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ISOLATION AND CHARACTERIZATION OF OLIGOSACCHARIDES CONTAINING D-FRUCTOSE FROM JUICES OF THE JERUSALEM ARTICHOKE. KINETIC CONSTANTS FOR ACID HYDROLYSIS

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ABSTRACT

Liquid chromatography and ^{13}C-n.m.r. spectroscopy have been used to isolate and characterize the fructo-oligosaccharides present in the juice of Jerusalem artichoke. Oligosaccharides having d.p. 2–7 were purified individually. A kinetic study was performed to determine the dependence of the acid-hydrolytic rate-constants on the degree of polymerization. These data are important in optimizing conditions for hydrolysis of the vegetable juice.

INTRODUCTION

Considerable recent research has been devoted to the use of organic material produced by photosynthesis as an energy source for liquid fuels. Among possible substitutes for exhaustible resources, the fructans from Jerusalem artichoke have received little attention[1,2]. However the carbohydrate reserves of this plant are substantial and its crop yield good. Furthermore the plant is highly resistant to insects and diseases, and offers a distinct advantage in areas that have a high risk of premature frost[3].

The tubers of Jerusalem artichokes may also be considered for production of fructose in the food industry, either chemically[3–6] or by enzymic methods[3,7].

To provide a rational basis for uses of the plant extract, a structural analysis was undertaken by means of liquid chromatography and ^{13}C-n.m.r. spectroscopy. In particular, the various oligosaccharides were isolated and characterized. From these purified compounds, rate constants for acid hydrolysis were determined and their dependence upon the degree of polymerization (d.p.) established. These results are of great importance for optimizing conditions for hydrolysis of the vegetable juice according to its relative carbohydrate composition (resulting from changes observed during growth, storage, or sprouting) and to the final product to be prepared.

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EXPERIMENTAL

Preparation of plant extracts. — A white variety (K 8) of Jerusalem artichoke from I.N.R.A., Rennes (France), was used. Tubers were washed, skinned, and ground, and then centrifuged with a Braun MP 50 apparatus. The juice was recovered and analyzed for proteins and carbohydrates. When not used immediately, it was frozen at −20° or freeze-dried.

Chromatographic methods. — Analysis of juices and separation of oligosaccharides were performed either by gel-permeation chromatography (g.p.c.), or by high-performance liquid chromatography (h.p.l.c.)[8].

Gel filtration chromatography was performed on Bio-Gel P2 (200–400 mesh) with water as the eluent at 65°. The preparative column was 4.5 × 150 cm and the analytical column was 1.5 × 210 cm.

Columns used for analytical h.p.l.c. were: μBondapak C18 (Waters Assoc.) eluted with water, and μBondapak carbohydrate (Waters Assoc.) eluted with 17:3 acetonitrile–water. Oligosaccharide samples fractionated on a preparative column of Bio-Gel P2 were purified on a column of Partisil 10 ODS (Whatman).

Detection was accomplished throughout by means of a differential refractive-index (r.i.) monitor (R 401, Waters Assoc.).

Hydrolysis of oligosaccharides. — Hydrolysis was effected at 70° in a glycerol bath. Samples (20 mg) were dissolved in 2 mL of sulfuric acid (pH 2) in a 5-mL flask fitted with a reflux condenser. At intervals, aliquots were withdrawn and made neutral. Products of hydrolysis were analyzed by h.p.l.c. and the rate constants determined directly from chromatograms according to a procedure already described[9].

N.m.r. spectroscopy. — Samples were dissolved in D_2O (60 mg/mL). The deuterium resonance was used as a field–frequency lock. Experiments were performed with complete proton-decoupling at 62.8 MHz on a Cameca spectrometer equipped with Fourier transform and on a Bruker XM-250 spectrometer equipped with an Aspect 2000 computer. The sample temperature was 30°. Free-induction decays were accumulated with a 6,000-Hz spectral width on a 16 K data base (resolution: 0.735 Hz per point). Chemical shifts are expressed in p.p.m. downfield from DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) with acetone (5%) as the internal standard (31.07 p.p.m. from DSS). Assignments were made on the basis of the data of Angyal *et al.* [10] and Pfeffer *et al.*[11] for D-fructose. For inulin oligosaccharides, peaks were assigned according to Binkley *et al.*[12] and Jarrell *et al.*[13].

RESULTS AND DISCUSSION

Fractionation of fructan oligosaccharides. — The extracted juice from Jerusalem artichoke was desalted with mixed resin (Amberlite MB-3). Without any other treatment, it was submitted to different liquid-chromatographic procedures to determine its carbohydrate composition (which totals ~80% of the dry matter[3,14]).

Separation of fructo-oligosaccharides by chromatography has been widely studied[15–17], but with the exception of gel filtration[16], the other methods are not sufficiently rapid and accurate for routine use. With the development of high-performance liquid chromatography, new supports are now available for liquid-partition chromatography[8].

Fig. 1 shows the chromatograms obtained. Chromatogram (*a*) is by gel permeation on Bio-Gel P2; the various components are fractionated according to their degree of polymerization (1<d.p.<9). Proteins and other compounds of high molecular weight are eluted first.

Trace (*b*) is a partition chromatogram in the normal phase on a μBondapak carbohydrate column, with 17:3 acetonitrile–water as eluent. This system permits elution and separate determination of glucose and fructose (corresponding to d.p. = 1) as well as sucrose and inulobiose (corresponding to d.p. = 2), when the latter is present. However, for quantitative measurements, caution must be observed as oligosaccharides having d.p. >3 tend to precipitate at concentrations of acetonitrile required for efficient separation.

Chromatogram (*c*) is by the reverse-phase process on C_{18} support. With water as eluent, rapid information may be obtained on the oligosaccharides of

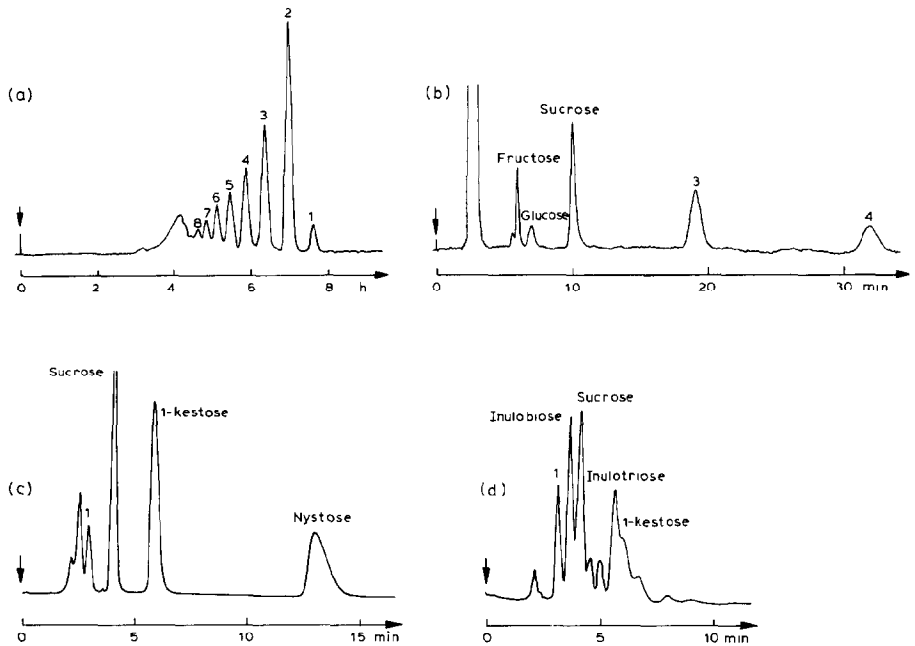

Fig. 1. Fractionation of oligosaccharides contained in desalted Jerusalem artichoke juice. (*a*) by gel permeation on Bio-Gel (Bio-Gel P2, 200–400 mesh; column: 210 × 1.5 cm; T = 65°; eluent: water; flow-rate: 35 mL/h; detector: r.i.). The numbers correspond to the degree of polymerization. (*b*) by partition chromatography in normal phase (column: μBondapak carbohydrate; eluent: 17:3 acetonitrile–water; flow-rate: 1 mL/min; detector: r.i.). (*c*) by reverse-phase process on C_{18} support (column: C-18 Radial pak; eluent: water; flow-rate: 1 mL/min; detector: r.i.). (*d*) the chromatogram obtained for a hydrolyzate of inulin (d.p. <3 limited). Experimental conditions as in (*c*).

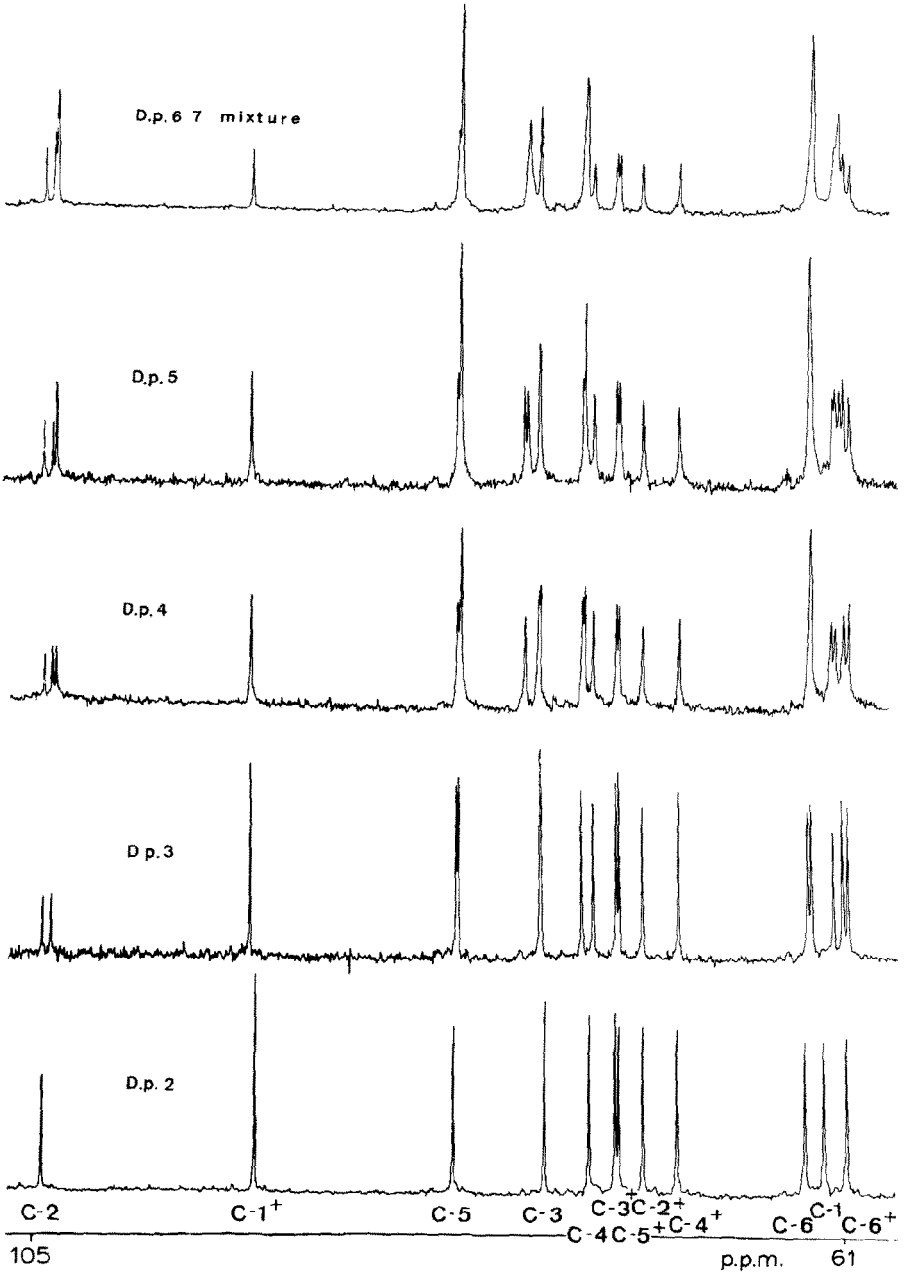

Fig. 2. ^{13}C-N.m.r. spectra at 62.8 MHz in D_2O of the purified oligosaccharides from Jerusalem artichoke juice. The symbol (+) denotes signals of the D-glucopyranosyl group of sucrose.

TABLE I

^{13}C CHEMICAL SHIFTS[a] (P P M) FOR FRUCTAN OLIGOSACCHARIDES[b]

Compound	*Unit*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
D.p. 2	α-D-glucopyranosyl	92 92	71.85	73.36	70.01	73.16	60.93
(sucrose)	β-D-fructofuranosyl	62.14	104 45	77.19	74.74	82.17	63.11
D.p. 3	α-D-glucopyranosyl	93.28	72.00	73.43	70.08	73.26	60.98
(1-kestose)	β-D-fructofuranosyl	61.78	104.52	77.52[d]	74.64	82.09[e]	63.14[f]
	β-D-fructofuranosyl′	61.27	104.06	77.48[d]	75.30	81.97[e]	62.92[f]
D.p. 4	α-D-glucopyranosyl	93.31	72.02	73.40	70.08	73.28	60.98
(nystose)	β-D-fructofuranosyl	61.70	104.48	77.63[d]	74.69	82.12	63.06
	β-D-fructofuranosyl′	61.92	103.84	78.43	75.18	81.95	63.06
	β-D-fructofuranosyl″	61.27	104.04	77.70[d]	75.27	81.95	63.06
D.p. 5	α-D-glucopyranosyl	93.28	72.02	73.40	70.08	73.28	60.98
	β-D-fructofuranosyl	61.73	104.50	77.60	74.67	82.09	62.99
	β-D-fructofuranosyl′	61.87[c]	103.84	78.43[d]	75.13	81.92	62.99
	β-D-fructofuranosyl″	61.49[c]	103.84	78.23[d]	75.13	81.92	62.99
	β-D-fructofuranosyl‴	61.29	104.04	77.60	75.27	81.92	62.99

[a]Chemical shifts are relative to acetone as the internal standard (31.07 p.p.m.). [b]The symbol ′ refers to a β-D-fructosyl group attached to sucrose, ″ refers to a β-D-fructosyl group attached to 1-kestose (d.p. 3), and so on. [c–f]Within a column, ^{13}C assignments relative to different β-D-fructofuranosyl groups may be interchanged.

lower d.p. (1<d.p.<6). This method was used to monitor transformations in the juice during hydrolysis. Monosaccharides (glucose and fructose) are not separated.

By these methods the composition of the juice was established as follows: 120 mg/mL total carbohydrates, including 3.6% monosaccharides (64% fructose and 36% glucose), 31.3% sucrose, 19.3% d.p. 3, 12.8% d.p. 4, 8.9% d.p. 5, 6.5% d.p. 6, and 17.6% d.p. >6). Fresh juice contains very little inulobiose. On chromatograms b and c (Fig. 1), which both reveal good separation of low-d.p. sugars, the absence of oligosaccharides containing only (2→1)-linked β-D-fructofuranose residues is evident, and this point is proved by comparison with chromatograms obtained with a hydrolyzate of inulin (see Fig. 1d, which shows clear separation of sucrose and inulobiose, and of 1-kestose and inulotriose). This corroborates the results of Quillet[15], who was able to differentiate glucose-containing fructans from pure fructose-containing fructans of equal molecular weight by two-dimensional chromatography of an inulin hydrolyzate.

Preparative-scale isolation of fractions according to their d.p. was achieved by chromatography on Bio-Gel P2. Each fraction was then purified by h.p.l.c. on a column of Partisil 10-ODS.

^{13}C-N.m.r. spectroscopy of the purified fractions confirms the existence of pure glucose-containing fructo-oligosaccharides (d.p. 2 corresponds to sucrose, d.p. 3 to 1-kestose, d.p. 4 to nystose, and so on).

^{13}C-N.m.r. analysis. — The ^{13}C-n.m.r. spectra of oligosaccharides having d.p. 2–5 and of a mixture with d.p. 6 and d.p. 7 are shown in Fig. 2. Table I records chemical-shift data for the corresponding α-D-glucopyranosyl group and β-D-fructofuranosyl residues.

The resonance lines of the α-D-glucopyranosyl carbon atoms (distinguished by the " + " symbol in the text) for all compounds appear independent of the chain length except for C-1$^+$, which shows a downfield shift of 0.36 p.p.m. in passing from sucrose to d.p. 3 or d.p. 4.

In the case of 1-kestose (and so, for a compound in which a β-D-fructofuranosyl group is attached to sucrose), only C-3 and C-3′ (see Table I for symbols), C-5 and C-5′, and C-6 and C-6′ resonate respectively very close to each other (chemical-shift difference <0.22 p.p.m.). In contrast, C-2′ is shifted upfield by 0.46 p.p.m. relative to the corresponding C-2, and C-1′ resonates 0.50 p.p.m. upfield of C-1, whereas C-4′ is shifted downfield by 0.66 p.p.m. The first two shifts may be explained in terms of α or β effects, but the case of C-4′ is more complicated and may involve conformational changes affecting the molecular backbone, which is surprisingly similar to a polyethylene oxide chain[22].

The attachment of a second β-D-fructofuranosyl group to sucrose (as in nystose) produced additional features. The C-6, C-6′, and C-6″ signals of the β-D-fructofuranosyl groups are no longer differentiated. The C-5′ and C-5″ signals are also not resolved but the C-5 signal of the β-D-fructose residue adjacent to the D-glucose group is clearly visible and resonates at a slightly lower field (0.17 p.p.m. downfield). In contrast, and for all different β-D-fructofuranosyl groups, lines for C-1, -2, -3, and -4 are separated, showing that all of these carbon atoms are sensitive to the chain conformation. Furthermore, the special location shown by the β-D-fructosyl group adjacent to two other β-D-fructosyl residues may explain the chemical shift of C-3′, clearly distinct from those of C-3 and C-3″. However this phenomenon is not observed for C-4′.

From the product of d.p. 5, a logical evolution of signals may be observed. All C-1 signals are still very sensitive to the conformation of the chain, whereas those of C-6 are not. The other carbon atoms are also influenced by the same factor, according to their distance from the chain. Except for C-6 (and in part for C-5), carbon atoms from the β-D-fructosyl residue adjacent to the D-glucose group and from the terminal β-D-fructosyl residue are clearly distinguished. However both C-3 and C-3‴ show very close chemical shifts.

In the case of d.p. 6 and 7, the phenomenon is much clearer. All C-6 signals are undifferentiated whereas the other carbon peaks show at least one distinct line corresponding to the β-D-fructosyl group linked to the glucose residue. For the carbon atoms of the polyoxyethylene-like backbone and for the adjacent ones, still more signals are detected. While all other C-3 atoms resonate at the same frequency, the C-3 atom of the D-fructosyl group adjacent to glucose still resonates at a higher field.

Consideration of chemical shifts alone does not permit definite conclusions concerning conformational changes in these molecules as a function of increasing d.p. For this purpose, more data are needed, including ^1H and ^{13}C relaxation studies.

Kinetic studies. — The purified oligosaccharides were hydrolyzed at 70° by

TABLE II

RATE CONSTANTS (±10%) FOR HYDROLYSIS OF FRUCTAN OLIGOSACCHARIDES BY SULFURIC ACID (T = 70°, pH 2)

Oligosaccharide	*Sucrose*	*Inulobiose*[a]	*1-Kestose (d.p. = 3)*	*Nystose (d.p. = 4)*	*D.p. 5*	*D.p. 6*	*D.p. 7*
Rate constant $k \times 10^{+2}$ (min^{-1})	2.0	9.0	8.5	10.8	11.3	12.2	13.0

[a]This compound, β-D-Fru*f*-(2→1)-D-Fru, was prepared by hydrolysis of inulin[18].

sulfuric acid (pH 2). Kinetic experiments were performed by h.p.l.c. Assuming that the hydrolysis (with an excess of acid) follows pseudo-first-order kinetics, apparent rate-constants were directly determined from chromatograms according to a method already described[9]. Rates of reaction are expressed as the rate of disappearance of the oligosaccharide under consideration, obtained from the ratio of peak areas. The rate constants (k) obtained are shown in Table II. In the case of sucrose, the value found is not significantly different from comparable literature data[19,20]. However our value determined for inulobiose ([β-D-Fru*f*-(2→1)-D-Fru], prepared by hydrolysis of inulin[18]) is higher than that recorded by Pazur *et al.*[19], whose preparation could have been contaminated by a small amount of sucrose[21].

Although many investigations on the inversion of sucrose have been performed using various acids, concentrations, and temperatures[22–28], few of these are of mechanistic significance. Two positions of bond fission are possible, glucosyl–oxygen fission and fructosyl–oxygen fission. However fructosyl–oxygen fission seems more likely as this would lead to a tertiary oxocarbonium ion (in the case of ketofuranosides), whereas glucosyl–oxygen fission leading to a secondary ion generally occurs with glycopyranosides[26].

Comparisons between the hydrolytic rate-constants for methyl α-D-glucopyranoside, *tert*-butyl β-D-glucopyranoside, inulobiose, and sucrose, respectively, might show that the hydrolytic mechanism involved is the same for the last two compounds, namely, fructosyl–oxygen fission, without, however, eliminating the possibility of equilibration occurring during the splitting.

Our data show the glucose–fructose bond of sucrose to be 4–5 times more resistant than the fructose–fructose bond of inulobiose. This explains the difficulty in detecting inulobiose during the course of hydrolysis of higher-d.p. oligosaccharides (see Fig. 3 in the case of d.p. 3 and d.p. 4). Accordingly inulobiose is scarcely observed during the acid hydrolysis of Jerusalem artichoke juice. All of these experiments clearly show that, during complete hydrolysis to monosaccharides, hydrolysis of the terminal fructose–glucose bond constitutes a somewhat limiting step, particularly from an industrial point of view.

The rate constants increase, with increasing degree of polymerization, but not to any great extent (<10%). This result differs from that found for oligosaccharides from various other series (malto-, cello-, and xylo-oligosaccharides)[29–34].

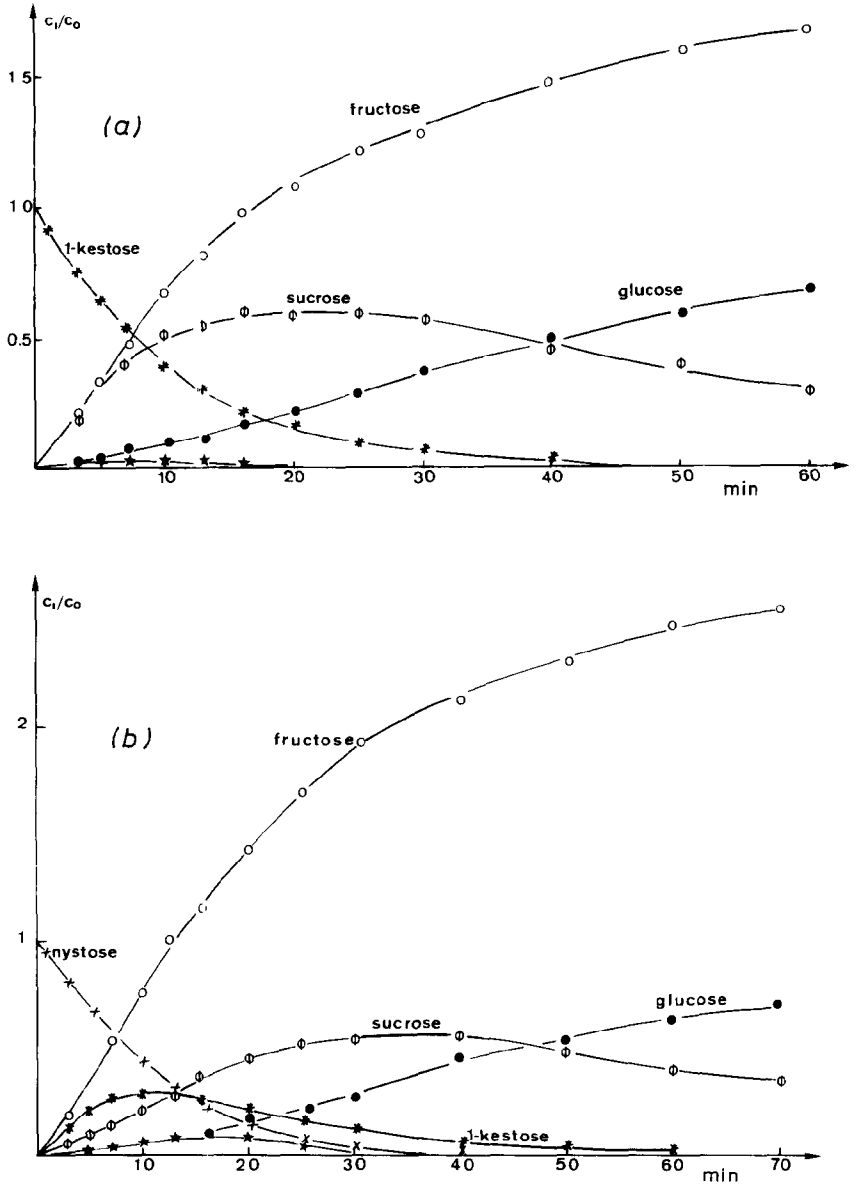

Fig. 3. Time courses of hydrolysis of d.p 3 (*a*) and d.p. 4 (*b*) by sulfuric acid. Temperature 70°; pH 2. Plots were obtained by monitoring the concentration, expressed in mol per liter (C_i), of the sugars in the mixture, relative to the initial concentration (C_0) of the oligosaccharide considered. The symbol (★) denotes inulobiose. Inulotriose, found in very low concentration, is not indicated.

The acid hydrolysis of these oligosaccharides has been described by using two rate constants; a constant k_1 for the glycosidic bond at the nonreducing end of the chain and a smaller constant (k_2) for each of the remaining glycosidic bonds. The rate of splitting k of an oligomer (d.p. = n) may thus be expressed by a relation of the following type:

$$k = k_1 + (n - 2)\, k_2.$$

The present results for the fructan oligosaccharides are slightly more complicated, as two types of linkage must be taken into account. Thus three rate-constants could be envisaged to describe the hydrolysis: two values relative to the respective terminal linkages and a third one for internal linkages. From the chromatograms obtained and also from Fig. 3b, it is clear that the terminal fructose–fructose bond is cleaved faster than the other bonds (for example during the hydrolysis of a compound having d.p. = n, the oligomer having d.p. = $n - 1$, containing only fructose residues, is barely detected as this product is formed at a lower rate than the corresponding glucose-containing oligosaccharide).

In order to check these assumptions, additional work is needed, including mathematical treatment capable of explaining the different curves obtained and the rate constants measured. Such a treatment should also show clearly the trend followed by these oligosaccharides with increasing d.p. and take into account the factors affecting the mechanism. The dependence of k on d.p. may be caused, as mentioned by Szejtli *et al.*[35], by several factors, the most important being an electrostatic shielding-effect (impeding protonation during hydrolysis). However, we also consider that conformational effects may be taken into account, as well as factors relative to solubility and intramolecular interactions[34,36].

CONCLUSION

From the data reported here, cleavage of the glucose–fructose linkage is shown to constitute the main limiting step during the acid hydrolysis of fructan oligosaccharides from juice of Jerusalem artichoke. This should be taken into account from the economic view point, to optimize hydrolytic conditions for production of fructose from the vegetable juice, or for the preparation of such chemicals as alcohols, obtained by fermentation of the partially hydrolyzed juice[37].

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