Polyfructan and Oligofructans Synthesized from Sucrose by Conidia of *Aspergillus sydowi* IAM 2544

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When conidia of *Aspergillus sydowi* IAM 2544 was incubated with sucrose, polyfructan and oligofructans were synthesized concomitantly in the incubation mixture. The polyfructan isolated from the incubation mixture was shown to have a molecular weight of the order of $2 \times 10^6$ which was comparable to those of microbial levans. But it was comprised of chains of $\beta-2\prime\rightarrow1\prime$ linked $\beta$-D-fructofuranoside residues as in inulin of higher plants. This polyfructan was considered to be a very unique polysaccharide which differed from any other fructans ever known. Oligofructans were characterized as fructans of 1-kestose series having the general formula of $((1F-(fructosyl)n-sucrose))$.

More than 50 years ago, Kopeloff and Kopeloff1) and Kopeloff et al.2) observed that the conidia of *Aspergillus sydowi* produced from sucrose a D-fructose polymer which they called “levan.” Later, Loewenberg and Reese3) reexamined this “levan” and reported, on the basis of the following results, that this substance was not the so-called levan, having mainly or exclusively $\beta-2\rightarrow6\prime$-fructofuranosidic linkages, but was probably an inulin having $\beta-2\rightarrow1\prime$-fructofuranosidic linkages: a) Partial acid or enzymic hydrolyzates of this substance gave the same paper chromatographic patterns as those of inulin, b) the IR spectrum of this substance was similar to that of inulin.

Inulin has been found only in higher plants and its biosynthetic pathway has remained obscure, although biosynthetic pathways of almost all other reserve polysaccharides have recently been elucidated.4) As to the mechanism of inulin biosynthesis two hypotheses have been proposed, namely, transfructosylation utilizing the pre-existing glycosidic bonds as in sucrose and fructosylsucrose,5,6) and the one supposing the involvement of UDP-fructose.7,8) If the polyfructan synthesized by *Asp. sydowi* has truely the same structure as that of inulin synthesized by higher plants, this fungus may afford a better means than the higher plants for the study of inulin biosynthesis.

The present paper describes an extensive characterization of this polyfructan as well as of oligofructans synthesized concomitantly.

**MATERIALS AND METHODS**

**Materials.** Inulin prepared from dahlia tubers was purchased from Nakarai Chemicals Ltd. Levan was prepared from timothy haplocorm according to the method of Suzuki.9) $\beta$-fructofuranosidase (150 units/mg) prepared from yeast was purchased from Boehringer Mannheim Japan Co.

**Microorganisms.** *Asp. sydowi* IAM 2009, 2078, 2514 and 2544 were obtained through the courtesy of Prof. Hiroshi Iizuka, The Institute of Applied Microbiology, The University of Tokyo, and *Asp. sydowi* QM 31c through the courtesy of Dr. Elwyn T. Reese, U.S. Army Quartermaster Research and Development Center. Preliminary experiments revealed that *Asp. sydowi* IAM 2544 was the most excellent among these strains in growth rate of mycelia and productivity of fructan by their conidia. Therefore this strain was used exclusively in the following experiments.

**Preparation of conidia from Asp. sydowi IAM 2544.** *Asp. sydowi* was grown on a potato dextrose agar (one liter of potato broth prepared from 200 g of fresh potato containing 10 g glucose and 17 g of agar) in petri dishes with diameter of 23 cm. After incubation at 30°C for

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7~10 days, conidia were harvested from the agar surface and suspended in distilled water. The conidial suspension was filtered through gauze to remove contaminating mycelia, and then through a MF-Millipore RA filter (pore size 1.2 μm). Conidia collected on the filter were stored in deep freeze until used. The conidial suspension having the optical density of 1.0 at 660 nm contained 3×10^6 cells/ml.

Preparation of polyfructan. To 0.01 M phosphate buffer (pH 5.4) containing 10% of sucrose and 0.01% of thimerosal (sodium ethylmercurithiosalicylate) was added conidia of Asp. sydowi to a final concentration of 1 g (wet wt.)/liter. After incubation for 7 days at 30°C, the culture was filtered through the MF-Millipore RA filter to remove the conidia, and the polyfructan in the filtrate was precipitated by adding 4 volumes of ethyl alcohol. The precipitated polyfructan was collected by centrifugation at 10,000 g for 20 min and after being redissolved in a minimum volume of water, it was dialyzed, successively, against tap water and then distilled water. The dialyzed solution containing polyfructan was treated again with 4 volumes of ethyl alcohol and the resulting precipitate was dried up in vacuo at 60°C to a constant weight. Usually the polyfructan was recovered in a yield corresponding to about 10~13% of sucrose.

Preparation of the oligofructan fraction. The same incubation mixture as described above was incubated at 30°C for 2 days. After removal of conidia and polyfructan as described above, the resulting 80% ethyl alcohol solution was concentrated to a syrup by a rotary evaporator to give 80% ethyl alcohol-soluble oligofructan fraction.

Ultracentrifugation. The rate of sedimentation was determined with a Hitachi Analytical Ultracentrifuge, Model UCA-1A, at a rotor speed of 21,410 rpm at 20°C. Measurements were made at several concentrations of polyfructan (2~14 mg/ml). The photographs were taken at 2 min intervals.

Viscometry. The viscosity of the polyfructan solution was determined with an Ostwald type viscometer in a thermostatted water bath at 20.00±0.02°C. The reduced viscosity (ηsp/c) was determined at several concentrations of polyfructan (2~10 mg/ml) and the intrinsic viscosity (η) was obtained by plotting the reduced viscosity against the concentration of polyfructan and extrapolating to zero concentration.

Molecular weight. The molecular weight of the polyfructan (M) was calculated by the equation,(10)

\[ M = \frac{\eta_p^{3/2}(\rho)^{1/2}S^{3/2}N^{3/2}}{f\beta\alpha(1-\rho_P)^{3/2}} \]

where \( \eta_p \) is the intrinsic viscosity of the solvent (water), \( S \) is the sedimentation coefficient of the polyfructan, \( N \) is Avogadro's number and \( \rho \) is the density of the solvent (water). \( \beta \) is a constant concerning to the axial ratio of the molecule and varied from 2.12×10^6 (for a spherical molecule) to 2.5×10^6 (for a linear molecule).\(^{11} \) \( \alpha \) is the partial specific volume of the polyfructan and was determined to be 0.67 according to the method of Schachman.\(^{10} \)

Methylation and methanolysis. Methylation of the polyfructan and oligofructan was carried out according to the modified method\(^{12} \) of Hakomori\(^{13} \) using 20~25 mg of the polyfructan or 5 mg of oligofructan as starting materials. The methylated samples were methanolyzed by methanolic 0.01 N hydrogen chloride in sealed tubes at 100°C for 30 min. After cooling, the methanolic hydrogen chloride was evaporated off and the remaining materials were dissolved in methyl alcohol.

Gas-liquid chromatography. Gas-liquid chromatography was carried out by using a Hitachi K83 Gas Chromatograph equipped with a column (3 x 950 mm) of 15% butan-1,4-diol succinate polyester on acid-washed celite (80~100 mesh)\(^{14} \) and a hydrogen flame ionization detector and operated at 175°C. Nitrogen was used as the carrier gas at a flow rate of 45 ml/min. As the medium peak of methyl 1,3,4,6-tetra-O-methylfructoside appeared in all samples tested, the relative retention time (Tg) of this peak was taken as 1.26 according to Aspinall\(^{14} \) and Tg of all other peaks were calculated in relation to this peak.

Paper chromatography and paper electrophoresis. Paper chromatography was carried out on Tōyō filter paper No 51A by the descending method at 23°C using the following solvent systems,

A) n-butyl alcohol: acetic acid: water (2:1:1)\(^{15} \)
B) n-butyl alcohol: acetic acid: water (4:1:5)\(^{14} \)
C) isopropyl alcohol: acetic acid: water (67:10:23)\(^{3} \)
D) n-propyl alcohol: ethyl acetate: water (6:1:3)\(^{6} \)

For the detection of ketose, aldose and reducing ketose sugars on the dried paper were detected using the following solvent systems,

A) n-butyl alcohol: acetic acid: water (2:1:1)\(^{14} \)
B) n-butyl alcohol: acetic acid: water (4:1:5)\(^{14} \)
C) isopropyl alcohol: acetic acid: water (67:10:23)\(^{3} \)
D) n-propyl alcohol: ethyl acetate: water (6:1:3)\(^{6} \)

For the detection of ketose, aldose and reducing ketose sugars on the dried paper were detected using the following solvent systems,
latter was used for the accurate analysis of oligofructans. Glucose was determined using Glucomesser (Tokyo Zoki Kagaku Co.) as follows: To 0.24 ml of hydrolyzed and neutralized sample was added 1.0 ml of the enzyme solution and the reaction mixture was incubated at 37°C for 1 hr. Incubation was terminated by adding 1.0 ml of 50% sulfuric acid to the reaction mixture and the absorbancy (530 nm) of the reaction mixture was determined by a Beckman DB spectrophotometer. Protein was determined by the method of Lowry et al.26) Phosphate was determined by the modified method27) of Bartlett28) after digestion of the sample with sulfuric acid.

RESULTS

Structure of polyfructan

When conidia of Asp. sydowi were incubated with 10% sucrose for 1 day and the culture filtrate was applied to a column of Sephadex G–200, the resulting elution pattern (Fig. 1) shows that two peaks of fructans are eluted. Paper chromatographic analysis revealed that Peak I contained a fructan which remained at the origin and that Peak II contained some oligofructans in addition to glucose, fructose and sucrose. These results indicated that the conidia synthesized from sucrose a polyfructan of high molecular weight and oligofructans of low molecular weight and that no fructan having the intermediate molecular weight was synthesized in a detectable amount.

![Fig. 1. Gel Filtration on Sephadex G–200 of Polyfructan and Oligofructans Synthesized by Conidia of Asp. sydowi IAM 2544 from Sucrose.](image)

Conidia of Asp. sydowi IAM 2544 was incubated with 10% sucrose solution at 30°C for 1 day. After the conidia were removed by filtration, 5 ml of the filtrate was applied on a column (3.2 × 60 cm) of Sephadex G–200. Fructans were eluted by distilled water in 5 ml fractions and monitored by the p-anisidine colorimetric method. The arrow shows the elution peak of Blue Dextran 2000.

Table I. Chemical Analysis of the Polyfructan

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.04</td>
</tr>
<tr>
<td>Total phosphate</td>
<td>0.01</td>
</tr>
<tr>
<td>Fructose</td>
<td>108.4</td>
</tr>
<tr>
<td>Glucose⁴</td>
<td>0</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>0</td>
</tr>
<tr>
<td>Reducing sugar after hydrolysis⁴</td>
<td>105.2</td>
</tr>
</tbody>
</table>

⁴ For the determination of glucose and total reducing sugar after hydrolysis, the sample was previously hydrolyzed in 0.01 M HCl at 100°C for 20 min and neutralized with 1 n NaOH.

The polyfructan prepared as described in the Methods section was soluble in water to yield a pale turbid solution. Chemical analysis of this polyfructan (Table I) revealed that it was composed exclusively of fructose and no glucose could be detected (at least below 0.1% of this polysaccharide). This agreed well with the results that glucose could not be detected by paper chromatography (using solvent system C) and by paper electrophoresis in the acid hydrolyzate of this polyfructan.

From the elution pattern of Fig. 1, the polyfructan was supposed to have a molecular weight very near to or higher than $2 \times 10^6$. To ascertain this, it was then subjected to the gel filtration on a Sepharose 2B column as shown in Fig. 2. The elution pattern of Blue Dextran 2000 (also shown in this figure) showed that apart from the main peak having the molecular weight of about $2 \times 10^6$, a minor peak appeared at the position of the void volume (the exclusion limit of this gel is reported to be $20 \times 10^6$ for polysaccharides). Apparently, the polyfructan was eluted also in two peaks, one at the position of the void volume and the other at the position between the void volume and the main peak of Blue Dextran 2000. However, this result is considered to show that this polyfructan is a mixture of homologous polysaccharides of very high and somewhat
FIG. 2. Gel Filtration of the Polyfructan on Sepharose 2B.

About 6 mg of polyfructan in 1 ml of distilled water was applied on a column (2.1 × 75 cm) of Sepharose 2B from which fine particles were previously removed by decantation. The sample was eluted by 0.025 M phosphate buffer (pH 7.5) and collected in 5 ml fractions. Fructose was determined by the p-anisidine colorimetric method. After the fructan was eluted completely, Blue Dextran 2000 was run in the same way and monitored by determining the absorbance of the eluate at 260 nm. ○−○, fructan; •−•, Blue Dextran 2000.

diverse molecular weight of the order of $10 \times 10^6$.

The molecular weight of the polyfructan was further investigated by ultracentrifugation and viscometry. The sedimentation pattern (Fig. 3a) showed that this polyfructan sedimented rapidly as a single peak. The sedimentation constant ($S_0$) was calculated to be 179.5 S by plotting the sedimentation coefficient against the concentration and extrapolating to zero concentration (Fig. 3b). The intrinsic viscosity ($\eta$) was calculated to be 0.15 dl/g. Calculation of the molecular weight from these data using the equation presented in the Methods section gave a value of $19 \times 10^6$ ($\beta = 2.5 \times 10^3$) or $26 \times 10^6$ ($\beta = 2.12 \times 10^3$) for this polyfructan. Although the precise molecular weight of this polyfructan is not known because of the lack of knowledge on the molecular structure of this polyfructan, it is supposed to be of the order of $20 \times 10^6$ which is very close to those of microbial levans (for instance, M.W. of $16 \sim 23 \times 10^6$ for a levan of Streptococcus salivarius, $17 \times 10^6$ for a levan of Aerobacter aerogenes and $25 \times 10^6$ for a levan of Bacillus subtilis had been reported) and far higher than those of inulins (M.W. of about 5000 – 6000) from higher plants.

To determine whether this polyfructan is of a levan type or of an inulin type in its glycosidic linkage, it was then subjected to partial acid hydrolysis followed by paper chromatography of the hydrolysis products. Inulin from dahlia tubers and levan from timothy haplocorm were treated in the same way and were used as reference standards. The results given in Fig. 4 show that the chromatographic pattern of the hydrolyzate of polyfructan was similar to that of inulin hydrolyzate in both solvent systems. Levan hydrolyzate and oligofructans obtained from the incubation mixture showed clearly different chromato-
TABLE II. GAS-LIQUID CHROMATOGRAPHIC ANALYSES OF THE METHANOLYSIS PRODUCTS OF PERMETHYLATED POLYFRUCTAN AND OLIGOFRUCTAN

$T_g$ of the medium peak of methyl 2,3,4,6-tetra-O-methylglucoside and the strong peak of methyl 1,3,4,6-tetra-O-methylfructoside were very near and could not be resolved in these experiments.

<table>
<thead>
<tr>
<th>Methylated sugars</th>
<th>Relative retention times ($T_g$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose and Fructose</td>
<td>0.95 1.26 1.40</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.01 1.26 1.40</td>
</tr>
<tr>
<td>Inulin</td>
<td>1.01 1.26 1.42 2.64 3.92</td>
</tr>
<tr>
<td>Levan</td>
<td>1.01 1.26 1.42 1.86 2.45 3.80 4.22</td>
</tr>
<tr>
<td>Polyfructan</td>
<td>1.02 1.26 2.64 3.95</td>
</tr>
</tbody>
</table>

Graphic patterns from that of the hydrolyzate of polyfructan. These results suggested that the polyfructan is of the inulin type in its glycosidic linkages.

More direct evidence about the nature of the glycosidic linkage of the polyfructan was obtained from gas-liquid chromatography of methanysis products of the permethylated polyfructan. As shown in Fig. 5 and Table II, the polyfructan gave relative retention times ($T_g$) of 1.02 and 1.28 corresponding to methyl 1,3,4,6-tetra-O-methylfructoside and $T_g$ of 2.64 and 3.95 corresponding to methyl 3,4,6-tri-O-methylfructoside in accordance with the case of inulin. In view of the high molecular weight of this polyfructan, the existence of the methyl tetra-O-methylfructoside, i.e. the non-reducing end, may be taken to suggest that this polyfructan has a high degree of branching or ramifications. However, as oligofructans derived from partial acid hydrolysis of this polyfructan showed the same chromatographic patterns as those derived from the linear saccharide inulin (as already shown in Fig. 4), it is hasty to make such a conclusion from the above results. We are rather of the opinion that this may be due to the presence of low molecular fructans in the polyfructan sample used in this particular experiment (80% ethyl alcohol precipitate of the culture filtrate was used) and that more detailed experiments are needed to decide whether this polyfructan has any branching or not. Any way, these results indicate that fructose is linked successively by 2→1'-fructosidic linkages in this polyfructan. The absence of peaks corresponding to methyl 2,3,4,6-tetra-O-methyl-glucoside indicated again the absence or extreme minority, if at all, of a terminal glucose residue in this polyfructan.

The polyfructan was not hydrolyzed by $\beta$-fructofuranosidase probably because of its high molecular weight. But when subjected to partial acid hydrolysis followed by treatment with $\beta$-fructofuranosidase, this polyfructan gave fructose as the sole reaction product as shown in the paper chromatogram of Fig. 6, whereas the acid hydrolysis products without
Further enzyme treatment gave a series of oligofructans on the same paper chromatogram. It is clear that in oligofructans and hence in the polyfructan from which the oligofructans were derived, the fructose molecules are linked to each other in a β-fructofuranoside configuration.

Structure of oligofructans

As already shown in Figs. 4a and 4b, the paper chromatographic patterns of the oligofructans isolated from the culture filtrate were clearly different from those of oligofructans formed by partial acid hydrolysis of polyfructan, inulin and levan. When the relative mobilities (Rf) of each sugar of these oligofructans in the paper chromatograms developed with solvent systems B) (data were not shown), C) (Fig. 4a) and D) (Fig. 4b) were plotted against the degree of polymerization (D.P.) of each sugar according to the equation of French and Wild, a linear relationship between log Rf/(1−Rf) and D.P. was obtained in any solvent systems used. This result indicated that these oligofructans contained a series of fructans having the same linkage.

When the paper chromatograms of the oligofructans developed in the same solvent system as used in Fig. 4a was sprayed with a reagent that could detect only reducing fructose residues, oligofructans from the culture filtrate showed only one spot corresponding to that of fructose whereas oligofructans formed from the partial acid hydrolysis of the polyfructan, inulin and levan showed substantially the same pattern as in Fig. 4a (data were omitted). This result indicated that, unlike other oligofructans, the oligofructans from the culture filtrate had no reducing fructose residues. In these oligofructans the reducing fructose terminals are supposed to be attached by glucose as in sucrose.

To characterize these oligofructans more decisively, each sugar of these oligofructans was separated by gel filtration using Sephadex G-15 as shown in Fig. 7. Paper chromatogram of these peak fractions (fraction numbers 68, 71, 77, 83 and 90) as summarized in Table III indicates that each of all peak fractions contains a single fructan except fraction 90 which is a mixture of fructose and glucose.

Chemical analysis of these peak fractions (shown in Table IV) indicated that each fructan contained in peak fractions 68, 71, 77 and 83
The polyfructan (246 µg) was hydrolyzed in 0.01 N HCl at 100°C for 3 min. After neutralization with 0.11 N NaOH, the sample was divided into 3 portions. One portion (designated as 2) was freeze-dried and spotted on the paper. The second portion (designated as 3) was freeze-dried, incubated with 0.04 ml of 0.1 M acetate buffer (pH 5.5) for 10 min at 27°C, again freeze-dried and spotted on the paper. The third portion (designated as 4) was treated as in the case of the second portion except that it was incubated with 0.1 M acetate buffer (pH 5.5) containing 86 µg of β-fructofuranosidase.

1 in the figure shows the polyfructan without any treatment. The solvent system used was A) and ketoses on the paper were detected by the naphthoresorcinol-phosphate reagent.

had one molecule of glucose and 4, 3, 2 and 1 molecules of fructose, respectively, per molecule. The result that they had no reducing power before hydrolysis agreed well with the supposition described above that the reducing terminals of these fructans were blocked by glucose residues as in sucrose. It was shown that peak fraction 90 contained free fructose and free glucose with a molar ratio of about 1:2.

All these oligofructans were shown to be hydrolyzed into fructose and glucose on treatment with β-fructofuranosidase as summarized in Tabel III (Although glucose was not detected in the hydrolysis products of peak fractions of 68, 71 and 77, this may perhaps be due to the low sensitivity of the spary reagent used against glucose and to the relatively low content of glucose in these oligofructans).
TABLE IV. SUGAR COMPOSITION OF OLIGOFRUCTANS SEPARATED BY SEPHADEX G-15

Fructose was determined by the modified method of Dische and Borenfreund. Reducing sugar was determined by the method of Park and Johnson reduced to 1/5 scale.

<table>
<thead>
<tr>
<th>Fructans</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Reducing sugar Before hydrolysis</th>
<th>Reducing sugar After hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>1.00</td>
<td>3.98</td>
<td>0</td>
<td>4.82</td>
</tr>
<tr>
<td>71</td>
<td>1.00</td>
<td>3.00</td>
<td>0</td>
<td>3.89</td>
</tr>
<tr>
<td>77</td>
<td>1.00</td>
<td>2.01</td>
<td>0</td>
<td>2.88</td>
</tr>
<tr>
<td>83</td>
<td>1.00</td>
<td>1.00</td>
<td>0</td>
<td>1.71</td>
</tr>
<tr>
<td>90</td>
<td>1.00</td>
<td>0.52</td>
<td>1.37</td>
<td>1.39</td>
</tr>
</tbody>
</table>

These results indicate that in these oligofructans the fructose molecules are linked to each other in a β-fructofuranoside configuration.

The gas-liquid chromatogram of the methanolysis products of permethylated oligofructan (the tetrasaccharide fraction separated by paper chromatography of ligofructans was used in this experiment) gave the same pattern as that of inulin and revealed the presence of methyl 2,3,4,6-tetra-O-methylglucoside (Tg of 1.02 and 1.41), methyl 1,3,4,6-tetra-O-methylfructoside (Tg of 1.02 and 1.26) and methyl 3,4,6-tri-O-methylfructoside (Tg of 2.74 and 4.11) (Results are summarized in Experiment II of Table II). These results clearly indicate that the interglycosidic linkage of this oligofructan and hence of other oligofructans of this series are C2→1′.

From the results described above, it was concluded that the oligofructans synthesized by conidia of Asp. sydowi were consisted of fructans of 1-kestose series having the general formula of (1°-(fructosyl)ₙ-sucrose) but having different values of n from 1 to 3.

DISCUSSION

Conidia of Asp. sydowi IAM 2544 produced the polyfructan which was characterized as a polysaccharide having chains of 2→1′ linked β-D-fructofuranoside residues as in inulin. But it had a molecular weight of the order of 20×10⁶ which was far higher than those of inulins synthesized by higher plants and comparable to those of levans synthesized by various microorganisms. Furthermore, the fact that this polyfructan was synthesized extracellularly from sucrose agreed well with the case of levan biosynthesis but greatly differed from the case of inulin biosynthesis. However, it was synthesized by non-growing conidia whereas levan was synthesized by growing cells. Thus, this polyfructan proved to be a very unique polysaccharide which differed from any fructans ever known. The mechanism of biosynthesis of this polyfructan with special reference to the mechanism of inulin biosynthesis and its physiological meanings are interesting problems to be studied.

In addition to polyfructan, oligofructans of 1-kestose series (D.P. 3~5) were also synthesized concomitantly by conidia of Asp. sydowi IAM 2544. This may suggest the possible role of these oligofructans as precursors in the biosynthesis of polyfructan.

The mechanisms of biosynthesis of polyfructan and oligofructans and the relationship between these mechanisms are under further investigations.

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195 (1919).


