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The production of a new water-soluble polysaccharide by *Acetobacter xylinum* NCI 1005 and its structural analysis by NMR spectroscopy

K. Tajima *, N. Uenishi, M. Fujiwara, T. Erata, M. Munekata, M. Takai

Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo 060, Japan

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Abstract

A new water-soluble polysaccharide (WSP) was isolated from a culture of *Acetobacter xylinum* NCI 1005 grown on sucrose. The structure of the WSP was analysed by nuclear magnetic resonance spectroscopy and determined to be a β-(2 → 6)-linked polyfructan, which is structurally different from the polymer synthesized from glucose instead of sucrose by the same strain. The discovery of this new polysaccharide has revealed that the bacterium is able to synthesize two different kinds of water-soluble polysaccharides. © 1998 Elsevier Science Ltd.

Keywords: Water-soluble polysaccharide; Levan; Levansucrase; *Acetobacter xylinum*; NMR spectroscopy

1. Introduction

Some species of the Gram-negative aerobe *Acetobacter xylinum* are able to synthesize cellulose as an extracellular polysaccharide [1], whereas other species of *Acetobacter* are able to produce either water-soluble polysaccharides (WSPs) [2–4] or both cellulose and WSPs [5,6]. Tayama et al. [4] have reported that, when grown on glucose, *A. xylinum* NCI 1005 produces a new type of water-soluble acidic heteropolysaccharide, tentatively designated as AM-1. The polysaccharide AM-1 is composed of D-glucose, D-galactose, D-mannose, and D-glucuronic acid. We have observed that *A. xylinum* NCI 1005 produces a WSP which is structurally different from AM-1 when grown on a sucrose medium. These results suggest that the synthesis of a WSP can be controlled by changing the carbon source of the medium.

In this study, the structure of the WSP has been analysed in detail by nuclear magnetic resonance (NMR) spectroscopy. In addition, we have examined the WSP synthesis of *A. xylinum* NCI 1005 when grown on a sucrose medium.

2. Results and discussion

Acidic hydrolysis of a water-soluble polysaccharide.—In the HPLC pattern of the acidic hydrolysate of the WSP, only one peak was observed (data are not shown). The retention time for the peak was the same as that of fructose. By comparison of the ^{1}H NMR spectra for fructose and the hydrolysate, it was

* Corresponding author. Fax: +81-11-706-7118; e-mail: tajima@moby.hokudai.ac.jp.

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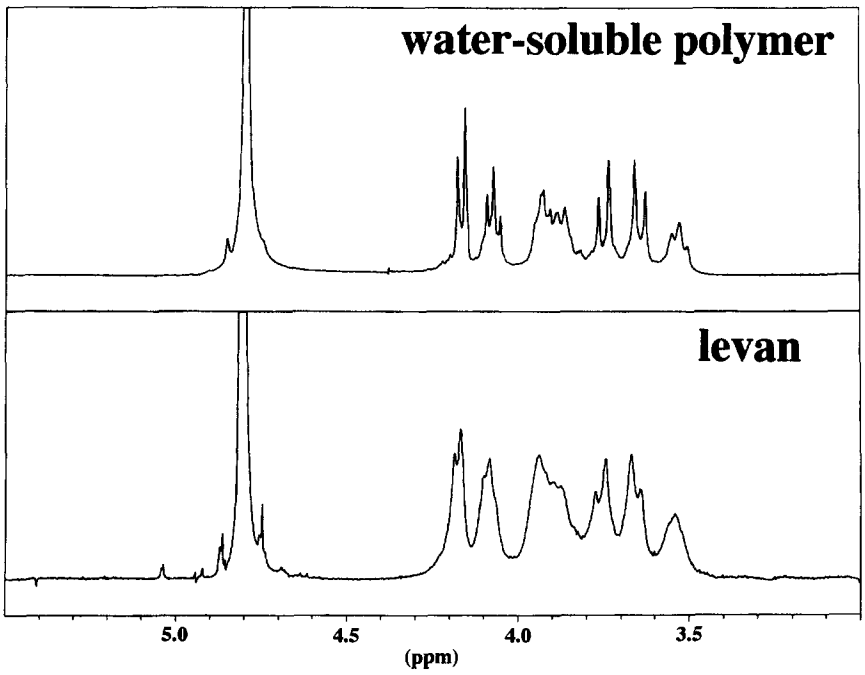

Fig. 1. ^{1}H NMR spectra of the water-soluble polymer and levan from *Erwinia herbicola*.

confirmed that the hydrolysate was fructose (data are not shown). These results suggest that the WSP synthesized by *A. xylinum* NCI 1005 from sucrose is a homopolymer of fructose, polyfructan. Polyfructans are generally subdivided into two main classes: levan, in which the main linkage is β-(2 → 6), and inulin, in which the main linkage is β-(2 → 1). To determine the structure of the polyfructan, ^{1}H, ^{13}C, ^{1}H–^{1}H, and ^{13}C–^{1}H NMR spectra were measured.

Structural analysis of water-soluble polysaccharides by NMR spectroscopy.—Fig. 1 shows ^{1}H NMR spectra of the WSP and levan from *E. herbicola*

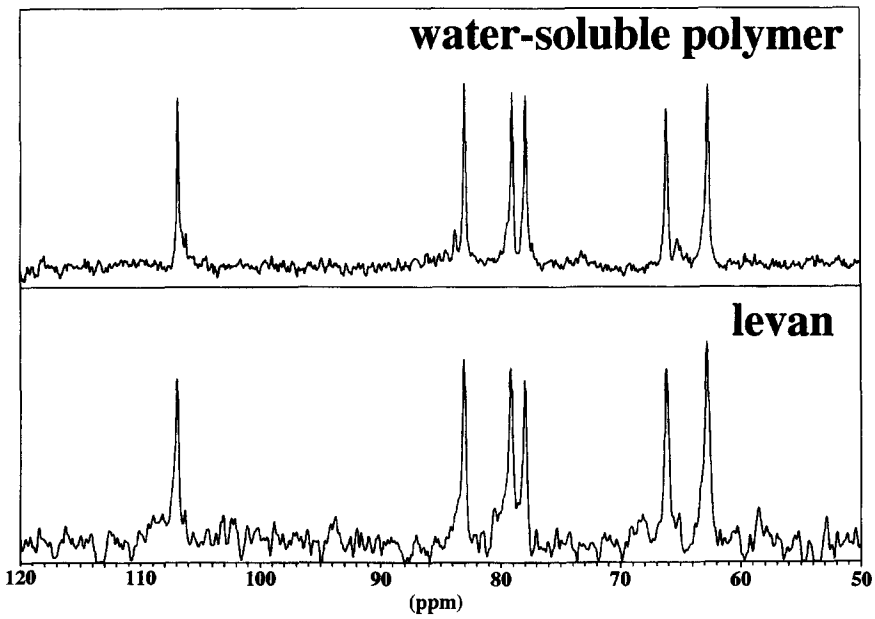

Fig. 2. ^{13}C NMR spectra of the water-soluble polymer and levan from *E. herbicola*.

(Sigma Chemical Co.). No difference was observed between these spectra, except that the spectrum of levan was broadened slightly due to its high molecular weight. Peaks corresponding to the glycosidic proton were not observed in the ^{1}H NMR spectrum of WSP, which suggests that there are very few or possibly no glycosidic protons occurring in the WSP. The ^{13}C NMR spectra of the WSP and of levan are the same (Fig. 2). There are six peaks in the spectra, and these peaks correspond to the carbons constructing the fructose residues. The simplicity of the spectra reflects that the WSP is a homopolymer with fructose. From the comparison of the proton-decoupled and undecoupled ^{13}C NMR spectra of the WSP, it could be suggested that the glycosidic carbon (106.88 ppm) has no linkage with the hydrogen. This is consistent with the absence of an apparent glycosidic proton in the ^{1}H NMR spectra of WSP. The peaks at 82.97, 79.00, and 77.89 ppm and those at 66.08 and 62.62 ppm correspond to the CH and CH_2 carbons, respectively.

To determine the assignments for the ^{1}H, the ^{1}H–^{1}H 2D NMR (COSY) chemical shift correlation, was measured (Fig. 3). The peak at the highest field appears to be the 6″-proton, and the assignment of the 6′- and 5′-protons can be determined by correlation with the 6″-protons. In the same manner, the assignment of 4′- and 3′-protons can be determined by their correlation with the 5′- and 4′-protons, respectively. The peaks at 3.6–3.8 ppm do not correlate with the other protons, and, therefore, they can be assigned to the 1′- and 1″-protons, respectively. The difference in chemical shift between the 6′- and 6″-protons is very large, which could be due to the shielding effect of the electron cloud of oxygen. Oxygen in the fructose residue and glycosidic oxygen takes an sp^2 hybrid orbital, and the peak for the 6″-proton appears at the highest field due to the shielding of the $2p^z$-electrons in the glycosidic oxygen.

Fig. 4 shows the ^{13}C–^{1}H 2D NMR spectrum of the WSP. From the correlation between C–H, each peak has been assigned as follows: C-1 (62.6 ppm), C-6

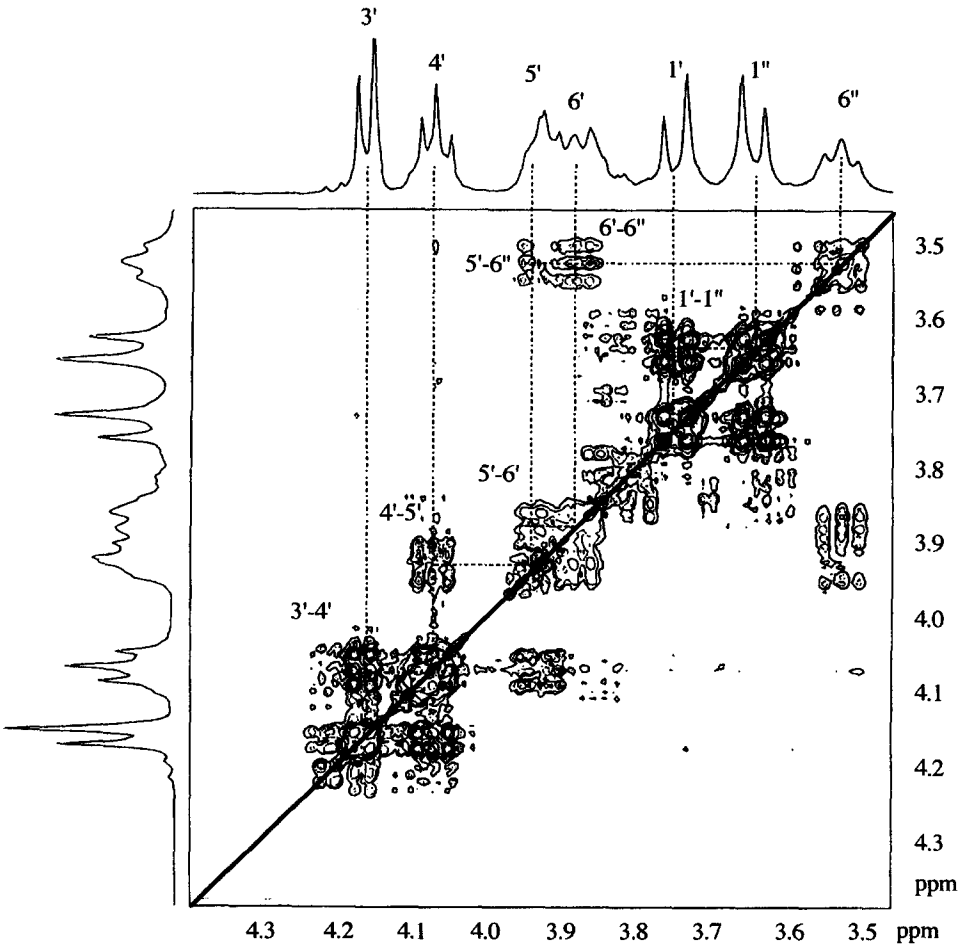

Fig. 3. ^{1}H–^{1}H chemical shift correlation NMR spectrum (COSY) of the water-soluble polymer.

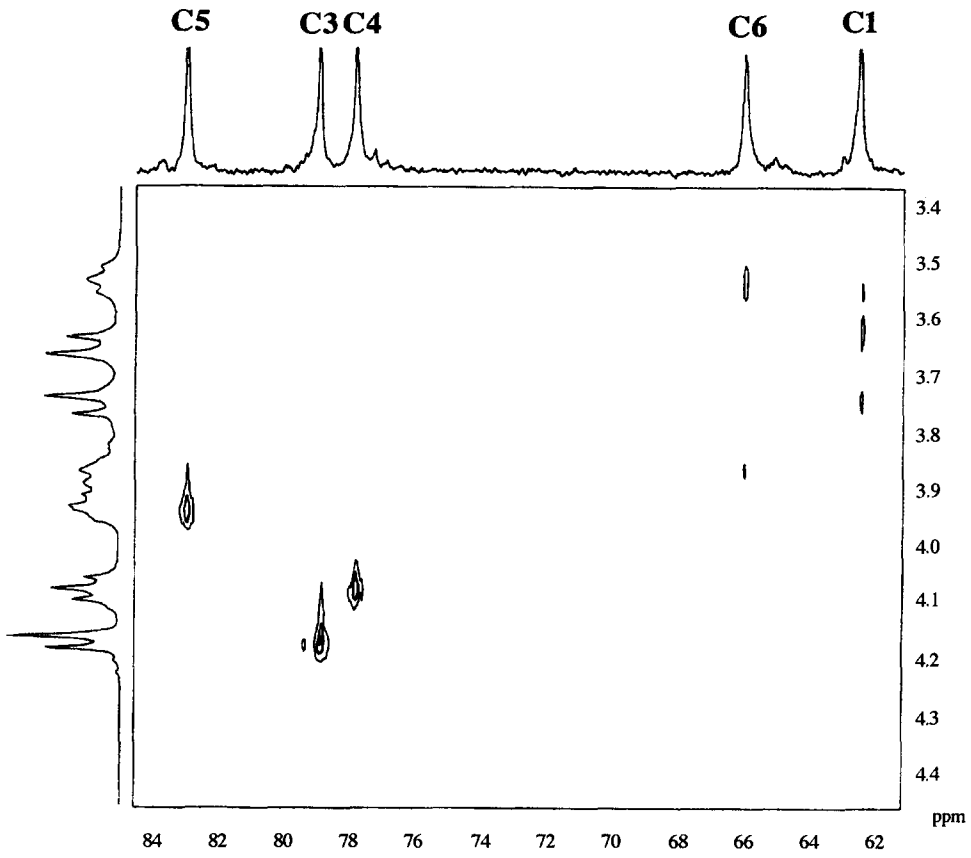

Fig. 4. $^{13}C-^{1}H$ 2D NMR spectrum of the water-soluble polymer.

(66.0 ppm), C-4 (77.9 ppm), C-3 (79.0 ppm) and C-5 (83.0 ppm). C-1 and C-6 each correlate with two protons, which corresponds with results from the nonproton-decoupled ^{13}C NMR spectrum. C-4, C-3 and C-5 each correlate with one proton.

From these results, the structure and the peak assignments for the ^{13}C NMR spectra of the WSP have been determined, as shown in Fig. 5 and Table 1, respectively. The differences in the chemical shifts of C-5 in inulin, β-(2 → 1) polyfructan, and levan, β-(2 → 6) polyfructan, were the most distinct, with a difference of 6.1 ppm, and the chemical shift of the WSP from NCI 1005 was closer to that of levan. Thus, a combination of NMR techniques has demonstrated that the WSP synthesized by *A. xylinum* NCI 1005 from sucrose is a β-(2 → 6)-linked polyfructan, namely levan.

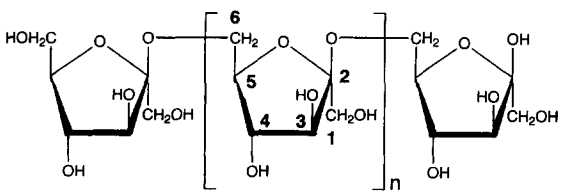

Fig. 5. Structure of the water-soluble polymer.

Production of a water-soluble polysaccharide.— Fig. 6 shows the time-course changes in the pH, the absorbance at 540 nm (which is an index of cell number), and the concentration of each component (levan, fructose, glucose, and sucrose) in the culture of *A. xylinum* NCI 1005. Levan, glucose, and fructose appear along with a rapid decrease in sucrose. That glucose and fructose were detected in the culture suggests that sucrose was hydrolysed by extracellular or membrane-binding enzymes. The sucrose levels decreased rapidly, and levan began to appear in 3 days. The levels of glucose and levan increased gradually and reached their maximal concentrations in 5 and 7 days, respectively. After reaching its maximum, the amount of levan decreased gradually, while the glucose disappeared in 9 days. The fructose levels remained constant throughout the incubation time, and it was observed that the concentrations of glucose and fructose in the culture were not the same. Because sucrose is composed of both glucose and fructose residues, the difference in the time-course changes of their concentrations suggests that their metabolic pathways in the bacterium are different. The culture pH fell from 6.8 to 3.0, indicating the formation of acidic substances. The levan had two

Table 1
^{13}C NMR chemical shifts of the WSP from NCI 1005, inulin, levan, and model compounds

Compound	Chemical shift (ppm)					
	Carbon atom					
	C-1	C-2	C-3	C-4	C-5	C-6
WSP from NCI 1005	62.6	106.9	79.0	77.9	83.0	66.0
Inulin [12]	62.2	104.5	78.5	76.6	87.4	63.4
Zymomonas levan [12]	61.4	105.1	77.5	76.6	81.3	64.6
Methyl α-D-fructofuranoside [13]	58.7	109.1	81.0	78.2	84.0	62.1
Methyl β-D-fructofuranoside [13]	60.0	104.7	77.7	75.9	82.1	63.6

components, with low- and high-molecular-weight fractions. The high-molecular-weight fraction disappeared at an earlier period, while the low molecular weight fraction remained even at later periods. The molecular weight and the yield of levan decreased with the incubation time after 5 and 7 days, respectively. This could be due to the hydrolysis of levan by acidic metabolic substances secreted from the bacterium, because levan is hydrolysed rapidly in an acidic solution (pH 3.0), and the bacterium has a weak ability to metabolize levan.

Many researchers have reported that levan is produced from sucrose by plants [7] as well as a variety of microorganisms such as *Streptococcus salivarius* [8], *Bacillus polymyxa* [9], *Zymomonas mobilis* [10], *Acetobacter diazotrophicus* [11], and so forth. These bacteria have levansucrase (EC 2.4.1.10), which specifically reacts with sucrose, synthesizing levan and releasing glucose. In our experiment, it was observed that levan, glucose, and fructose appeared in the culture as sucrose levels rapidly decreased, suggesting that *A. xylinum* NCI 1005 expresses an extracellular or membrane-binding levansucrase.

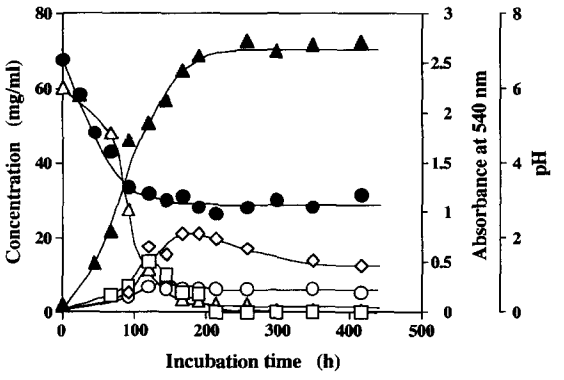

Fig. 6. Time-course changes in the pH (●), the absorbance at 540 nm, which is a index of cell number (▲), and the concentration of each component (levan (○), fructose (□), glucose (◇) and sucrose (△)) in the culture of *A. xylinum* NCI 1005.

Tayama et al. [4] have reported that *A. xylinum* NCI 1005 synthesizes an acidic water-soluble heteropolysaccharide (AM-1) from glucose. Similarly, some quantity of the AM-1 was observed in our experiment, although the amount was smaller than that obtained from glucose. This could, however, be due to a low concentration of glucose in the culture when sucrose was used as a carbon source. We have observed that *A. xylinum* NCI 1005 produces levan, which is structurally different from AM-1 when grown on a sucrose medium. Therefore, it is clear that *A. xylinum* NCI 1005 is a very interesting bacterium which can be produce two kinds of water-soluble polymers, suggesting that the synthesis of WSP can be controlled by changing the carbon source of the medium.

3. Experimental

Preparation of the water-soluble polysaccharide. —*A. xylinum* NCI 1005 was available from Nakano Vinegar Co., Ltd. A total of 50 mL of a 72-h seed culture of *A. xylinum* NCI 1005 was inoculated into 1500 mL of SYP medium (6% sucrose, 1% yeast extract, and 0.5% polypeptone in distilled water) and incubated in a shaking culture at 120 strokes/min or in a static culture at 28 °C for 7 days. Cells were removed by centrifugation at 8000 rpm for 15 min. Ethanol (four times the volume as that of the supernatant) was added to the supernatant, and the mixture was maintained at 4 °C overnight. A precipitate formed and was collected by centrifugation at 8000 rpm for 15 min, and the precipitated WSP was dissolved in 15 mL of deionized water. Activated charcoal was added into the solution to remove residual organic compounds, and the solution was stirred for 10 min. The activated charcoal was removed by centrifugation and ultrafiltration, and the filtrate was

dialysed against deionized water at 4 °C for 2 days. The dialysed solution was lyophilized.

Acidic hydrolysis of the water-soluble polysaccharide.—The purified polysaccharide was hydrolysed with 0.5 N H_2SO_4 solution at 70 °C for 3 h. The hydrolysate was filtered through a 0.45-μm membrane filter, and the filtrate was analysed and fractionated by high-performance liquid chromatography (HPLC) with a SP 8010 column (Showa Denko K.K.) and G4000PW column (Tohso Co., Ltd), respectively.

Structural analysis of the water - soluble polysaccharide by NMR spectroscopy.—NMR spectra of the hydrolysate and WSP were obtained at 100 MHz for ^{13}C and 400 MHz for proton with a Bruker MSL400 spectrometer. ^{13}C NMR spectra were accumulated with a 90° pulse, 5.3 μs, 25,000 Hz spectral width, and a 2-s repetition rate. ^{1}H decoupled NMR spectra were accumulated with a 90° pulse, 4.0 μs, 3000 Hz spectral width, and a 4-s repetition rate.

Homonuclear shift-correlated (COSY) spectra were obtained using spectra widths of 850 Hz and 425 Hz, with 1 K and 256 data points in the F2 and F1 dimensions, respectively, and zero-filled to 2 K and 512 data points, respectively. The recycle time was 2 s and the total accumulation time was approximately 1 h on a deuterium oxide sample solution in a 5-mm o.d. spinning sample tubes at 20 °C. Heteronuclear shift-correlated two-dimensional (2D) experiments were carried out using an F2 spectral width of 2500 Hz and an F1 width of 250 Hz; 64 time points were accumulated with 1 K and 128 data points in F2 and F1, respectively. The recycle time was 1.5 s. The accumulation time was over 2 h, and the 2D matrix was zero-filled (256 × 2 K) and converted with a Gaussian and Lorentz function in the F1 and F2 dimensions, respectively.

Determination of molecular weight.—The molecular weight of polysaccharide was determined by gel-permeation chromatography (GPC) with $G4000PW_{XL}$ + $G2500PW_{XL}$ (Tohso Co., Ltd).

Determination of the culture component.—The concentration of each component in the culture was determined by HPLC with a SP 8010 column (Showa Denko K.K.).

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References

[1] H. Hestrin and M. Schramm, *Biochem. J.*, 58 (1954) 345–352.
[2] H. Minakami, E. Entani, K. Tayama, S. Fujiyama, and H. Masai, *Agric. Biol. Chem.*, 48 (1984) 2405–2414.
[3] K. Tayama, H. Minakami, S. Fujiyama, H. Masai, and A. Misaki, *Agric. Biol. Chem.*, 49 (1985) 959–966.
[4] K. Tayama, H. Minakami, S. Fujiyama, H. Masai, and A. Misaki, *Agric. Biol. Chem.*, 50 (1986) 1271–1278.
[5] R.O. Couso, L. Ielpi, R.C. Garcia, and M.A. Dankert, *Eur. J. Biochem.*, 123 (1982) 617–627.
[6] S. Valla and J. Kjosbakken, *Can. J. Microbiol.*, 27 (1981) 599–603.
[7] C.C. Carpita, T.H. Housley, and J.E. Hendrix, *Carbohydr. Res.*, 217 (1991) 127–136.
[8] P.J. Simms, W.J. Boyoko, and J.R. Edwards, *Carbohydr. Res.*, 208 (1990) 193–198.
[9] Y.W. Han and M.A. Clarke, *J. Agric. Food Chem.*, 38 (1990) 393–396.
[10] K.D. Barrow, J.G. Collins, P.L. Rogers, and G.M. Smith, *Eur. J. Biochem.*, 145 (1984) 173–179.
[11] L. Hernandez, J. Arrieta, C. Menendez, R. Vazquez, A. Coego, V. Suarez, G. Selman, M. Petit-Glatron, and R. Chambert, *Biochem. J.*, 309 (1995) 113–118.
[12] J. Tomasic, H.J. Jennings, and C.P.J. Glaudemans, *Carbohydr. Res.*, 62 (1978) 127–133.
[13] S.J. Angyal and G.S. Bethel, *Aust. J. Chem.*, 29 (1976) 1249–1265.