Carbohydrate Polymers 2 (1982) 135–144

^{13}C NMR STRUCTURAL INVESTIGATION OF SCLEROGLUCAN

MARGUERITE RINAUDO

Centre de Recherches sur les Macromolécules Végétales, Laboratoire Propre du CNRS Associé à l'Université Scientifique et Médicale de Grenoble, 53 X, 38041, Grenoble-Cédex, France

& MARC VINCENDON

Laboratoires de Chimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85 X, 38041 Grenoble-Cédex, France

(Received: 24 March, 1982)

ABSTRACT

This paper concerns the ^{13}C NMR signal assignment in the DMSO of a neutral polysaccharide, scleroglucan. The previously proposed chemical structure is confirmed. The ^{13}C NMR spectrum shows that scleroglucan is a regular poly (A, B, C, D) type glucan. The relaxation times of the different series of carbon atoms demonstrate that a single, pendant glucose group is attached to each third monomer along the main chain of what is a β(1→3)-glucan. Partial acid hydrolysis gives a spectrum analogous to that of the β(1→3)-D-glucan, curdlan, and confirms the structure of the polymer backbone.

In aqueous solution, no signal has been obtained due to the existence of a rigid, ordered conformation as demonstrated by optical rotation; in the presence of sodium hydroxide, a conformational transition is produced just as with curdlan. The conclusion is that the behaviour of scleroglucan in solution is similar to that of other β(1→3)-D-glucans even though it is more soluble.

INTRODUCTION

The subject of this paper is the structural analysis of scleroglucan: a β(1→3)-D-glucan with a single, pendant glucose group attached through a β(1→6) linkage. Scleroglucan is a neutral, exocellular polysaccharide secreted by a fungus from the genus Sclerotium

Carbohydrate Polymers 0144-8617/82/0002-0135/$02.75 –

(*Sclerotium rolfsii*) (see, for example, Sandford, 1979; Rodgers, 1973; Griffith & Compere, 1972).

This water soluble polysaccharide has been used as a suspending and gelling agent; antitumour activity has also been suggested by Hamuro & Chinara (1973). In general, its properties are similar to those of other $\beta(1 \rightarrow 3)$-glucans and specially to the well known curdlan (see Blum & Sarko, 1977; Harada, 1977, 1979; Marchessault *et al.*, 1977; Ogawa *et al.*, 1972, 1973; Saito *et al.*, 1976, 1977, 1978, 1979; Takeda *et al.*, 1978). In particular, it exhibits a gel-like structure in aqueous solution at low temperature.

The chemical structure was first established by Johnson *et al.* (1963) for the polysaccharide produced by *Sclerotium glucanicum*; hydrolysis by an exo-laminarase produces 1 mol gentiobiose for every 2 mol glucose and therefore its structure may be represented as in Fig. 1.

This paper reports a ^{13}C NMR analysis of the natural and acid modified polymers. Structural information obtained by other methods is correlated with NMR data and a definitive structure is proposed.

EXPERIMENTAL

Scleroglucan is manufactured by CECA (Velizy, France). The $\beta(1 \rightarrow 6)$-D-glucose units can be split off by partial acid hydrolysis; the acid modified polymer must be a $\beta(1 \rightarrow 3)$-D-glucan, analogous to the gel forming curdlan. For that purpose, a 1% (w/v) N H_2SO_4 solution was boiled under reflux for 6 h, neutralised and the polymer recovered by precipitation with ethanol.

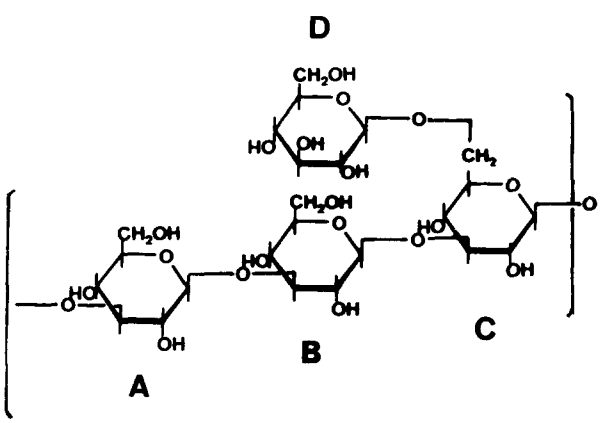

Fig. 1. Structural unit of scleroglucan.

The sample of curdlan (kindly supplied by T. Harada) was a gel forming β-1,3-glucan which is produced by *Alcaligenes faecalis* var. *myxogenes* IFO 13140 (Harada, 1979).

Total hydrolysis of scleroglucan with H_2SO_4 confirmed that glucose was the only monomeric unit in the polysaccharide.

Enzymic hydrolysis was performed with a commercial 'cellulase' which possesses a β(1 → 3)-D-glucan hydrolase activity; samples with various degrees of polymerisation were obtained after different times of hydrolysis because of the presence of an endo-hydrolase. The partially depolymerised polymers were recovered by precipitation with ethanol. This method makes it possible to decrease the viscosity of solutions for NMR investigations.

NMR measurements were performed in DMSO-d_6 solution at 60°C on a Cameca 250 spectrometer operating at 62·86 MHz for ^{13}C. The pulse Fourier transform technique was used with complete proton decoupling, a spectral window of 12 500 Hz, a pulse width of 12 μs (90°) and digitisation in 16K data points giving a digital resolution of 1·5 Hz. Generally 100 000 scans were necessary to obtain a ^{13}C spectrum. The chemical shifts were taken from external HMDS (hexamethyldisiloxane) and were converted to external TMS by adding +1·8 ppm to the values obtained. T_1 relaxation time measurements were made by the inversion-recovery method using the sequence $(180\text{-}t\text{-}90\text{-}T)_n$ with seven t values and the average of three experiments on a Bruker WM 250 spectrometer; the variability of T_1 values is within 15%. The number of sequences, n, necessary to have a sufficient signal/noise ratio was 5000–7000 with this spectrometer. The polymer concentration in all experiments was 50 mg ml^{-1}.

Optical rotatory measurements were performed at 300 nm with a Fica Spectropol 1 spectropolarimeter equipped with a 10 cm quartz cell; the polymer concentration was 0·72 mg ml^{-1}.

RESULTS AND DISCUSSION

(A) ^{13}C NMR Spectra

The macromolecule can be assumed to be a polymer of four monomeric units (A, B, C, D) shown in Fig. 1.

The ^{13}C NMR spectrum shown in Fig. 2A was recorded in DMSO-d_6 solution at 60°C; 24 signals could be expected for a regular polymer containing a group of four D-glucopyranosyl residues as a repeating unit; 18 different signals are visible in the spectrum of Fig. 2A, some of them clearly being more intense than others. The integral intensity of the different signals obtained on an antigated ^{13}C NMR spectrum gave: a 4:1 ratio for the signals at 104 ppm and individual signals in the 87 ppm region, a ratio of 2:1 for the signal at 77·3 ppm compared to signals at 77·55 or 77·05 ppm and a ratio of 4:1 for the complex signal at 69·4 and that at 71·15 ppm. Taking into account these superimposed signals, 24 carbons could then be identified.

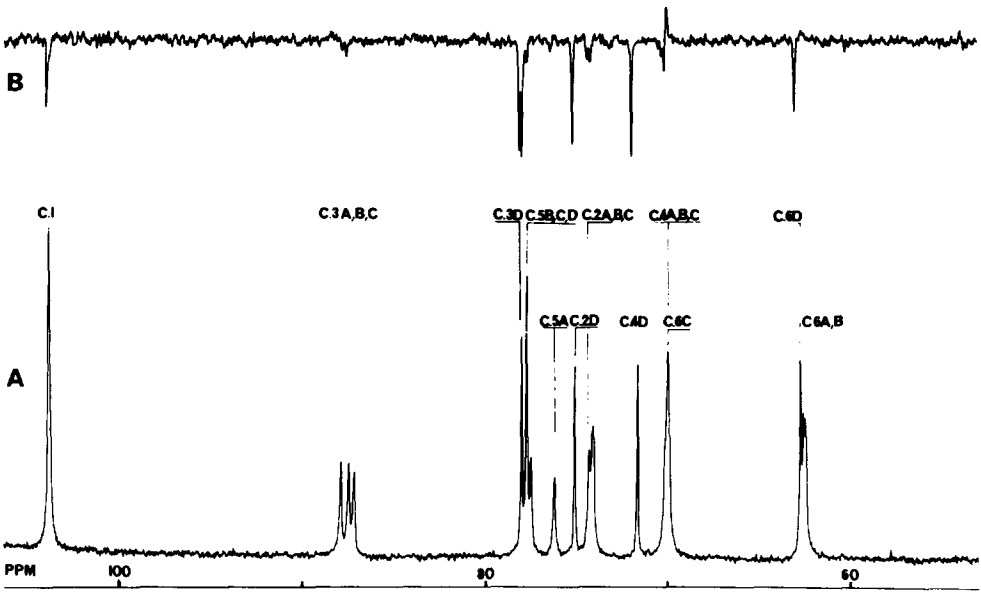

Fig. 2. (A) ^{13}C NMR spectrum of scleroglucan in DMSO-d_6 solution at 60°C. (B) ^{13}C NMR spectrum of the partially relaxed atoms (same conditions).

This confirms that scleroglucan is a regular polymer with a tetrasaccharide repeating unit.

Surprisingly, all C-1 signals have the same chemical shift (104 ppm) whereas anomeric carbons are generally quite sensitive to the magnetic environment as, for example, in lichenin (a β-linked glucan with a trisaccharide as repeating unit) in which three C-1 signals were observed (Gagnaire & Vincendon, 1977).

The three different signals in the 87 ppm region are due to C-3 carbons involved in the $\beta(1 \rightarrow 3)$ glycosidic linkage and are more sensitive to the magnetic environment. The hydroxymethyl C-6 carbon involved in the $\beta(1 \rightarrow 6)$ linkage (C-6C) is found at 69·4 ppm while signals from the three others may be observed in the 62 ppm region on the hydrogen coupled spectrum.

Assignment of all the carbon signals was achieved by comparison of chemical shifts with the corresponding $\beta(1 \rightarrow 6)$ and $\beta(1 \rightarrow 3)$-glucans linear homopolymers (Table 1) and by the use of the rule that glycosylation of a carbon induces an important downfield shift of the α-carbon (+7 ppm) while the β-carbon is shifted upfield (−1 ppm); the results are given in Table 1.

An important feature of the spectrum in Fig. 2 is the great difference in peak height (and thus of line width) for the different single signals. These could be separated into two groups – small signals: C-3A, C-3B, C-3C, C-5C, C-5A, C-2A, C-2B, C-2C; and high signals: C-3D, C-2D, C-4D, C-6D.

TABLE 1
^{13}C NMR Chemical Shift δ^{+a} of Scleroglucans and Homopolymers in DMSO Solution at 60°C

Compound:		C-1	C-2	C-3	C-4	C-5	C-6
Scleroglucan	A	103·9	73·8 (172)[b]	87·65 (155) [10][b]	69·45	75·7 (150) [9]	61·85 (110)[b]
	B	103·9	73·65[b]	87·15 (160) [10][b]	69·4	77·05 (165) [9][b]	61·90 (105)[b]
	C	103·9	73·65[b]	86·9 (141) [9·5][b]	69·4	77·3[b]	69·4
	D	103·9	74·6 (230) [5]	77·55 (215) [5·5]	71·15 (230) [5]	77·3	62·05 (140)
Hydrolysed scleroglucan		103·8	73·8	87·0	69·3	77·2	61·8
Curdlan (linear $\beta(1\rightarrow 3)$-glucan)		103·7	73·6	86·9	69·2	77·1	61·7
Pustulan (linear $\beta(1\rightarrow 6)$-glucan)		104·15	74·3	76·45	70·9	77·4	69·45

[a] From external TMS.
[b] Assignment could be reversed. T_1 relaxation time (ms) is given in parentheses. Line width at half height (Hz) is given in square brackets.

This is due to the different relaxation times of the carbon species in parts of the molecule having different mobility. Regarding the structure of the polymer, the (1 → 6)-D-glucose pendant unit is expected to be more mobile and therefore to give sharper signals. The T_1 relaxation times of those signals have been determined and are given in Table 1; the high signals of the pendant unit D have an average T_1 value of 230 ms, and the others, corresponding to the main chain (units A, B, C), 150 ms.

The assignment based on chemical shift comparisons agrees with the assignment based on T_1 values. The choice between C-6 hydroxymethyl carbons was made on T_1 considerations. The one with the highest T_1 value has been assigned to the C-6 atom of the pendant group D.

Figure 2B gives the spectrum of a partially relaxed experiment during the measurement of T_1 by the inversion-recovery method with the pulse sequence $(180\text{-}t\text{-}90\text{-}T)_n$ corresponding to a t value of 100 ms. This figure clearly shows the difference in relaxation time for the carbon atoms of the D unit. While practically all the carbon atoms of the chain show a null intensity, the intensity of the six carbon atoms of the D-glucopyranosyl unit is still negative. It is also noticeable that the only signal with a positive intensity is the C-6 hydroxymethyl carbon atom of the C unit corresponding to the branching point of the chain. It was not possible to determine the T_1 value of this signal, because of the overlapping signals. However, this value is less than 100 ms, showing that this carbon atom is located in a more rigid part of the chain than the two other hydroxymethyl carbon atoms C-6A and C-6B. This is probably due to the hindered rotation about the C-5/C-6 linkage of the C-unit in solution in DMSO.

Treatment of scleroglucan with a 1 N solution of H_2SO_4 produces a specific splitting of the pendant groups.

Figure 3 gives the ^{13}C NMR spectrum of the hydrolysed polymer. Six main signals are visible, and minor signals (about 10%) corresponding to the initial tetrasaccharide repeating unit are still present. A comparison of the chemical shifts of the major peaks with the ^{13}C NMR signals of curdlan, the $\beta(1 \rightarrow 3)$ glucan, is shown in Table 1. This result confirms that under acidic treatment there is a preferential cleavage of the $\beta(1 \rightarrow 6)$-D-glucopyranosyl groups producing mainly a $\beta(1 \rightarrow 3)$ linear D-glucan.

(B) Influence of the Solvent on the NMR Spectrum

Scleroglucan was dissolved in D_2O at an elevated temperature and low concentration (0·5% w/v). The solution forms a gel at room temperature. No ^{13}C signals can be detected even at 90°C and the absence of ^{13}C signals is attributed to the presence of an ordered helical conformation which is also observed by optical rotatory measurements.

In Fig. 4, the dependence of the specific rotatory power $[\alpha]^{300}$ is given as a function of the NaOH content of the solvent. It is clear that an ordered conformation exists in neutral or weakly basic solvents, followed by a conformational transition at about N/6 NaOH. In DMSO, the optical rotatory power is of the same magnitude as in NaOH over 0·2 M and corresponds in both solvents to a random coil conformation. Disappearance of the NMR signals due to partial ordering of polymer chains has

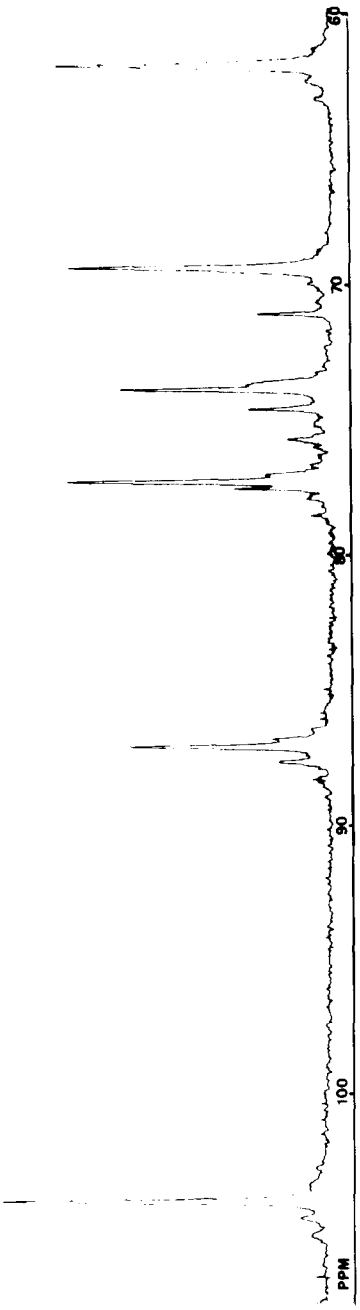

Fig. 3. ^{13}C NMR spectrum of acid hydrolysed scleroglucan (same conditions as in Fig. 2A).

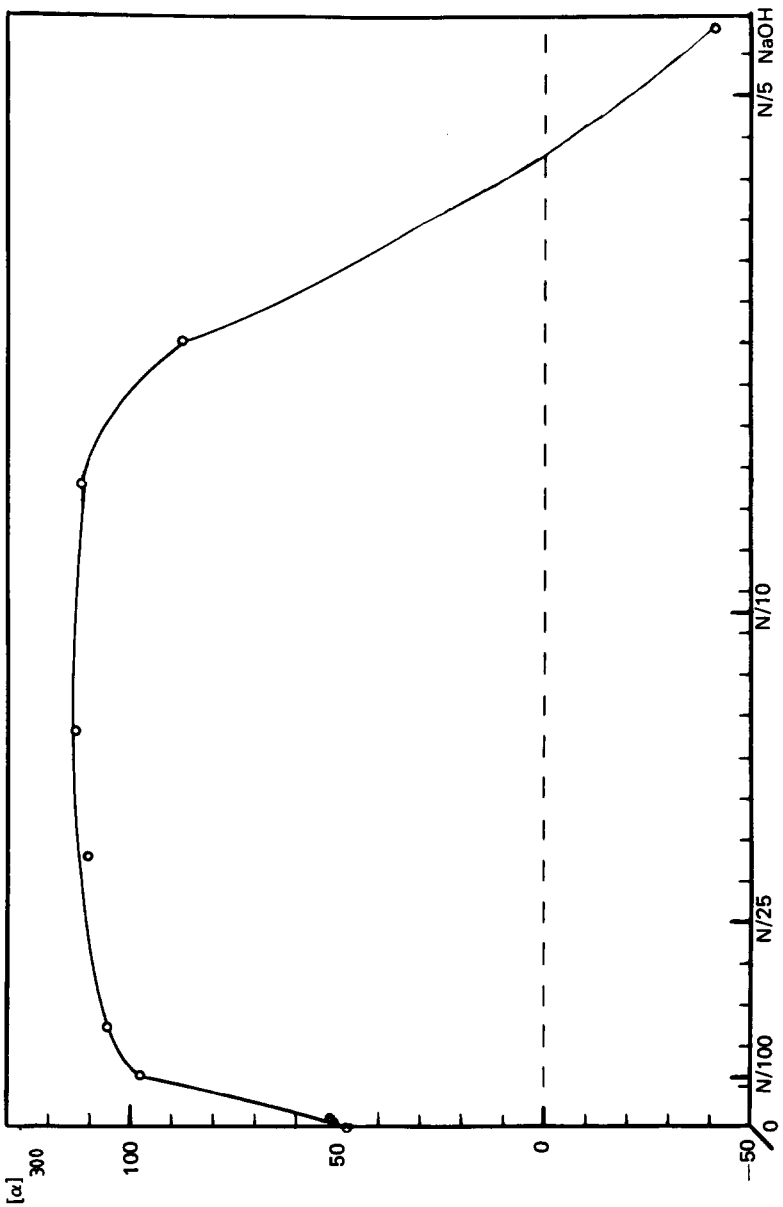

Fig. 4. Optical rotatory power $[\alpha]_{20}^{300}$ of scleroglucan as a function of NaOH concentration.

previously been noticed for gelatin (Eagland *et al.*, 1974), ι-carrageenan (Bryce *et al.*, 1974), polygalacturonic acid in the presence of Ca^{2+} ions (Rinaudo, 1980) and for κ-carrageenan in the K^+ form (Rochas *et al.*, 1980).

By contrast, scleroglucan in 0·2 N NaOD solution gives a modified ^{13}C NMR spectrum shifted upfield by 1 ppm from that of the DMSO solution. Most of the ^{13}C signals are affected and anomeric signals particularly are split into two groups.

Changes in the nature of the solvent may therefore produce a significant modification in the polymer conformation and therefore in the ^{13}C NMR spectra.

Nearly identical behaviour was obtained previously with curdlan, by Saito *et al.* (1978), Harada (1979) and Kasai & Harada (1980) and other β(1 → 3) glucans: lentinan (Saito *et al.*, 1977, 1978); schizophyllan (Saito *et al.*, 1979); polysaccharide A_3 from *P. ostreatus* (Saito *et al.*, 1976). In DMSO or NaOD solution, the ^{13}C NMR spectra are well defined; in D_2O, a partial disappearance of the signals is attributed to gel formation with junction zones by triple helix formation. In addition, the authors suggest monochain helix formation for the rest of the polymer with a chemical displacement of the C-3 and C-1 signals in the conformational transition range.

These NMR data are different from ours, as we observed a complete disappearance of the signals when ordered conformation is established; in addition, we were never able to observe chemical shift variations of the signals in the range of conformational transition. This result seems to favour the gelling mechanism proposed by Fulton & Atkins (1980) who suggest association of rigid, triple helices.

CONCLUSION

The ^{13}C NMR spectra of scleroglucan obtained in DMSO solution demonstrate the regular structure of this polymer. The ^{13}C NMR spectra are in agreement with the chemical analysis and confirm the proposed structure: scleroglucan is a β(1 → 3)-D-glucan with a degree of branching of one pendant glucose unit every three monomeric units of the main chain, as shown in Fig. 1. In addition it has been shown that there is little or no microheterogeneity on the chain (at least <10%) as no extra signals are visible on the NMR spectrum. Enzymic hydrolysis by an endo-mechanism gives segments whose ^{13}C NMR spectra are the same as the one for the polymer. The partial acid hydrolysis of the scleroglucan allows the isolation of a polymer with a structure mainly of the β(1 → 3)-glucan type.

The assignment of the ^{13}C NMR signals has been performed mainly by correlating the results with those of the corresponding homopolymers which were used as models. Relaxation time measurements show the presence in solution of a difference of mobility for the different glucose units; the carbon atoms of the pendant unit have higher relaxation time values corresponding to a higher mobility of these units. The relaxation time of the C-6 hydroxymethyl carbon atom involved in the glycosidic linkage of the pendant unit and corresponding to the branching point of the chain

shows a lower value than the same free hydroxymethyl carbon of the chain, indicating a hindered rotation of this group due to the bulky substituent.

In DMSO as well as in NaOH solutions (over 0·2 M), a scleroglucan chain presents a random coil conformation, which allows ^{13}C NMR measurements with a good signal-to-noise ratio. In water solution and in slightly basic media, the NMR spectrum disappears due to the stabilisation of an ordered conformation which is also detected by an increase in the optical rotatory power. A multichain interaction mechanism can be proposed to explain this conformational transition due to solvent effect and pH.

The influence of the solvent allows us to conclude that the behaviour of the scleroglucan in solution is similar to that of other $\beta(1 \rightarrow 3)$-glucans and specially that of curdlan even if the solubility of the former is better in water.

REFERENCES

Bluhm, T. L. & Sarko, A. (1977). *Can. J. Chem.* **55**, 293.

Bryce, T. A., McKinnon, A. A., Morris, E. R., Rees, D. A. & Thom, D. (1974). *Farad. Discuss. Chem. Soc.* **57**, 221.

Eagland, D., Pilling, G. & Wheeler, R. G. (1974). *Farad. Discuss. Chem. Soc.* **57**, 181.

Fulton, W. S. & Atkins, E. D. T. (1980). In: *ACS Symposium Series no. 141*, chapt. 25, p. 385.

Gagnaire, D. & Vincendon, M. (1977). *Bull. Soc. chim.* 479.

Griffith, W. L. & Compere, A. L. (1972). In: *Development in Industrial Microbiology*, Society for Industrial Microbiology, chapt. 60.

Hamuro, J. & Chinara, G. (1973). *Nature* **245**, 40.

Harada, T. (1977). Extracellular microbial polysaccharides, in: *ACS Symposium Series no. 45*, chapt. 20, p. 265.

Harada, T. (1979). *Polysaccharides in Food*, ed. J. M. V. Blanshard and J. R. Mitchell, Butterworths, London, chapt. 18, p. 283.

Johnson, J., Kirkwood, S., Misaki, A., Nelson, T. E., Scaletti, J. V. & Smith, F. (1963). *Chem. Ind. (London)*, 820.

Kasai, N. & Harada, T. (1980). *ACS Symposium Series no. 141*, chapt. 24, p. 363.

Marchessault, R. H., Deslandes, Y., Ogawa, K. & Sundarajan, P. R. (1977). *Can. J. Chem.* **55**, 300.

Ogawa, K., Watanabe, T., Tsurugi, J. & Ono, S. (1972). *Carbohydr. Res.* **23**, 399.

Ogawa, K., Tsurugi, J. & Watanabe, T. (1973). *Carbohydr. Res.* **29**, 397.

Rinaudo, M., Ravanat, G. & Vincendon, M. (1980). *Makromol. Chem.* **181**, 1059–70.

Rochas, C., Rinaudo, M. & Vincendon, M. (1980). *Biopolymers* **19**, 2165.

Rodgers, N. E. (1973). *Industrial Gums*, ed. R. L. Whistler, Academic Press, New York, chapt. 22, p. 499.

Saito, H., Ohki, T., Yoshioka, Y. & Fukuoka, F. (1976). *FEBS Letters* **68**, 15.

Saito, H., Ohki, Takasuka, N. & Sasaki, T. (1977). *Carbohydr. Res.* **58**, 293.

Saito, H., Miyata, E. & Sasaki, T. (1978). *Macromolecules* **11**, 1244.

Saito, H., Ohki, T. & Sasaki, T. (1979). *Carbohydr. Res.* **74**, 227.

Sandford, P. A. (1979). *Polysaccharides in Food*, ed. J. M. V. Blanshard and J. R. Mitchell, Butterworths, London, chapt. 16, p. 251.

Sandford, P. A. (1979). *Advances in Carbohydrate Chemistry and Biochemistry*, vol. 36, Academic Press, New York, p. 305.

Takeda, H., Yasuoka, N., Kasai, N. & Harada, T. (1978). *Polymer J.* **16**, 365.