Studies on the Conformational Aspects of Inulin Oligomers

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The conformational studies of inulin oligomers from G-F_2 to G-F_9, which isolated from *Platycodon grandiflorum*, suggested a plausible conformational change between G-F_7 and G-F_8 from the trends in their chemical shift patterns and molecular rotation; the oligomers higher than G-F_8 would form some secondary conformations more rigid than shorter oligomers. On the other hand, spin-lattice relaxation (T_1) studies of the protons proposed through-space interactions of 2- and 4-H's of glucose moiety in G-F_5, presumably with some atom(s) of the terminal fructose moiety. This would reflect that the inulin molecule adopts a 5/1 helix.

Keywords inulin; inulin oligomer; conformation; molecular rotation; NMR; spin-lattice relaxation time (T_1)

Inulin is a glucofructan with β-2→1 linked D-fructose, and occurs mainly in the underground parts of some members of the Compositae and Campanulaceae families. Next to starch, this polysaccharide is the most abundant carbohydrate reserve in the plant kingdom. Because of its inertness to the human body, it is extensively used for determining glomerular filtration rates in renal physiological examinations.[1] It should be noted that inulin or its degradation product has recently attracted attention in the immunological sciences because of its immunological activities.[2,3]

Since the polydispersity and degree of polymerization of inulin depend highly on the species, the vegatational periods of the original plant, and preparation methods,[4,5] the conformation of inulin and even its fundamental physicochemical properties such as molecular weight are still obscure because of the difficulty in obtaining pure compounds.[6–8] Thus, the preparation of pure inulin oligomers is prerequisite for any type of investigation of inulin.

A successful isolation of inulin oligomers (G-F_n, n=2—9, see Fig. 1) allowed us to investigate the conformation of the inulin oligomers mainly by using 500-MHz nuclear magnetic resonance (NMR). The information about these oligomers should be indispensable for understanding the structure and conformational properties of inulin itself.

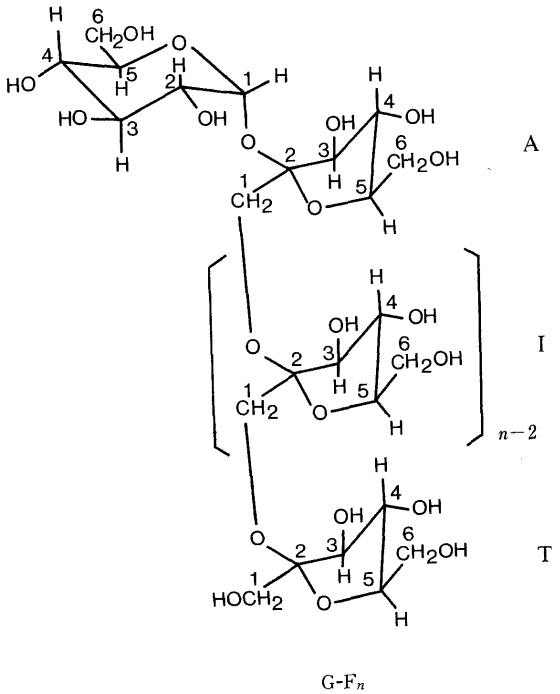

Fig. 1. Structure of Inulin Oligomer (G-F_n)

A, fructose unit adjacent to glucose; I, internal fructose unit; T, terminal fructose unit; n, degree of polymerization.

Experimental

Isolation of Inulin Oligomers The inulin oligomers were isolated from Chinese bellflower (*Platycodon grandiflorum* A. DC.) collected at the herb garden in the university as follows: The fresh root material was minced and extracted with 70% aqueous ethanol. The extract was evaporated to dryness *in vacuo*, redissolved in water and deionized with mixed-bed ion exchange resin (BioRad AG501 × 8). Active charcoal was added to the deionized aqueous solution and the charcoal was packed in a column, eluted successively with a water–ethanol mixture in the stepwise gradient of ethanol from 0 to 100% in 10% steps. The 10–30% ethanol eluate, which contained the desired oligomers (G-F_n, n=2—12), was chromatographed on a charcoal column (Darco G-60 and Celete 535 1:1 mixture, 4 cm i.d. × 20 cm), and eluted with aqueous ethanol to recover the oligomers, G-F_2 to G-F_{12}, in 15% ethanol eluate (*ca.* 800 ml). This oligomer mixture was then successfully separated into its components by medium pressure column chromatography with aminopropyl-bonded silica gel (YMC-NH_2, 1.7 cm i.d. × 75 cm) with acetonitrile–water (1:1) as shown in Fig. 2. Each component was further purified on a semi-preparative μ-Bondapack NH_2 column (0.78 cm i.d. × 30 cm, Waters Associates, Shodex RI detector model SE-11) with acetonitrile:water=7:3 to obtain pure oligomers (G-F_2—G-F_9). G-F_2 to G-F_9 were used for the following experiment, as the amounts of G-F_{10} to G-F_{12} were insufficient.

Prior to the NMR studies, each oligomer was treated with D_2O, followed by lyophilization; this was repeated twice. The amounts of each sample used for the NMR experiments were *ca.* 7 mg of each oligomers dissolved in 0.5 ml of high purity D_2O (99.96 atom% D, Merck).

Instruments High resolution NMR spectra were obtained on a GN-500 (General Electric) with a 1280 data processor and a 16 bit ADC. H,H-correlated spectroscopy (COSY) spectra were obtained by the so-called COSY45 pulse sequence in the absolute value mode, and H,C-COSY spectra were obtained in a phase-sensitive manner by the modified pulse sequence of A. Bax *et al.*[9] which was provided from General

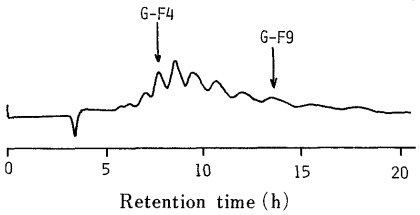

Fig. 2. Elution Profile of Inulin Oligomers (G-F_2—G-F_9)

Column, aminopropyl-bonded silica gel (YMC-NH_2, 1.7 cm i.d. × 75 cm); mobile phase, acetonitrile–water (1:1).

Erectronic NMR Instruments. Chemical shifts were measured in ppm from the internal acetone signal (2.05 ppm, obtained under the same condition with interanl sodium 2,2,3,3-tetradeutero-3-trimethylsilyl-propionate (TSP) as the reference standard). The spin-lattice relaxation time (T_1) of the protons was estimated based on the inversion–recovery method.

Molecular rotations of the oligomers were measured as an aqueous solution on a JASCO mode IDIP-181 polarimeter at 24 °C at the D line.

Results and Discussion

Several analytical methods are available for studying the conformation of compounds in question. X-Ray crystallography is the most direct method when the compound is crystallized. As a solution, the optical rotation is related to the spatial contribution of all functional groups, taking the conformational features of the molecule into account. Therefore, plotting the molecular rotation, *i.e.*, the optical rotation calculated with the molecular weight factor, can be a practical method to detect conformational changes among homooligomers in solution. As long as each monomeric unit in the oligomer takes continuous and constant spatial conformation independent of the degree of polymerization, molecular rotation and the degree of polymerization should be in an almost linear relationship.[10] On the other hand, whenever a conformational change occurs at a certain degree of polymerization, there should again be a linear relationship, but with a different slope from the previous one, following a particular degree of polymerization. Thus, there would be a point of discontinuity in the relation. In fact, in the case of inulin oligomers up to G-F_9, the 10-mer, the molecular rotation decreased almost linearly up to G-F_7, then the slope became drastically different for G-F_8 and higher oligomers, as shown in Fig. 3. This would probably be due to a conformational change; oligomers smaller than G-F_7 take a non-rigid conformation and may exist as a dynamic equilibrium of some energetically stable conformers, *e.g.*, folded or extended types, whereas the oligomers larger than G-F_8 start taking a certain, rather rigid conformation, with a limited back-bone chain movement.[11] In addition to this drastic change, a small change at around G-F_3/G-F_4 was observed.

High-field, high-resolution NMR is also one of the most powerful tools for conformational studies in a solution, compensating for X-ray studies when combined with computing logic: so-called distance geometry analysis.

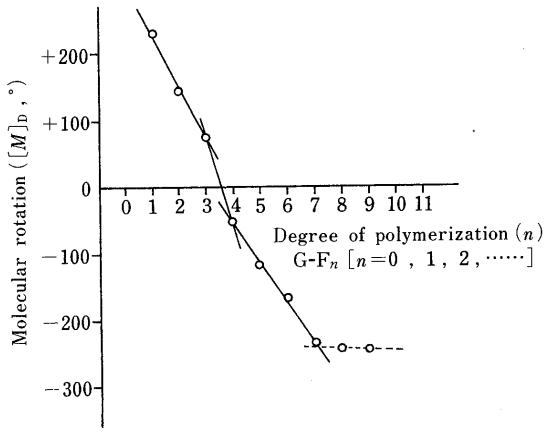

Fig. 3. Relationship between Molecular Rotation and Degree of Polymerization in Inulin Oligomers (G-F_2—G-F_9)

This technique is extensively applied to conformational studies of oligo- and polypeptides,[12] particularly of those with important physiological activities. However, direct application of the same logic to the conformational analyses of peptides to oligosaccharides is more difficult because in contrast to oligopeptides, whose protons are rather well spread in a wider range in chemical shifts, many magnetically similar protons exist in oligosaccharide molecules, and most of them would be overlapped in its ^{1}H-NMR spectra. Actually, in the case of inulin oligomers, only a few protons, 3- and 4-H's of fructose units and 1-, 2-, and 4-H's of glucose residue, were well resolved and therefore practically suitable for studying the conformational properties of the oligomers. On the other hand, the rest of the protons were all overlapped, hence almost useless (for example see Fig. 4c). On top of that, conformational studies of inulin oligomers are particularly

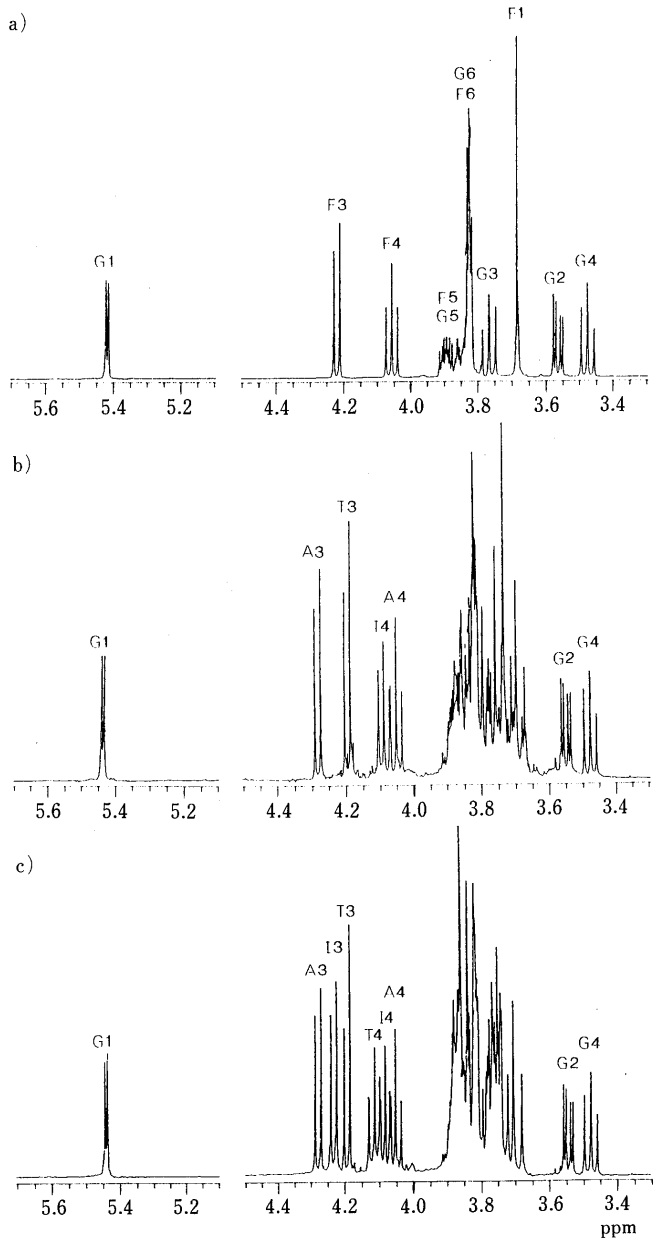

Fig. 4. 500-MHz ^{1}H-NMR Spectra of Inulin Oligomers in D_2O at 20 °C

a) Sucrose, b) kestose (G-F_2), c) nystose (G-F_3). Spectral width, ±3125 Hz. A, fructose unit adjacent to glucose; I, internal fructose unit; T, terminal fructose unit.

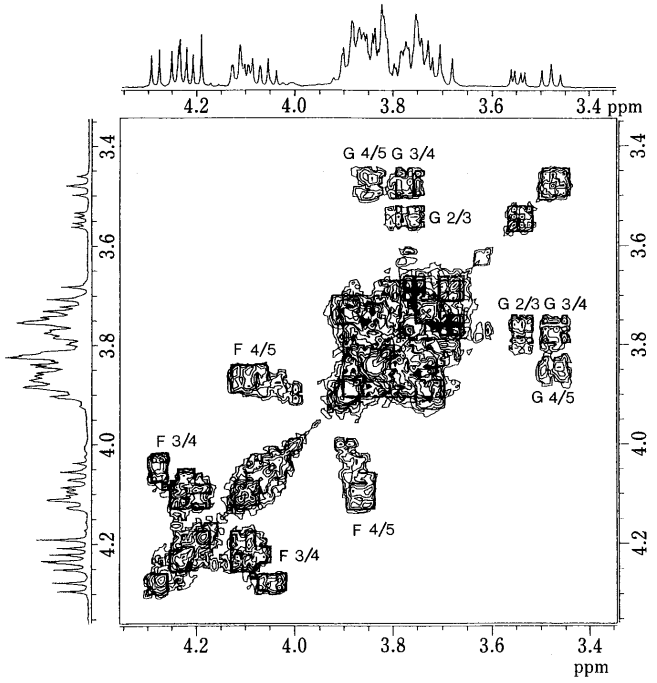

Fig. 5. H,H-COSY Spectrum of Nystose (G-F_3) in D_2O at 20 °C

Anomeric proton region was omitted. Spectral width, ±1000 Hz for ω_1 and ω_2.

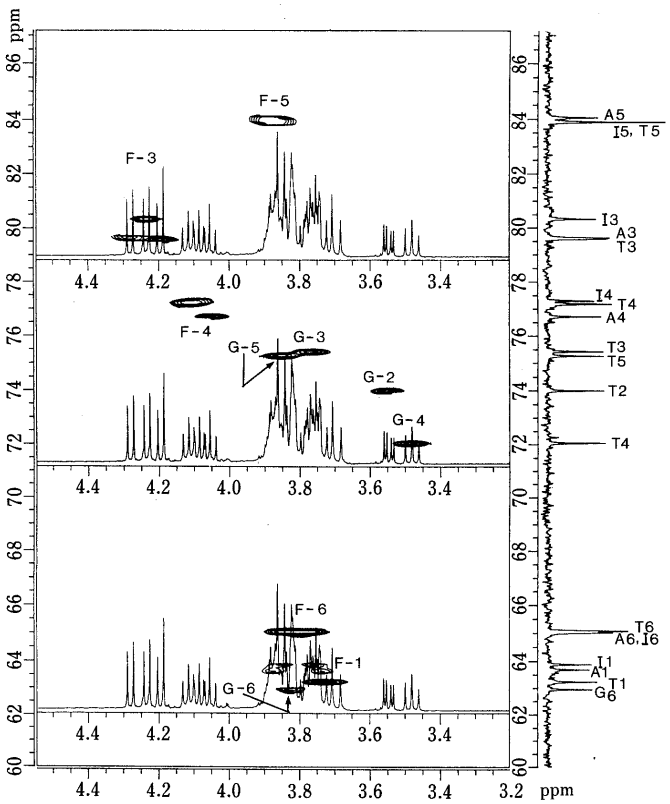

Fig. 6. H,C-COSY Spectrum of Nystose (G-F_3) in D_2O at 20 °C

Anomeric proton/carbon region was omitted. Spectral width, ±781 Hz for ω_1 and ±4167 Hz for ω_2. A, fructose unit adjacent to glucose; I, internal fructose unit; T, terminal fructose unit.

difficult because of a lack of anomeric protons which can work, if present, as reporters of the conformation around the glycosidic linkages participating in the formation of the back-bone (O–C2–C1–O) of the inulin molecule. Nevertheless, extensive studies were attempted, despite the limited number of exploitable proton signals, since somewhat distinct differences can be expected in any of the NMR parameters if any conformational changes occurred among the oligomers. H,H-COSY spectra, as well as normal spectra, were obtained for all the inulin oligomers, and some of the typical spectra are shown in Fig. 4 [normal spectra of G-F (*i.e.*, sucrose), G-F_2, and G-F_3] and Fig. 5 (H,H-COSY spectrum of G-F_3). Resonances for the α-D-glucopyranosyl residue of these oligomers were readily assigned by following the connectivities of protons starting from the anomeric doublet proton at 5.41, and the assignments were confirmed by comparing the data for sucrose.[13] On the other hand, all protons on the fructose moieties except 3- and 4-H's occur overlapped within a very narrow range (*ca.* 3.7—3.9 ppm), together with 3-, 5-, and 6,6,-H's of the glucose moiety, so that no first-order analysis was made for them. Therefore, only for the 3- and 4-H's of the fructose moiety were analyses of NMR parameters possible, and the same was true for the 1-, 2-, and 4-H's of glucose residue.

In G-F_2 (1-kestose) spectra (Fig. 4b), H,H-COSY indicated that the two sets of signals, 4.19(d)/4.09(t) and 4.28(d)/4.05(t), coupled to each other were assignable to 3-/4-H's of each fructose unit. The correlation of three doublets and three triplets in the G-F_3 spectrum were similarly obtained by H,H-COSY experiments (Fig. 5): the lowest-field doublet to the highest-field triplet, the middle doublet to the middle triplet, and the highest-field doublet to the lowest-field triplet. All of the 4-H's of the fructose moieties are coupled to 5-H's at *ca.* 3.88 submerged in the cluster of protons. However, the assignment of these three sets of connected protons belonging to three different fructose moieties is difficult. For example, answers to the questions, "which is of the terminal?," "which is of internal?," and "which is of the one next to glucose?," were not available without further detailed investigation. The H,C-COSY experiment was performed to assign these protons. ^{13}C-NMR spectra of G-F_3 have already been analyzed and the carbons were assigned.[14] Thus, it was possible to assign each protons of G-F_3 as follows: the lowest-field doublet at 4.28 and the highest-field triplet at 4.06 protons are coupled respectively to 79.61 ppm C-3 and 76.70 ppm C-4 of the fructose unit adjacent to glucose. Therefore, it is now clear that the proton set 4.28/4.06 is assignable to 3-/4-H's of the fructose adjacent to glucose. Similarly, the 4.23/4.08 protons are of the middle (internal) fructose unit, and 4.20/4.11 protons, which comprise the very inner proton pair, are of the terminal fructose residue. This trend, *i.e.*, the terminal fructose unit yields the very inner pair, whereas the fructose adjacent to glucose gives the very outer pair of 3- and 4-H's in ^{1}H-NMR, is still true for other higher oligomers, G-F_4 through G-F_9. In addition, from the H,C-COSY experiment, 3.69 and 3.77 AB-spin system protons (in G-F_3) were assigned to two 1-H's of the terminal fructose unit; in the case of sucrose, it appeared as a two-proton singlet. All the assignments of protons thus obtained for the inulin oligomers are summarized in Table I. The coupling constants are universally the same throughout the oligomers studied here, $J_{1,2}=4.0$, $J_{2,3}=10.0$, $J_{3,4}=9.5$, and $J_{4,5}=10.0$ Hz for glucose moiety, and $J_{3,4}=J_{4,5}=8.5$ Hz, for any fructose units. Thus no changes regarding

TABLE I. ^{1}H-NMR Chemical Shifts (δ) of Inulin Oligomer Ring Protons (500 MHz in D_2O)

Inulin oligomer	Glucose unit					Fructose units							
						F-1 (adjacent)			F-2			F-3	
	H1	H2	H3	H4	H5	H1	H3	H4	H1	H3	H4	H3	H4
G-F	5.409	3.564	3.766	3.475	3.820	3.682	4.221	4.057	3.683				
G-F_2	5.439	3.549	3.758	3.476	3.820	3.781	4.285	4.051	3.746	4.199	4.089		
G-F_3	5.440	3.546	3.768	3.479	3.820		4.280	4.054		4.235	4.085	4.196	4.114
G-F_4	5.442	3.546		3.480	3.820		4.286	4.054		4.229	4.086	4.242	4.109
G-F_5	5.440	3.548		3.481	3.820		4.292	4.054		4.234	4.086	4.241	4.106
G-F_6	5.443	3.549		3.481	3.830		4.290	4.054		4.240	4.086	4.250	4.106
G-F_7	5.444	3.551		3.482	3.830		4.290	4.054		4.241	4.085	4.250[a]	4.106
G-F_8	5.444	3.552		3.482	3.830		4.290	4.054		4.242	4.085	4.263[a]	4.107
G-F_9	5.445	3.552		3.482	3.830		4.290	4.055		4.242	4.085	4.256[a]	4.107

Inulin oligomer	Fructose units											
	F-4		F-5		F-6		F-7		F-8		F-9	
	H3	H4	H3	H4	H3	H4	H3	H4	H3	H4	H3	H4
G-F_4	4.200	4.111										
G-F_5	4.250	4.110	4.201	4.114								
G-F_6	4.250	4.106	4.250	4.106	4.199	4.110						
G-F_7	4.250[a]	4.106	4.250[a]	4.106	4.241[a]	4.106	4.199	4.111				
G-F_8	4.258[a]	4.107	4.254[a]	4.107	4.249[a]	4.107	4.246[a]	4.107	4.199	4.112		
G-F_9	4.265[a]	4.107	4.256[a]	4.107	4.250[a]	4.107	4.246[a]	4.107	4.242	4.107	4.200	4.112

a) Assignments may be reversed.

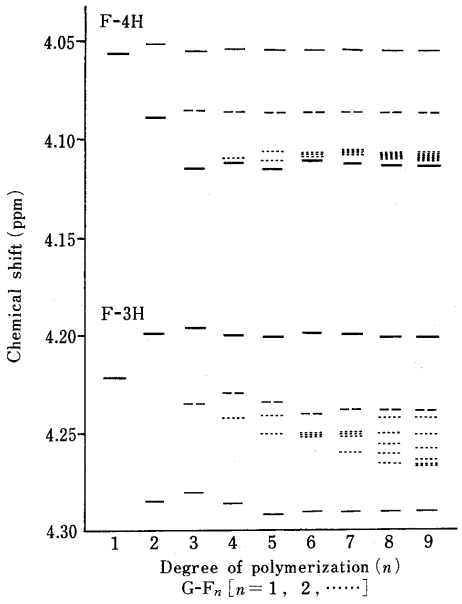

Fig. 7. Chemical Shift Variation of 3- and 4-Protons of Fructose Units for Inulin Oligomers (G-F—G-F_9)

——, terminal fructose unit; ——, fructose unit adjacent to glucose; — —, second fructose unit from glucose – – –, internal fructose unit.

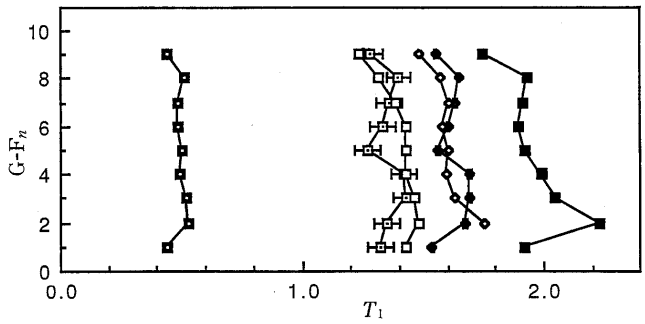

Fig. 8. T_1 Behavior of Several Proton Signals in Inulin Oligomers (G-F_2—G-F_9) in D_2O at 20 °C

◆, 4-H of glucose unit (G-4); ⊡, 2-H of glucose unit (G-2); ◇, 3-H of A (fructose unit adjacent to glucose unit) (F-3a); □, 4-H of A (F-4a); ■, 3-H of T (terminal fructose unit) (F-3t); ▪, 1-H of T (F-1t).

G-1 was used for normalizing T_1 values. Sample concentrations were about 1.5%. Standard deviations of T_1 for G-2 and G-4 were shown in this Figure. The coefficient of variation (CV)% of T_1 for any proton was less than 5%.

the ring conformation are plausible.

The chemical shifts of 3- and 4-H's of all the fructose moieties for the oligomers studied are depicted schematically in Fig. 7. The chemical shifts of two fructose units, the terminal unit and that adjacent to glucose, were almost independent of the degree of polymerization, and the 3- and 4-H's for internal frutose units appeared in between these two. The resonance positions of the 3-H of fructose moieties vary slightly according to the degree of polymerization. These tiny variations would suggest no obvious conformational changes up to G-F_9. However, some comments should be made on an apparent changes in pattern which shows the dispersion of 3-H's of the internal fructose units of G-F_4/G-F_5/G-F_6 and G-F_7/G-F_8. An insertion of one fructose unit to G-F_6 to form G-F_7 resulted in the addition of a new doublet at *ca.* 4.26, as far as 3-H's are concerned, whereas an insertion of another fructose unit to G-F_7 to form G-F_8 caused a separation of all the 3-H's of the internal fructose unit.

Since no changes in the pattern of the chemical shifts regarding 4-H's were observed, except for G-F_5/G-F_6, only a small influence on 4-H's appeared to be prompted from the back-bone conformational alterations, even if it occurred as is in the case of G-F_7/G-F_8 mentioned above. This could be mainly due to the fact that 4-H's are more remote from the back-bone chain than 3-H's. This change, observed on the 3-H's of internal fructose moieties, may be closely related to the observations of molecular rotations, suggesting the higher oligomer adopts a somewhat different, presumably more rigid, conformation than the smaller oligomers.

T_1 of each isolated proton, 1-, 3-, 4-H's of fructoses and 1-, 2-, 4-H's of the glucose unit was plotted *vs.* the degree of polymerization (Fig. 8). Only G-2 (2-H of glucose) and G-4 (4-H of glucose), which are on the same β side of the pyranose ring, showed significant irregularity at G-F_4/G-F_5/G-F_6, whereas no further changes were obvious on the other protons except at G-F_8/G-F_9. Considering the fact that T_1 is sensitive to the motility of the nuclei in question, the nuclei would be relaxed *via* other nuclei, particularly protons in its vicinity. Therefore, this significant change observed around G-F_5, the shortening of the T_1 value on G-2/G-4, accounts for an access of some nuclei, probably of the terminal fructose moiety, to glucose from its β side of the pyranoside ring by forming one pitch of a spiral conformation. Note also that this access apparently shortens the T_1 values of both G-2 and G-4 in oligomers larger than G-F_6. In this context, the approaching nuclei, if there are protons, should also reveal similar behavior (shortening) in their T_1 relaxation. However, we could not point out which proton should be responsible for this effect because of the poor resolution of protons at the range of 3.7—3.9, where quite a lot of protons appear overlapped.[15] Nevertheless, it should not be too speculative to assume from this T_1 experiment that five fructose residues can make one spiral. This is in good agreement with the results based on powder X-ray diffractometry and energy calculation of inulin, suggesting a 5/1 helix conformation with one pitch of 10.8 Å.[16] Thus, it is possible to estimate the dimension of the inulin molecule consisting of 25 mer to adopt a spiral cylinder with a 12—13 Å diameter and *ca.* 60 Å length, which agrees with a recent small angle X-ray scattering experiment indicating the average size of inulin molecule as a truncated cone with the mean dimensions of 16 Å in diameter × 59 Å in length and a funnel-shaped thicker end (smaller end; 12 Å diameter, and the other end; 23 Å diameter).[17] In this connection, of interest is the fact that the size of the binding domain in some anti-inulin immunogloblins has been estimated to be 14 × 14 × 7 Å or 15 × 14 × 10 Å,[2] fitting the inulin molecule with this 5/1 helix conformation.

In conclusion, we have obtained evidence suggesting a 5/1 helix even in aqueous solution, from the T_1 experiment. Thus, the detailed examination of T_1 changes would also be a useful technique for conformational analysis. Also, trends in the chemical shift pattern and molecular rotation of inulin oligomers from G-F_2 to G-F_9 clearly suggest conformational changes at G-F_7 and G-F_8. Although this conformational change cannot be simply accounted for by this helix formation, it is most likely that the oligomers higher than G-F_8 adopt more rigid conformations than the shorter oligomers by forming almost two units of the helix structure. In order to rationalize this implied confomational change, the crystallization of inulin oligomers is now under way for single-crystal X-ray studies.

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10) In the case of maltooligoitol (from maltitol to maltoheptaitol), degree of polymerization and molecular rotation are in linear relationship. Authors are grateful to Hayashibara Biochemical Laboratories, Inc., for the generous gift of maltooligoses and maltooligoitols.
11) Because the $[M]_D$ of G-F_7 is almost on a broken line, G-F_8—G-F_9, in Fig. 8, it is difficult to determine whether the conformation of G-F_7 is similar to that of the group of G-F_4—G-F_6 or G-F_8—G-F_9. However, a detailed comparison of the NMR splitting pattern among the oligomers suggests that G-F_7 resembles the group of G-F_4—G-F_6 in conformational properties, as will be mentioned later.
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15) Phase sensitive rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) experiment was performed to clarify which proton(s) interact(s) with G-2/G-4 of G-F_5. Some positive cross peaks were obscure at from 3.73 to 3.78 and from 3.75 to 3.88 where G-3,5,6,6, as well as F-1,1,5,6,6, exist, in addition to the positive cross peak for G-2/G-4, 1,3-diaxial protons.
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