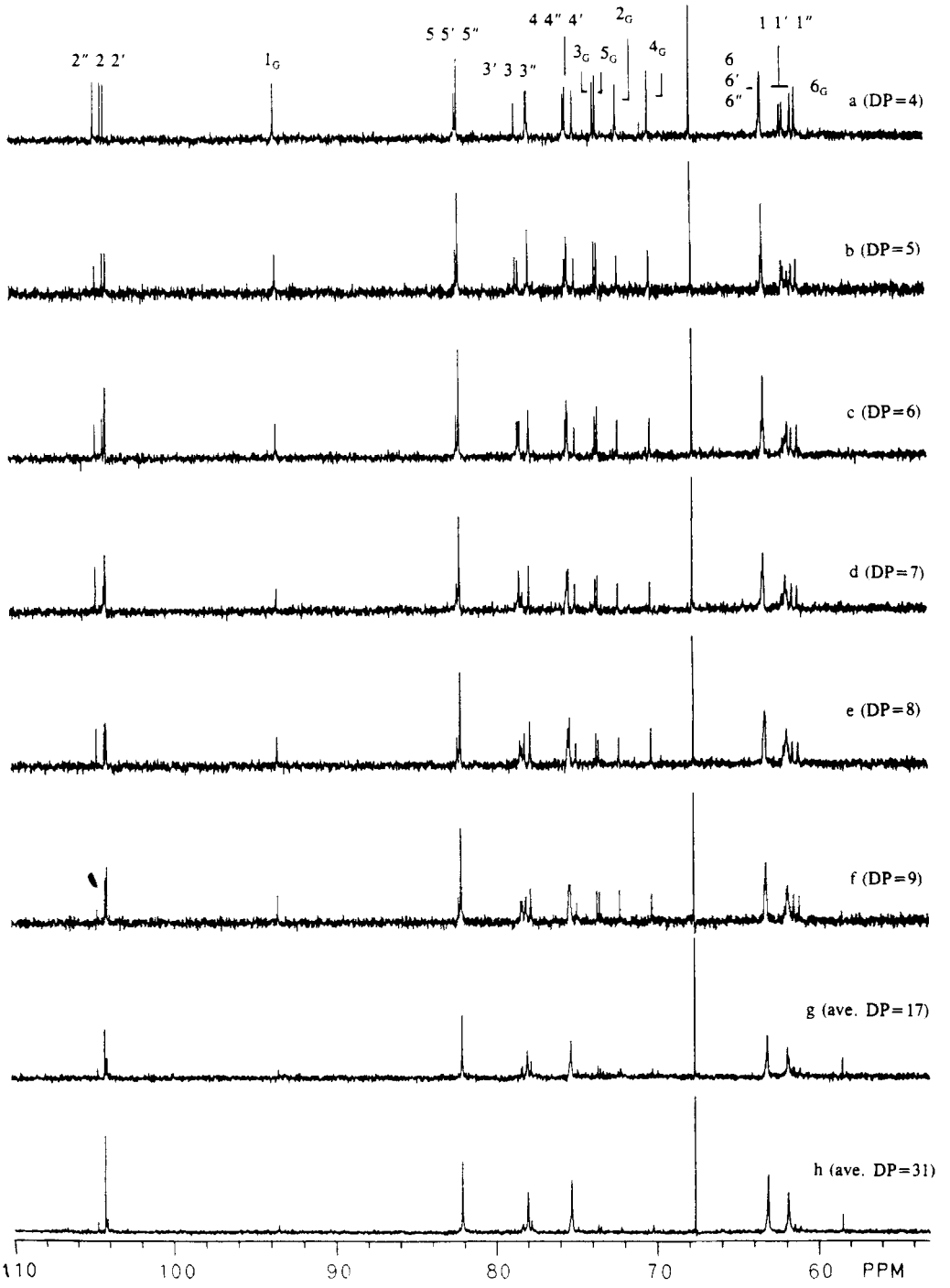

Figure 2. ^{13}C NMR spectra of the inulin oligomers: a) nystose, b) 1,1,1-kestopentaose, c) 1,1,1,1-kestohexaose, d) 1,1,1,1,1-kestoheptaose, e) 1,1,1,1,1,1-kestooctaose, f) 1,1,1,1,1,1,1-kestononaose, g) cold water soluble inulin, h) cold water insoluble inulin.

From Figure 2 one can see that signals from the C-5's of all the β-D-fructofuranosyl units except the terminal one have the same chemical shift starting from DP = 4. Signals from the C-6's of the β-D-fructofuranosyl units are similar at all DP values but not exactly the same (indicated by peak shoulders and line broadening). Signals from the C-2's and C-4's of the β-D-fructofuranosyl units form single but relatively broad peaks at the higher DP values. Signals from C-1's appear to start overlapping when the DP reaches 7. However, within the DP range of 4 through 9, none of the C-3 signals show any tendency to coalesce into one peak, even a broad peak.

Since C-3's of β-D-fructofuranosyl units are the ring carbons closest to the linkage bonds, they are strongly affected by the characteristics of both the linkage and the ring. Any perturbation on OH-3 (*e.g.*, variations in hydrogen bonding) will induce a change in the chemical shift of C-3 as well as possibly altering the fructofuranose ring shape. The variation in the chemical shifts of C-3's of the β-D-fructofuranosyl units clearly indicate such perturbation. This perturbation will also produce different H-3-H-4 coupling constants for each β-D-fructofuranosyl unit, and this was previously observed for DP = 5 (Ref. 7).

It is very interesting to notice that at DP = 6 and 7 the coincident peaks of the fructofuranose C-1, C-2 and C-3 shift downfield by about 0.1 ppm. At this same DP length, the proton T_1 relaxation times of the H-2 and H-4 of the α-D-glucopyranoside reported by Oka *et al.*[14] change very noticeably. The conclusion of those investigators was that at this point, a complete turn of the helix was formed. However there may be an alternate explanation for the changes observed in the ^1H and ^{13}C spectra, as noted below.

2. Molecular modeling of 1,1,1-kestopentaose.

For a molecule with the size of DP = 5, it is currently impractical to do a complete evaluation of all the geometry parameters by molecular mechanics. Some compromises have to be taken into account to obtain results of reasonable accuracy and with reasonable computer time. In this study, (a) the center bond ψ of the fructose-fructose linkages is set to close to 180° and allowed to relax during optimization. This is generally suitable for the three-bond inulin-type linkages—inulobiose,[19] 1-kestose[20] and nystose[21] models all prefer the *anti*-periplanar position. (b) The ring shape of each

residue in the molecule was set to the preferred $^4T_3$ and other possibilities were not tested.[23] (c) The orientations of secondary alcohol groups were treated as secondary factors and therefore not tested.

A kestopentatose (Figure 1, n = 2) starting structure model was assembled with in-house code and optimized by MM3(92). In the driver run (Run A), all linkage angles except the central angle (ψ) of the fructose-fructose linkage were driven through the three staggered positions with the exception of the glucose-fructose linkage angle Φ which was driven through three positions of -30°, 0° and 30°. There were 729 driver runs with 3 x 3 data points each, or 6561 structures in total.

Results from Run A were tabulated and sorted according to the steric energies calculated by MM3(92). Those structures with the steric energies within 15 kcal/mole of the lowest energy were further optimized without any restriction (Run B). The number of single structures optimized in Run B was 3117. This generated a list of low-energy structures in which one structure had the steric energy of 85.3237 kcal/mole (Table I). Since the next lowest energy structure is about 2.3 kcal/mole higher in steric energy than that of the lowest energy one, or less than 2% of its population, only the lowest energy structure was further studied in the side group orientation run (Run C). In Run C all the primary alcohol groups were driven through the three staggered positions, yielding 729 data points. The resulting structures were then further optimized without any restriction. The results of part of Run C are listed in Table II.

Results from Run B (Table I) show that the preferred linkage angles for glucose-fructose linkages are -60° for Ψ and mostly about -30° for Φ, which are in good agreement with the results from the modeling of 1-kestose[20] and nystose.[21] For all the fructose-fructose linkages, the ϕ angles have strong preferences toward -60° and the ω angles prefer 180° with some accessible structures preferring -60°. Similar to the case of inulobiose,[7] ϕ angles at 60° increase the interaction between the CH_2 groups on the linkages and the hydrogens on C6 of the same fructose units while ϕ angles at 180° put the linkage CH_2 groups close to the two alcohol groups on C1 and C3 of the fructose units. Contrary to the findings from inulobiose,[7] and 1-kestose,[20] the ω angles tend to populate one form due to the prolonged oligomer chain. Still, at the terminal linkage, the ω angle of F3-F4 is still flexible enough that any one of the three staggered positions is energetically accessible.

Table I A list of the unique structures from Run B, tabulating the energies and linkage angles of DP = 5 inulin oligomer (4 kcal/mole above the minimum).

Energy	Φ	Ψ	ω(F1-F2)	ϕ(F1-F2)	ω(F2-F3)	ϕ(F2-F3)	ω(F3-F4)	ϕ(F3-F4)
85.3237	-38.24	-69.65	178.38	-67.79	-176.60	-67.00	-179.53	-73.21
87.6260	-28.36	-59.85	-59.88	-66.23	-177.45	-66.04	-176.44	-68.14
87.6638	-36.78	-68.14	-179.52	-66.26	-176.57	-58.95	57.02	-59.18
87.7443	-9.10	-55.54	-60.57	-67.24	-177.61	-65.77	-176.00	-69.96
88.1807	-53.63	-93.43	-178.95	-75.62	-60.44	-60.87	57.04	-63.12
88.2840	-34.09	-68.09	-179.31	-63.92	-173.52	-60.51	-63.28	-62.88
88.4564	-20.19	-61.37	-178.20	-70.03	-175.11	-59.88	55.82	-60.04
88.6269	-38.93	-65.13	52.39	-61.49	-176.99	-69.53	-176.42	-69.64
88.7961	-17.71	-61.76	-177.59	-67.15	-172.24	-59.42	-64.21	-64.04
88.9798	-33.83	-68.05	-178.86	-62.74	-58.92	-54.87	59.30	-60.56
89.1169	-9.03	-54.96	-176.67	-70.18	53.03	-61.89	52.44	-62.48
89.1411	-39.39	-70.49	178.58	-67.47	-176.45	-66.88	179.24	52.34
89.1646	-27.11	-62.69	-178.31	-61.35	-61.84	-60.32	-177.50	-64.56
89.2259	-14.57	-57.54	-175.66	-66.46	-64.57	-60.47	-177.68	-63.86
89.3089	-7.77	-54.50	-177.00	-69.61	54.88	-65.29	-176.31	-65.59

Since there was one structure from Run B with a steric energy 2.3 kcal/mole lower than any other structure, only this one was selected for side-group orientation calculations (Run C). Table II indicates that there are many energetically accessible structures which differ only by the positions of those primary alcohol groups. The glucose χ_G angle prefers to be at -60° (gt) and 60° (gg). All the fructose χ-6 groups prefer 60°. Putting the χ-6 angles at -60° will place the primary alcohol groups on top of the fructose rings while an angle of 180° will create inter-residue repulsion between the side group and the secondary alcohol group on C3. The χ-1 angle on the terminal fructose unit is relatively free to rotate but for the same reason mentioned above, -60° is not preferred.

To see if a helical polymer could be formed, those structures with steric energies within 1 kcal/mole of the lowest energy structure in Table II were used to create polymers and calculate the helical structure parameters of the resulting inulin polymer, N, number of units per turn, and H, translation distance for each helical turn.[24] The helices were very similar and the average value for these particular linkage angles was N = 3.91 and H = 1.18 (left-handed). This small H value indicates that the proposed

Table II The evaluation of side group flexibility of DP = 5 inulin oligomer for final refinement of the global minimum, in energy rank order. The coordinates of the global minimum structure may be obtained at enos2.ucdavis.edu (128.120.76.24) in /pub/fruc8 *via* anonymous ftp on internet.

Energy	Φ	Ψ	ω(F1-F2)	ϕ(F1-F2)	ω(F2-F3)	ϕ(F2-F3)	ω(F3-F4)	ϕ(F3-F4)	χ(Glu.)	χ6(F1)	χ6(F2)	χ6(F3)	χ6(F4)	χ1(F4)
84.6569	-38.35	-73.10	-179.70	-68.24	-176.90	-68.37	179.68	-72.95	63.53	67.16	68.29	68.32	68.42	-178.67
84.9355	-38.48	-73.16	-179.77	-68.29	-176.97	-68.17	179.89	-73.12	63.49	67.18	68.28	68.31	68.38	-68.23
84.9483	-39.03	-70.01	178.53	-68.03	-176.85	-67.29	-179.00	-72.78	-68.03	66.45	68.27	68.22	68.25	-178.67
84.9799	-38.50	-73.15	-179.74	-68.22	-176.96	-68.34	179.49	-72.99	63.47	67.18	68.29	68.30	68.54	49.88
85.1465	-39.24	-70.02	178.70	-68.19	-177.04	-67.48	-179.55	-72.92	-68.34	66.41	68.25	68.22	68.23	-68.30
85.1975	-39.22	-69.99	178.71	-68.10	-177.01	-67.56	-179.97	-72.88	-68.42	66.41	68.25	68.20	68.37	49.76
85.4995	-38.69	-73.39	-179.34	-67.92	-176.95	-69.63	179.93	-73.10	63.56	67.24	68.31	-66.35	68.14	-178.61
85.5004	-38.32	-72.80	-179.70	-69.36	-176.45	-68.24	179.54	-73.33	63.30	67.07	-66.34	68.27	68.46	-178.57
85.5716	-38.78	-69.89	178.78	-68.23	-177.01	-67.79	179.91	-73.02	170.88	66.64	68.29	68.28	68.36	-178.65
85.6442	-38.76	-73.28	-179.59	-68.29	-176.48	-67.96	179.81	-74.85	63.50	67.25	68.29	68.36	-66.79	-178.51

helical structure is not practical. After a complete turn of the helix was formed, there would have been some short contacts between the first and the fifth, the second and the sixth residues, etc. These results are similar to French's who elaborated inulin polymers from crystal-based models of the inulobiose fragments linkages in nystose.[21] In that study, the F2-F3 linkage angles are similar to ours and result in collisions between the first and sixth residue. On the other hand French found that extending the F1-F2 linkage resulted in a collision-free helix, but that linkage has a ϕ angle of 62° and that linkage had such a high relative energy in our MM3 study that it was not found in any of our searches for low energy linkage forms.

DISCUSSION

Based on our model, an inulin oligomer would be expected to experience perturbations when DP = 6, and that is what is observed, both from the ^{13}C spectra as reported here, and from ^{1}H spectra.[14]

^{13}C NMR chemical shifts are insensitive to long range through space interactions. Thus, it is unlikely that the changes in carbon chemical shifts can be produced by the formation of a complete turn of a helix as proposed by Oka *et al.*, since this helix is proposed to have a distance of 10 Å between helix turns.[14] This is supported by the fact that the chemical shifts of the α-D-glucopyranoside carbons are unchanged.

Although T_1 relaxation time is more sensitive to long range through-space interactions, the proposed pitch of 10.8 Å is most likely too large to produce the strong interaction observed. In addition, if a regular helix were the predominant conformation, then the shortened T_1 relaxation time observed for DP = 6 would have persisted at larger DP values. However, the relaxation time of the aforementioned protons lengthened at DP greater than 6, suggesting that the DP = 6 case is unique.

Thus, it appears that at DP = 6 the length of the oligomer chain is of an appropriate length to allow the terminal β-D-fructofuranosyl unit to form intra-residue hydrogen bonds, most likely with one of the β-D-fructofuranosyl units, as the chemical environment of the glucose unit is unchanged. However, as the oligomer increases in size it appears that this specific interaction diminishes.

CONCLUSION

Coalescence of ^{13}C NMR signals does not establish a helical conformation in solution. The time-averaging process in the NMR time-scale could yield coalesced signals for completely mobile, randomly oriented molecules, as has been suggested for malto-oligomers.[22] However, the lack of coalescence seen here definitely indicates that up to DP = 9, a simple, regular helix is not the predominant conformation of inulin oligomers in aqueous solution, nor are they completely randomly oriented mobile oligomers.

The most likely explanation for all observations to date is the existence of a perturbed helix with a single conformation or a few populated conformations. Comparing the experimental data with the modeling results suggest the existence of segments of oligomer that have the low energy linkage form observed in our calculations, interrupted by breaks. It is conceivable that the observed variations in the C-3 NMR signals of each fructose unit could arise in a regular inulin helix simply from end effects, but at DP = 9, this explanation seems very unlikely. This observation is in stark contrast to (1→4) glucan oligomers which form a regular helix with as few subunits as four to five and have coalesced NMR signals.

If plants can increase their viability with a soluble source of respiration energy under cold conditions then fructans appear to provide such a source. This hypothesis is supported by the observation that in cold tolerant plants the average DP length of fructans drops at the onset of cold weather to produce material that is more cold water soluble.

EXPERIMENTAL

Pure fructan oligomers were separated from lyophilized Jerusalem artichoke tissues. Multiple 5 g samples of tissue were each extracted three times with boiling water (230 mL per extraction). Samples were centrifuged (30 min at 15,000 g) after each extraction and the supernatants were pooled. Soluble proteins were precipitated from supernatants by adding 1 mL of 10% lead acetate per 100 mL and heating to 80 °C. The samples were allowed to stand overnight, centrifuged (30 min at 15,000 g), concentrated

by flash evaporation and deionized with Dowex-1 and -50W. The ion exchange columns were previously preconditioned with 2 N NH_4OH and washed with deionized water until the pH was neutral. Fructan extracts were concentrated under negative pressure to about 50% W/V sucrose equivalents as estimated by refractive index.

Approximately 6-mL aliquots of the concentrated extract were applied to a Toyopearl HW-40S TSK gel column (4.8 cm x 240 cm). Deionized and degassed water was pumped through the column (bottom to top) at room temperature. The elution of fructan from the column was monitored with an inline Waters R403 differential refractometer. Fructans of DP = 4 through DP = 9 were separated as individual peaks. Eluted fractions from multiple runs were pooled by DP and rechromatographed three times or until preparations containing fructan of a single DP as determined by anion exchange chromatography and pulsed amperometric detection (Dionex Series 4000).

Cold water soluble and cold water insoluble fructan fractions were prepared by placing a complete Jerusalem artichoke fructan extract at 4 °C overnight. The insoluble fructan (average DP = 31) precipitated at 4 °C and was separated from the soluble fraction (average DP = 17) by centrifugation. Average DP was estimated by measuring the glucose to fructose ratio following hydrolysis. Chromatographic data showed a broad DP range for both the cold water soluble (DP 7-29) and cold water insoluble (DP 11-41) samples (Figures 3a and 3b).

For inulin oligomers with DP = 4 through 9, a 20 mg sample was dissolved in 1.5 mL of D_2O and transferred to a 5 mm NMR tube. For the cold water soluble and insoluble inulins, 50 mg samples were used. For the cold water insoluble sample, a warm water bath was used to dissolve the sample, and then it was allowed to reach room temperature. The NMR spectrum was then promptly taken before the sample precipitated.

^{13}C NMR spectra were recorded using a QE-300 (General Electric) NMR spectrometer with a 5-mm broad-band probe operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C. When carrying out the Fourier transform, neither line-broadening nor resolution enhancement was applied.

The initial structure of a DP = 5 inulin oligomer was optimized by MM3(92). Dihedral angle driver option 4 was used throughout the driver studies. The default termination criterion of 0.00008N kcal/mole, where N is the number of atoms in the

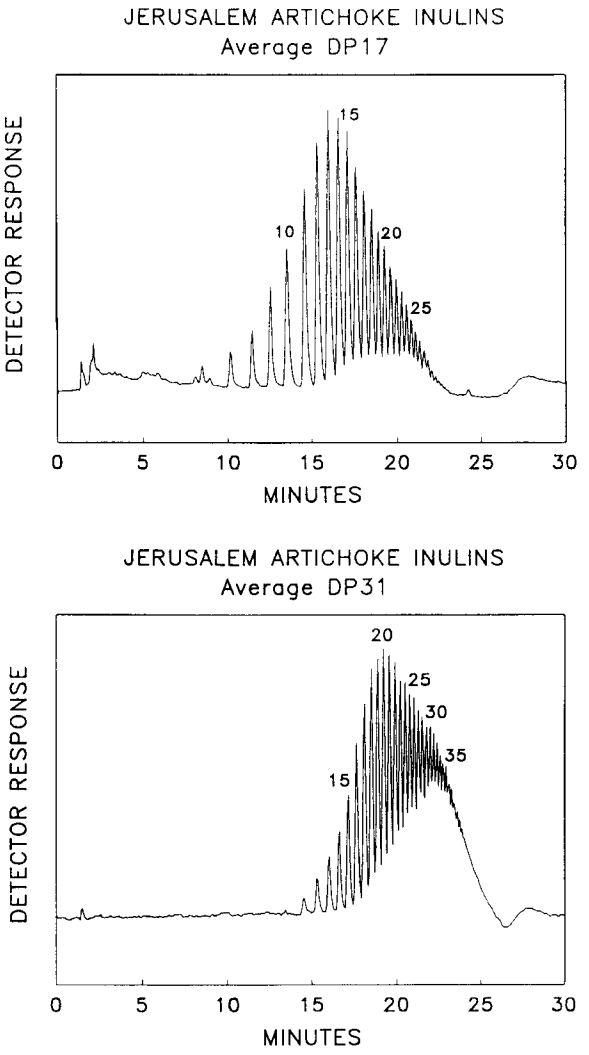

Figure 3. Chromatographic profile of the a) cold water soluble inulin (ave. DP = 17), and b) cold water insoluble inulin (ave. DP = 31), obtained from Jerusalem Artichoke.

molecule, was used. A dielectric constant of 80 was used in this study in order to take the water solution environment into consideration.[25] In all calculations, energy optimization options was used instead of geometry optimization. The MM3(92) steric energies were directly used to compare the different conformers and are the energies reported herein. All calculations were carried out on an IBM RISC/6000 350 Power

Station. The average CPU time for each 3 x 3 driver calculation was about 10 min and for each full optimization about 2 min.

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