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Characterisation of the high-molecular weight fructan isolated from garlic (*Allium sativum* L.)

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Abstract

A high molecular weight fructan was isolated from garlic and the structure determined by enzymatic, chemical and spectroscopic (NMR) methods. It was found that the garlic fructan belongs to the neokestose family. It has a (2 → 1)-linked β-D-Fru*f* backbone with (2 → 6)-linked β-D-Fru*f* side chains. A structural model was postulated for a degree of polymerisation of about 58. This model was substantiated using an *endo*-inulinase purified from *Aspergillus ficuum* and by ^{1}H and ^{13}C NMR spectroscopy. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Fructan; NMR; Reductive cleavage; *endo*-Inulinase

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1. Introduction

Garlic (*Allium sativum* L.) belongs to the botanic family of the Liliaceae, which store carbohydrates in cloves making up 75% of the dry matter [1]. In the early 1940s the polysaccharides from garlic were investigated and many publications about the structure of the fructan followed [2–5]. In the following years the D-fructan was isolated and a linear inulin-type was postulated [6,7] from the results obtained from methanolysis, hydrolysis, periodate oxidation, Smith degradation and enzymatic degradation. In 1981 the differences in the fructan content and synthesis in some *Allium* species [8–10] were investigated. The degree of polymerisation (dp) was determined by means of size-exclusion chromatography and for garlic a dp of about 50 was reported. The typical trisaccharides 1-kestose (1F-fructosylsucrose) and neokestose (6G-fructosylsucrose) were found in garlic [11]. Newer results show that garlic belongs to the neokestose-based fructans [12]. Previous works revealed a highly branched fructan, called sinistrin, in red squill (*Urginea maritima*, a Liliaceae from the Mediterranean area) [13,14]. Detailed investigations showed that the structure of the garlic fructan is very similar to the structure of the fructan from *U. maritima*.

2. Results and discussion

During one vegetation period the average dp of garlic was determined. The number average dp (dp_n) was measured with the glucose–fructose–sucrose kit (dp was calculated

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from the difference of free glucose, free fructose, free sucrose and hydrolysed fructan). Fig. 1 shows the results from 1 year. The highest dp was reached at the beginning of October (about 40 dp_n), then the depolymerisation started.

Samples from October were extracted with hot water to prepare the carbohydrates. The extract was then separated on a preparative size-exclusion chromatographic (SEC) system (Fig. 2) consisting of two columns filled with Biogel P2 and P4 (exclusion limit 6000 Da determined with dextran). The dp of the fractions was tested by TLC using an inulo-oligosaccharide standard. Fractions 82–98 contained mono- and disaccharides. Fraction 78 gave two spots for the trisaccharides 1-kestose and neokestose. Fraction 70 showed a spot in the tetrasaccharides range and the following fractions contained higher homologues of the fructan. Fractions 38–41 were pooled, lyophilised and used for further chemical, enzymatic and NMR analysis. Identification of all significant peaks was carried out according to previously obtained gas chromatography–mass spectrometry data [14]. In

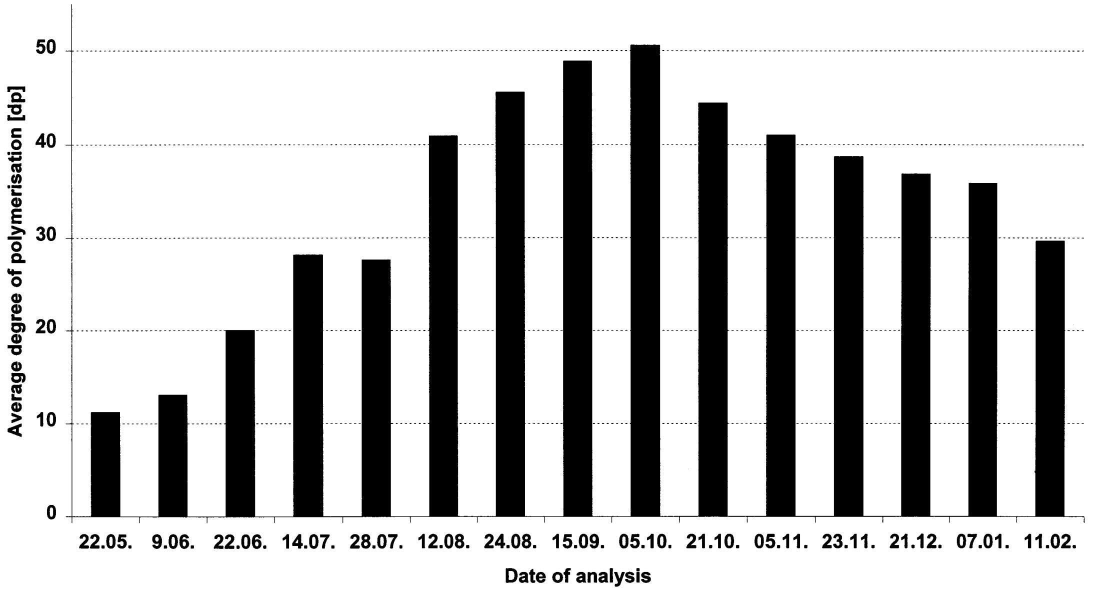

Fig. 1. Average dp of garlic measured enzymatically with the Boehringer-Mannheim test-kit during one vegetation period.

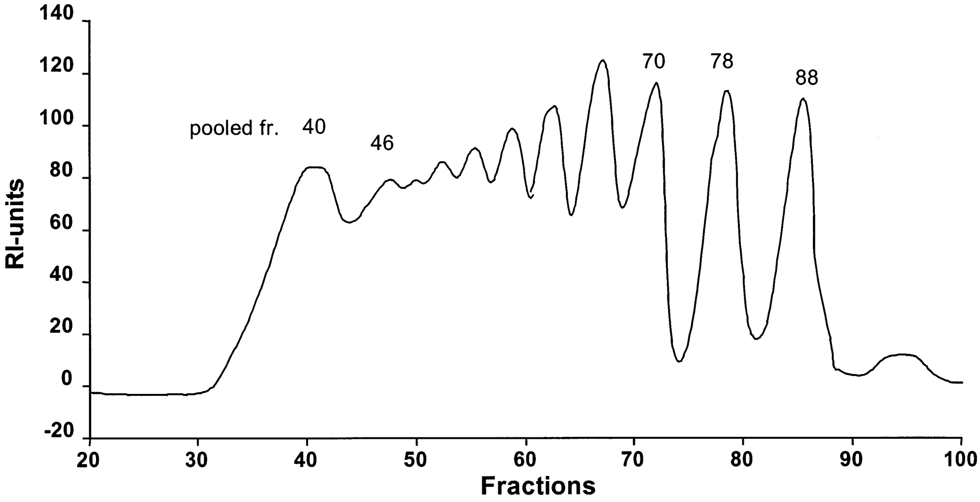

Fig. 2. SEC-chromatogram of the hot water extract from garlic, column material-Biogel P2 and P6, column dimension-1000 × 26 mm, eluent-water, flow-0.3 mL min^{-1}, RI-detector. fr. 95, monosaccharides; fr. 88, sucrose; fr. 78, trisaccharides; fr. 70, tetrasaccharides; lower fr., higher molecular weight fructans.

Table 1
Average percentage (%) and number (*n*) per molecule of β-D-Fru*f* and α-D-Glc*p* residues in the pooled SEC fractions obtain by the reductive cleavage method

Fraction		SEC78		SEC70		SEC64	
		%	*n*	%	*n*	%	*n*
β-D-Fru*f*	terminal	53.1 ± 1.2	1.6 ± 0.04	43.2 ± 1.5	1.6 ± 0.06	33.3 ± 1.0	1.7 ± 0.05
	1-linked	13.9 ± 1.5	0.4 ± 0.05	29.8 ± 0.9	1.1 ± 0.03	45.1 ± 1.8	2.3 ± 0.09
	6-linked	n.d.		n.d.		n.d.	
	1,6-linked	n.d.		n.d.		1.9 ± 0.5	0.1 ± 0.03
α-D-Glc*p*	terminal	6.6 ± 0.5	0.2 ± 0.02	5.4 ± 0.8	0.2 ± 0.03	3.9 ± 0.9	0.2 ± 0.05
	6-linked	26.4 ± 0.9	0.8 ± 0.03	21.6 ± 0.7	0.8 ± 0.03	15.8 ± 1.0	0.8 ± 0.05
dp		3.0		3.7		5.1	

		SEC53		SEC42		SEC40	
		%	*n*	%	*n*	%	*n*
β-D-Fru*f*	terminal	23.5 ± 1.3	2.8 ± 0.15	21.3 ± 1.1	3.2 ± 0.16	19.1 ± 1.3	11.1 ± 0.75
	1-linked	55.5 ± 2.0	6.6 ± 0.24	55.3 ± 3.0	8.2 ± 0.45	48.2 ± 2.8	28.0 ± 1.40
	6-linked	5.9 ± 1.0	0.7 ± 0.12	6.7 ± 1.9	1.0 ± 0.28	15.5 ± 2.1	9.0 ± 1.22
	1,6-linked	6.7 ± 0.9	0.8 ± 0.11	10.0 ± 0.9	1.5 ± 0.13	15.5 ± 2.0	9.0 ± 1.16
α-D-Glc*p*	terminal	n.d. [a]		n.d.		n.d.	
	6-linked	8.4 ± 0.4	1.0 ± 0.05	6.7 ± 0.2	1.0 ± 0.03	1.7 ± 0.2	1.0 ± 0.12
dp		11.9		14.9		58.0	
Degree of branching		0.07		0.10		0.16	

[a] n.d. = not detected.

order to obtain quantitative information (Table 1) peak areas were corrected by applying the effective-carbon response method [15,16]. The fraction of the trisaccharides (fr. 78) contained a terminal and a 6-O-linked α-D-Glc*p* residue and only one 1-O-linked β-D-Fru*f* residue. This confirms that 1-kestose and neokestose are present in the carbohydrates of garlic. In the fraction of the tetrasaccharides (fr. 70) two residues of terminal β-D-Fru*f*, one residue of 1-O-linked β-D-Fru*f*, one residue of 6-O-linked α-D-Glc*p* and no 6-O-linked β-D-Fru*f* residues were found. In the higher molecular weight fractions increasing amounts of 1,6-O-linked and 6-O-linked β-D-Fru*f* residues were found and no more terminal α-D-Glc*p* residues. The fraction with the highest dp_n consists of 48.2% of (2 → 1)-linked, 15.5% of (2 → 6)-linked, 19.1% of terminal and 15.5% of branched β-D-Fru*f* residues and 1.7% α-D-Glc*p* residues linked as neokestose.

The ^{1}H and ^{13}C NMR spectra of inulin (a), the garlic fructan (b) and sinistrin (c) dissolved in $\mathrm{Me_2SO}$-d_6 are shown in Figs. 3 and 4. To assist the signal assignments, 2D spectra (HH-DQF-COSY and inverse HC-COSY) were also recorded and used as recently described in an investigation of glycogen and amylopectin branching [17]. This resulted in clear correlations of the type OH-3↔H-3↔H-4↔OH-4, H-4↔H-5↔H-6↔OH-6 and H-1↔C-1, H-3↔C-3, H-4↔C-4, H-5↔C-5, and H-6↔C-6. The corresponding assignments are consistent with those recently achieved for inulin and sinistrin in aqueous solutions [14].

Small signals pertaining to the terminal fructan moieties can be discerned in this clearly resolved ^{1}H NMR spectrum (Fig. 3). Although the ^{1}H-signals of the garlic fructan and sinistrin are severely broadened due to a distribution of structurally different fructan units, the above-mentioned correlations and assignments are still valid. Comparison of the ^{13}C-spectra of garlic fructan (b) and sinistrin (c) shows that they have a close structural similarity with the inulin backbone (C-1–C-6)

(Fig. 4). The most significant difference between the garlic fructan (b) and sinistrin (c) is the relative intensity of the C-2 inulin backbone signal (→1)-Fru*f*-(2→ at 103.5 ppm and the group of three signals at about 104 ppm, from the anomeric carbon atoms to the branching units, →6)-Fru*f*-(2→, Fru*f*-(2→, and →1,6)-Fru*f*-(2→. This difference indicates a significantly lower degree of branching of the garlic fructan as compared with sinistrin. However, as indicated by the various relative signal positions and intensities there is a close structural similarity evident between the garlic fructan and sinistrin, which has been recently advanced [14].

The composition of the high molecular weight fructan of garlic is similar to sinistrin, the fructan from *U. maritima*. The amount of (2→6)-linked β-D-Fru*f* residues is more or less identical to the branched β-D-Fru*f* residues. The glucose is linked as neokestose-type only and the amount of (2→1)-linked β-D-Fru*f* residues is rather high (48.2%). Fig. 5 shows a model of the suggested structure from the high molecular weight fructan from garlic with a dp_n of 58. The calculated number of β-D-Fru*f* residues in this molecule is 27.9 (2→1)-linked, 9.0 (2→6)-linked, 11.1 terminal and 9.0 branched β-D-Fru*f* moieties. The calculated difference between the terminal and the branched fructofuranosyl residues is 2.1. This value is in agreement with only one glucopyranosyl residue being present in the neokestose position in each fructan molecule.

Purified *endo*-inulinase isolated from *Aspergillus ficuum* [18] has been found to hydrolyse only non-branched (2→1)-linked β-D-Fru*f* residues with a minimum chain length of 7 fructofuranosyl residues [19]. Table 2 shows the results of the degradation of different fructans with the purified *endo*-inulinase. Inulo-oligosaccharides up to a dp_n of 3–7 are not degraded by this *endo*-inulinase. Higher molecular weight fructans, (2→1)-linked and non-branched as inulin are hydrolysed yielding fructo-oligosaccharides with a dp_n from 3

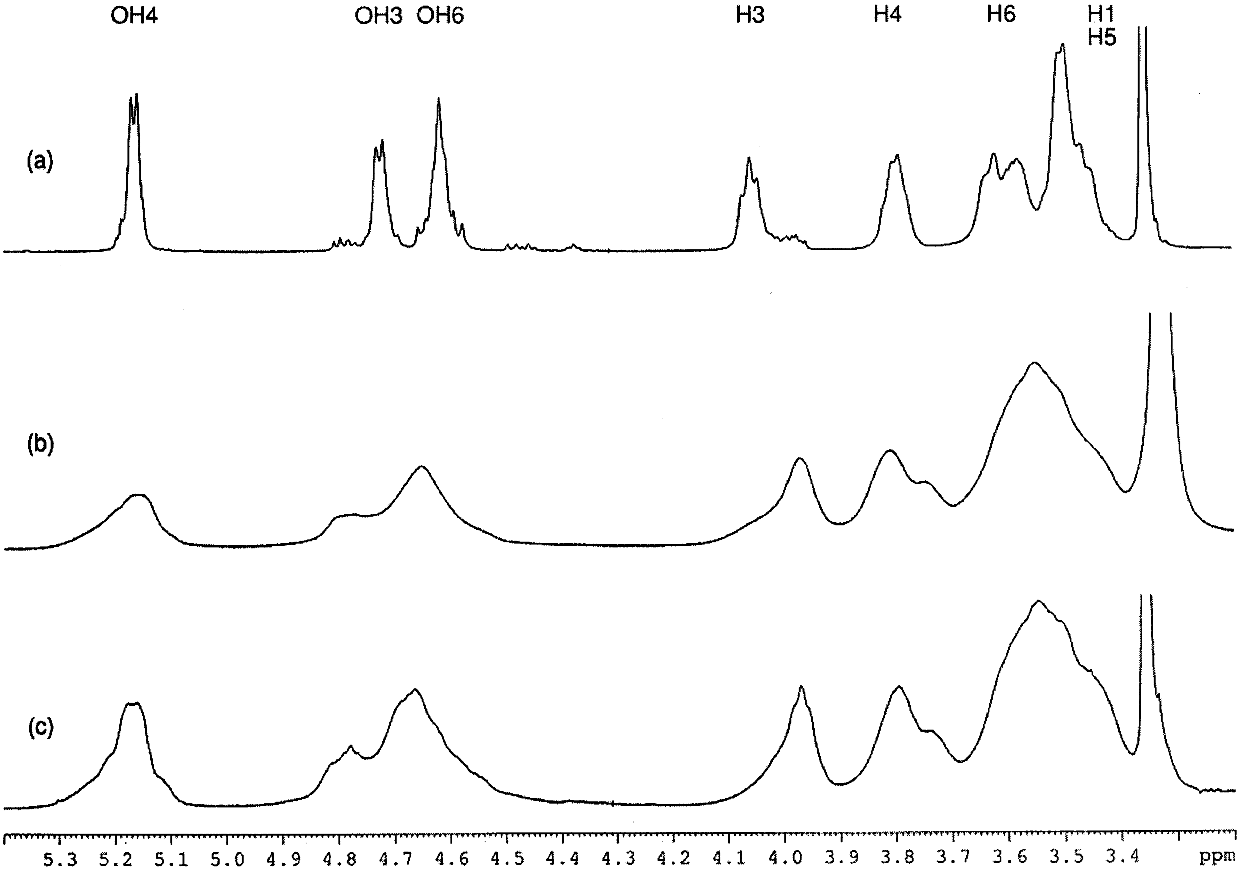

Fig. 3. ^{1}H NMR spectra of inulin (a), garlic fructan (b) and sinistrin (c) in $\text{Me}_2\text{SO-}d_6$.

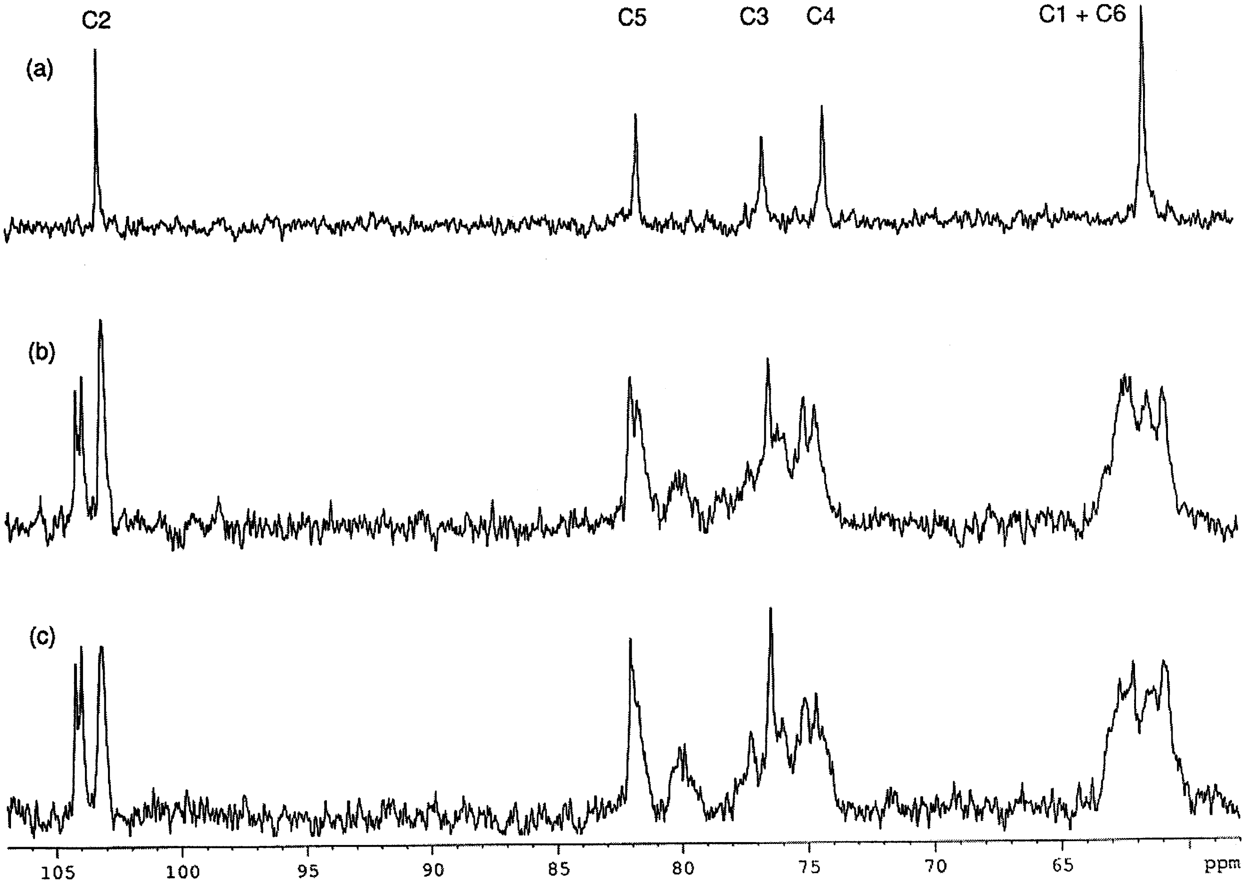

Fig. 4. ^{13}C NMR spectra of inulin (a), garlic fructan (b) and sinistrin (c) in Me$_2$SO-d_6.

to 7. Levan (mainly (2→6)-linked β-D-Fru*f* residues) and also sinistrin (mixed structure and highly branched) were not hydrolysed. In addition, after 24 h reaction time no monomers were released from the garlic fructan.

3. Experimental

Extraction of carbohydrates.—In a typical experiment the carbohydrates (50–100 g plant material) were extracted with 100 mL hot water. The mixture was left for 1 h at 80 °C, then boiled for 5 min, and filtered through a cotton cloth. The cloudy extract was centrifuged at 14,000*g* for 20 min. The clear extract was ready for use or was stored in small portions at −80 °C.

Enzymatic reaction mixtures for the activity determination of the inulinases.—A 2.5% inulin, levan, sinistrin and garlic solution was prepared in 0.01 N NaOAc buffer of pH 4.7. 5400 μL from this solution were mixed with 600 μL enzyme solution and incubated at 37 °C. 450 μL samples were drawn at several times and put in a 100 °C waterbath for 10 min to deactivate the enzymes. Samples were taken after 15, 30, 240 and 1440 min.

Enzymatic carbohydrate analysis.—The test combination for glucose, fructose and sucrose from Boehringer-Mannheim (Vienna, Austria) [20] was used for the enzymatic determination of the carbohydrates (test-kit no. 716260). At first the free glucose, free fructose and sucrose were measured. Afterwards the sample was hydrolysed. 1 mL of extract and 1 mL 0.1 N H_2SO_4 were incubated for 1 h at 80 °C. Afterwards 1 mL was withdrawn from the vial and neutralised with 10 mL satd $NaHCO_3$ soln. This solution was then used for the enzymatic determination of the total glucose and total fructose content. The calculations for the different sugars were made according to the instructions of the manufacturer. To calculate the average dp the amounts of free glucose, free fructose and half the sucrose were subtracted from the total glucose and the total fructose content. Afterwards the fructose content was divided by the glucose content, which resulted in the average dp of the fructans.

SEC.—Biogel P2 (fine, 45–90 μm, MW range 100–1800) and P4 (fine, 45–90 μm, MW range 800–4000; both Co. BioRad, Vienna, Austria; column dimensions: 1000 × 250 mm) were used with distilled water (filtered and degassed) as the eluent at a flow rate of 15 mL h^{-1}. An RI-detector (Knauer, Co. Berger, Linz, Austria) was used for the detection of the peaks. The fractions were collected with the Gradifrac and recorded with the REC 102 (both Pharmacia, Vienna, Austria). The fractions 76–80 (78), 69–72 (70), 62–65 (64) and 21–25 (23) were combined and analysed by GC.

GC.—The methylation and reductive cleavage were carried out as described [21–23]. GC was performed on a HP 8590 gas chromatograph with FID (Hewlett–Packard, Vienna, Austria). A Permabond OV1701 capillary column from Machery and Nagel (Düren, Germany; 25 m, i.d. 0.25 mm; 0.25 μm film thickness) was used with N_2 as carrier gas, temperature program 100→250 °C at

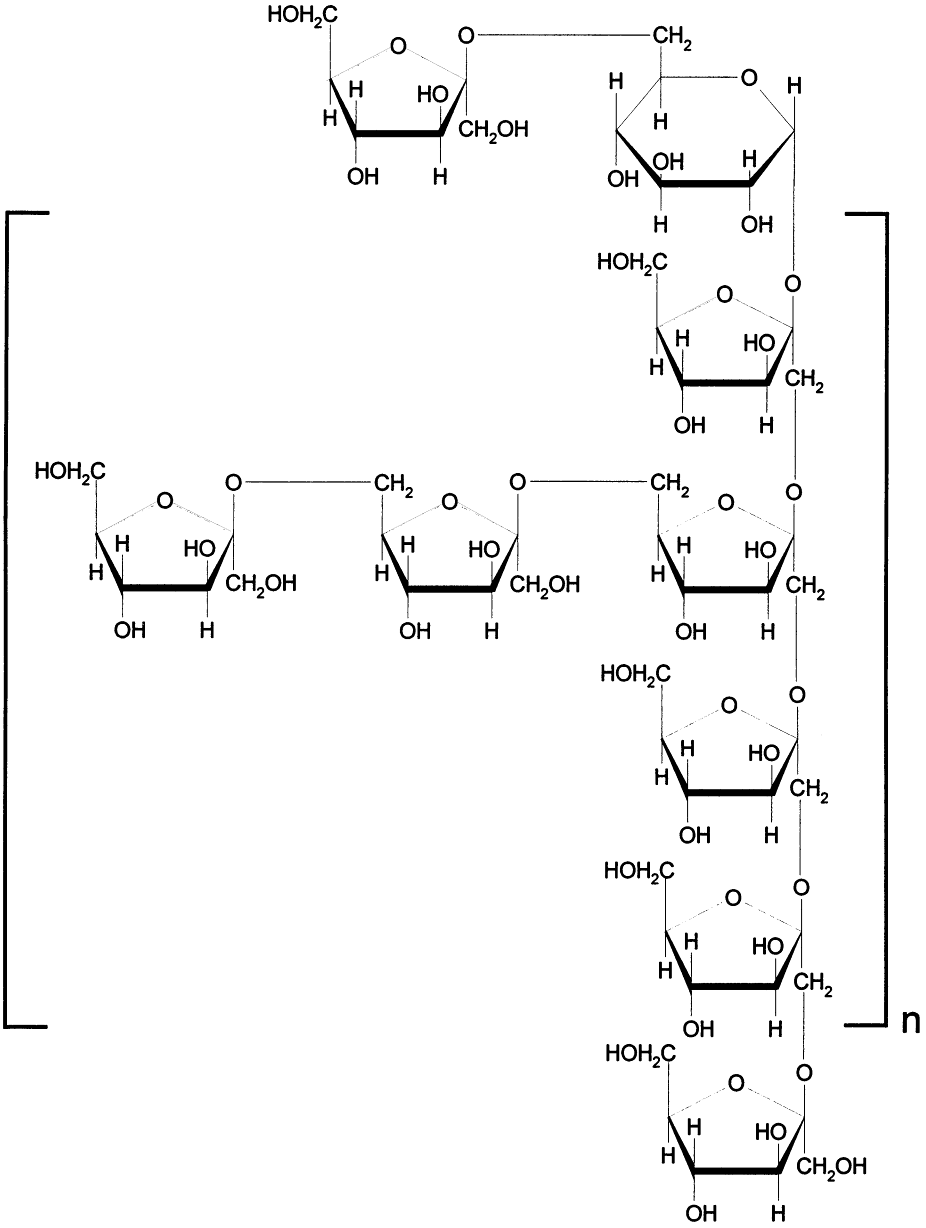

Fig. 5. Suggested structure for the garlic fructan; *n* = 9 for a dp of 58.

Table 2
Degradation products of inulo-oligomers and fructans after incubation with *endo*-inulinase from *A. ficuum*

Sample	TLC-detection of products (dp 3–7)	
	Incubation time	
	4 h	24 h
Inulo-oligosaccharides		
dp 6	−	−
dp 7	−	−
dp 8	+	+
Inulin	+	+
Levan	−	−
Sinistrin	−	−
Fructan of garlic	−	−

4 °C min^{-1}, injector temperature 230 °C and the detector temperature 300 °C. The samples were injected with a split ratio of 1:50.

NMR.—The samples were prepared by dissolving 20 mg of inulin, garlic fructan and sinistrin in 0.7 mL Me_2SO-d_6 (99.8% D, Eurisotop) at 60 °C for 5 min in 5 mm quartz sample tubes. The spectra were recorded at 298 ± 1 K on a Bruker DRX 500 instrument at a proton frequency of 500.13 MHz and a carbon frequency of 125.77 MHz under non spinning conditions. 2D (HH-COSY and HC-COSY) experiments were executed using the gradient enhanced HH-DQF-COSY technique [24] (2048 data points in F^2, 256 data points in F^1; 8 scans; 16 dummy scans; 3004.8 Hz spectral width in both dimensions; phase sensitive (TPPI); apodization function: Gaussian curve) and the inverse HC-COSY strategy (*ge*-HSQC [25]; 2048 data points in F^2, 512 data points in F^1; 8 scans, 16 dummy scans; 3004.8 Hz spectral width in F^2 and 18,865 Hz in F^1; phase sensitive (echo/antiecho); apodization function in F^2: Gaussian curve, in F^1: sinebell squared).

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