ELSEVIER

Carbohydrate Polymers 51 (2003) 223–228

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Acetyl substitution of glucuronan influences glucuronan cleavage by GlyA from *Sinorhizobium meliloti* M5N1CS (NCIMB 40472)

A. Da Costa[a], P. Michaud[a], A. Heyraud[b], P. Colin-Morel[b], B. Courtois[a], J. Courtois[a,*]

[a]*Laboratoire des Polysaccharides Microbiens et Végétaux, IUT/Génie Biologique, Université de Picardie Jules Verne, Avenue des Facultés, Le Bailly, 80025 Amiens Cedex 1, France*

[b]*CERMAV-CNRS Université Joseph Fourrier, 38041 Grenoble Cedex, France*

Received 24 October 2001; revised 10 May 2002; accepted 17 May 2002

Abstract

The *Sinorhizobium meliloti* M5N1CS strain produces during fermentation a polyglucuronan and oligoglucuronans β-(1 → 4) linked and partially acetylated. Following the detection of unsaturated oligoglucuronans in the culture media, a glucuronan lyase (GlyA) associated to the *S. meliloti* M5N1CS has been identified. In this work, we have studied the degradation by Glya of glucuronans with different degree of substitution by acetate and characterized the oligoglucuronans obtained. We have determined that acetyl residues on glucuronan influence the susceptibility of glucuronan to cleavage by the specific lyase and that 2,3-di-*O*-Ac-Glc*p*A residues, closed to the cleavage site, limited the depolymerization of a purified glucuronan, while the degradation of a nascent polymer was not affected.

Keywords: *Sinorhizobium meliloti*; Glucuronan lyase; Substituent; Acetate

1. Introduction

Polysaccharides can be degraded either by polysaccharide hydrolases (Sutherland, 1999) or polysaccharide lyases (Sutherland, 1995). These specific lyases known to cleave anionic polymers (i.e. alginate), yield products in which the non-reducing end is modified to form an unsaturated uronate (Sutherland, 1995). Specific polysaccharide lyases were recently described as active on neutral polysaccharides (i.e. α-(1,4)-glucan) (Yu, Christensen, Kragh, Bojsen, & Marcussen, 1997; Yu, Kenne, & Pedersen, 1993). The lyase cleaving mechanism consists of a β-elimination leading to the formation of a double bond in the newly generated non-reducing end, either between C-4 and C-5 in uronic polymers or C-1 and C-2 in neutral α-(1,4)-glucans. Polysaccharide lyases tested on neutral or anionic polymers led to the production of oligosaccharides with a degree of polymerization (dp) ranging from 2 to 5 (Brown & Preston, 1991; Hotchkiss, Revear, & Hicks, 1996; Sutherland, 1995; Yu, Bojsen, Svensson, & Marcussen, 1999).

Among the polysaccharides degraded by specific lyases, some of them are acylated; the most common substituents correspond to ketal-linked pyruvate or ester-linked acetyl groups. Polysaccharide lyase activity was described to be reduced on highly acetylated alginate (Schiller, Monday, Boyd, Keen, & Ohman, 1993). The acetylation of mannurosyl residues, on position C-2 and C-3 in alginate, inhibited the alginate lyase activities from different *Azotobacter* species (Skjak-Braek, Grasdalen, & Larsen, 1986). Similar results were found by Kennedy, McDowell, and Sutherland (1992) with alginate lyase tested on *O*-acetylated mannuronate blocks. Deacetylation of a preparation of *Pseudomonas aeruginosa* alginate increased 15-fold its susceptibility to alginate lyase action (Sutherland, 1995). Similar results were obtained with gellan lyase tested on natural acetylated gellans (Giavasis, Harvey, & McNeil, 2000; Hashimoto, Miki, Tsuchiya, Nankai, & Murata, 1998).

Recently, it has been proposed that highly 2,3-di-*O*-acetylated glucuronan (one acetyl for 0.7 residue) was not degraded by a glucuronan lyase from *Sinorhizobium meliloti* M5N1CS strain (NCIMB 40472) (Da Costa et al., 2001). A mainly 3-*O*-acetylated glucuronan (one acetyl for 1.3 residue) was degraded by the new lyase; in fact the best substrate was a deacetylated glucuronan.

* Corresponding author. Tel.: +33-3-22-53-40-99; fax: +33-3-22-95-62-54.

E-mail address: josiane.courtois@iut.u-picardie.fr (J. Courtois).

0144-8617/03/$ - see front matter
PII: S0144-8617(02)00170-4

The identification of oligoglucuronans containing 2,3-di-*O*-acetylated residues with dp ≥ 2, in fermentation media of the *S. meliloti* M5N1CS strain (Pirlet et al., 1998, 1999) may be correlated to a putative polysaccharide degradation by the glucuronan lyase. Comparaisons of oligoglucuronans obtained in vivo during EPS production by the *S. meliloti* M5N1CS strain, to those obtained in vitro by degradation of the purified glucuronan with the extracted glucuronan lyase, will confirm the origin of oligoglucuronans obtained during fermentation. These activities will inform us, if as for the succinoglycan degraded by ExoK and ExsH (York & Walker, 1997), glucuronan lyase activity is only influenced by acetyl residues (York & Walker, 1998b), and/or by polysaccharide physical structure varying from random coil to helical structure or aggregates (Burova et al., 1996), from nascent polymer to accumulated and purified polymer (York & Walker, 1998a).

2. Materials and methods

2.1. Bacterial strain and culture conditions

For glucuronan lyase production, the *S. meliloti* M5N1CS strain (NCIMB 40472) (Courtois, Courtois, Heyraud, Colin-Morel, & Rinaudo, 1993) was aerobically cultured in a 2-l erlenmeyer flask containing 1 l of tryptone yeast (TY) medium (pH 7.2) (Beringer, 1974) supplemented with sucrose (1% w/v) (TYS), during 75 h at 30 °C on a rotary shaker (100 rev/min). The inoculum was 100 ml of a *S. meliloti* M5N1CS suspension in the same TYS medium, grown 20 h at 30 °C under agitation (100 rev/min). A 10 ml bacterial suspension grown 20 h at 30 °C was used to inoculate the 100 ml TYS medium.

2.2. Glucuronan production

The *S. meliloti* M5N1CS strain was cultivated at 30 °C in a 6-l reactor (LSL Biolaffite, Saint-Germain-en-Laye, France) containing 4.5 l of *Rhizobium* complete (RC) medium (Courtois, Hornez, Courtois, & Derieux, 1983) supplemented with sucrose (1% w/v) (RCS). The inoculum was 0.5 l of RCS in a 1-l erlenmeyer flask inoculated with *S. meliloti* M5N1CS. After 20 h of incubation on a rotative shaker (100 rev/min) at 30 °C, the inoculum was transferred in the reactor and incubated as previously described (Michaud et al., 1994), with or without addition of 0.8 g/l/24 h $MgSO_4,7H_2O$ for the production of mainly 2,3-di-*O*-acetylated or 3-*O*-acetylated (highly acetylated) glucuronans. The two high-molecular-weight (HMW) glucuronans were produced during two 75-h fermentations.

2.3. Glucuronan isolation, purification and characterization

After 75-h fermentations the broths were centrifuged (33,900 × *g*, 40 min, 20 °C) and the HMW polysaccharides were concentrated from the cell-free broths by tangential ultrafiltration on a 1×10^5 normal-molecular-weight cut-off (NMWCO) polysulfone membrane (0.1 m^2) (Sartorius, Goettingen, Germany). The concentrates were diluted with one volume of distilled water and ultrafiltered as earlier; this step was repeated five times, the last concentrated products were then dried under vacuum. The substitution degree controlled by ^{1}H NMR spectroscopy was one acetate for 1.3 residue and one acetate for 0.7 residue for the mainly 3-*O*-acetylated and highly acetylated glucuronans, respectively (Courtois et al., 1994).

2.4. Glucuronan lyase purification

Four litres of a 75-h *S. meliloti* M5N1CS culture broth were centrifuged (13,880 × *g*, 30 min). No glycosidase activity was detected on the cell free broth (Da Costa et al., 2001). The pellet was suspended in 80 ml of a 10 mM Tris–HCl buffer (pH 8) and sonicated at 20 kHz (13-mm-diameter standard probe) for 30 min, with an ultrasonicator (Vibra-Cell model VCX 600 W; Danbury, Conn.). After centrifugation (50,000 × *g*, 1 h) of the cell lysate, the supernatant corresponding to the crude enzyme solution was purified by two steps of anion exchange chromatography on a Sartobind Q100 Membrane Adsorber (Sartorius, Goettingen, Germany), leading to pure glucuronan lyase (12.9 μM). The purity of the preparation was tested by SDS-PAGE 12.5% (w/v) polyacrylamide gels followed by staining with Coomassie Brilliant R-250 and a single band was detected. One unit (U) of the enzyme activity was defined as an increase of 1 unit per hour in the absorbance at 235 nm (Da Costa et al., 2001).

2.5. Fractionation of oligoglucuronates

Oligoglucuronates obtained by enzymic degradation were size-fractionated by preparative gel filtration on a glass chromatographic column (2.5 × 100 cm) packed with Bio-Gel P-6 (Bio-Rad), eluted with a 50 mM $NaNO_3$ solution, the flow rate was 50 ml/h. Detection was achieved with a R-403 Waters differential refractometer. Fractions (10 ml) were collected with a 201–202 model Gilson collector. Fractions belonging to a same peak were combined, concentrated, desalted by HPLC on a Toyopearl HW 40F/50F (Interchrom) column and freeze-dried.

2.6. NMR studies

^{1}H NMR analysis were performed at 85 °C with an AC-300 Bruker (Bruker Spectrospin, Wissembourg, France) Fourier transform spectrometer, with a 5 mm ^{1}H ^{13}C dual probe, according conditions described previously by Dantas et al. (1994). The polymerization degree was estimated by comparison of the integral of the H-1 or H-4 signals of the unsaturated unit, to the integral of the other H-1 signals.

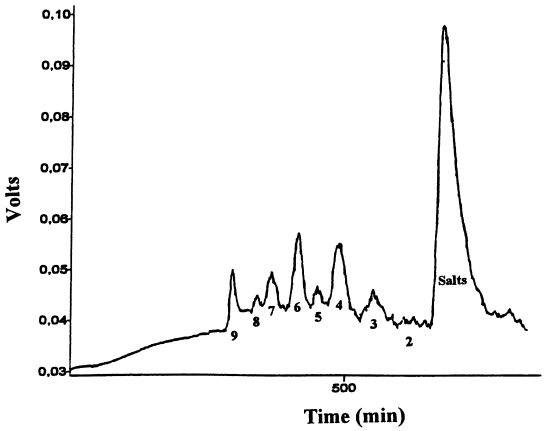

Fig. 1. Gel permeation analysis of a mainly 3-*O*-acetylated glucuronan incubated with *S. meliloti* M5N1CS glucuronan lyase. The degree of polymerization is indicated by number under the peaks. The chromatography was performed on a Bio-Gel P-6 column, 100 by 2.5 cm; the flow rate was 50 ml/h; the eluent was 50 mM $NaNO_3$; the detector was refraction index detector.

3. Results and discussion

3.1. Glucuronans degradation

A mainly 2,3-di-*O*-acetylated glucuronan (2% w/v) in 50 mM KH_2PO_4 buffer (pH 6.5) was incubated 15 h with 0.46 U of glucuronan lyase, at 50 °C. Then, the reaction mixture was analysed by size exclusion chromatography on a Bio-Gel P-6 column, and the elution profile was compared to those obtained with a sample corresponding to a mainly 2,3-di-*O*-acetylated glucuronan solution, incubated without enzyme. We noted the elution profiles of the glucuronan mainly 2,3-di-*O*-acetylated incubated with and without enzyme were similar (data not shown). Same results were obtained when the glucuronan lyase concentration was increased.

A mainly 3-*O*-acetylated glucuronan (2% w/v) in 50 mM KH_2PO_4 buffer (pH 6.5) was incubated 15 h with 0.46 U of enzyme at 50 °C before analysis by size exclusion chromatography as previously. No oligosaccharides were detected with the enzyme-free sample (data not shown), while the elution profile of the reaction mixture (Fig. 1) was significant of the polymer degradation by GlyA. The elution profile compared to those obtained after enzymic degradation with Celluclast (Novo Nordisk, Danemark) of a deacetylated glucuronan (Dantas et al., 1994) revealed the presence of oligoglucuronans with dp ≥ 2 in the reaction mixture. Pure oligosaccharides dp 2–9 were collected from 270 to 640 min elution. In the fraction eluted first, low molecular weight glucuronan with a dp average of 11 was detected.

3.2. ^{1}H NMR analysis of oligoglucuronans

The absence of oligomers, from a substrate corresponding to the highly acetylated glucuronan, was confirmed by ^{1}H NMR studies as signals characteristic of H-4 of unsaturated unit were not detected. This result confirmed, as for alginate (Kennedy et al., 1992; Skjak-Braek et al., 1986; Sutherland, 1995) or gellan (Giavasis et al., 2000;

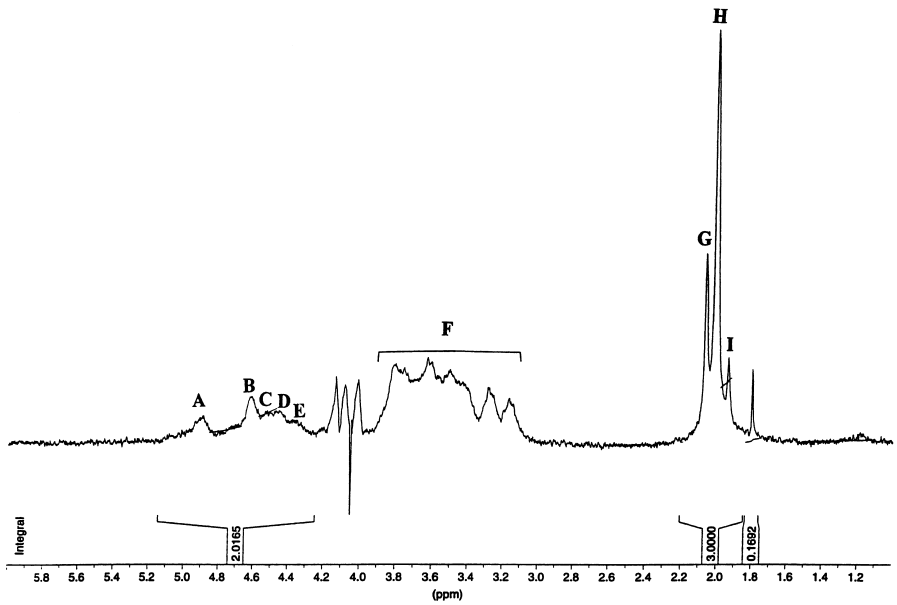

Fig. 2. ^{1}H NMR spectrum of HMW mainly 3-*O*-acetylated glucuronan in D_2O (at 300 MHz; *T*, 85 °C). (A) H-3 of 3-*O*-acetylated residues; (B) H-1 and H-2 of 2-*O*-acetylated residues; (C) H-1 of 3-*O*-acetylated residues; (D) H-1 of unacetylated residues; (E) H-1 of unacetylated residues before and after acetylated residues; (F) H-2, H-3, H-4 and H-5 of the repeating unit; (G) protons of the acetyl group at C-2 in the 2-*O*-acetylated residues; (H) protons of the acetyl group at C-3 in the 3-*O*-acetylated residues and protons of the acetyl group at C-2 in the 2,3-di-*O*-acetylated residues; (I) protons of the acetyl group at C-3 in the 2,3-di-*O*-acetylated residues.

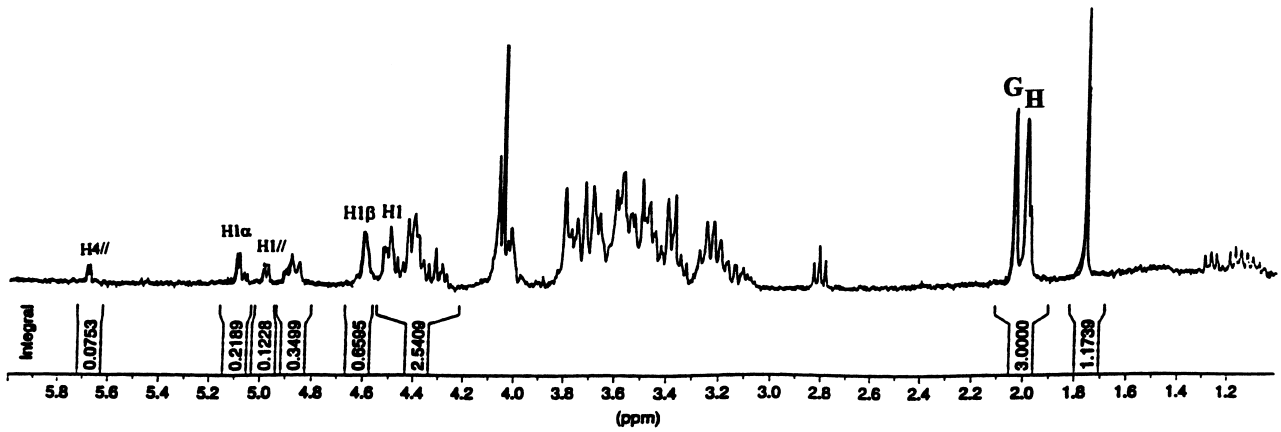

Fig. 3. ^{1}H NMR spectrum of acetylated oligoglucuronans (dp 4) in D_2O (at 300 MHz; *T*, 85 °C). (H4//) H-4 of the unsaturated non-reducing end; (H1//) H-1 of the unsaturated non-reducing end; (H1α) H-1α of the reducing end; (H1β) H-1β of the reducing end; (H1) H-1 of the repeating unit; (G) protons of the acetyl group at C-2 in the 2-*O*-acetylated residues; (H) protons of the acetyl group at C-3 in the 3-*O*-acetylated residues and protons of the acetyl group at C-2 in the 2,3-di-*O*-acetylated residues.

Hashimoto et al., 1998) that the presence of acetyl groups in the glucuronan reduces the specific lyase activity.

After degradation of the mainly 3-*O*-acetylated glucuronan by the glucuronan lyase, each oligoglucuronans was collected. ^{1}H NMR spectrum from each fraction was compared to those of the polymer substrate (mainly 3-*O*-acetylated glucuronan) (Fig. 2). We noted the presence of doublets at 5.70 ppm (Fig. 3), characteristic of H-4 of an unsaturated unit, and signals at 4.95 and 4.50 ppm assigned to H-1 of unsaturated residues and to H-1 of non-terminal residues. Signals in the 2 ppm region characteristics of protons from *O*-acetyl groups were detected on all spectra. However, oligoglucuronans obtained in this study by glucuronan degradation with the pure GlyA, compared to those detected in *S. meliloti* M5N1CS fermentation medium (Pirlet et al., 1999) revealed some differences mainly in the acetyl region.

The substitution degree average was determined by comparison of the total H-1 resonances from oligoglucuronans (from 4.3 to 5.1 ppm) to protons in the acetyl region (from 2.1 to 1.8 ppm) (Courtois et al., 1994) and the molar ratio of the different species of glucuronic residues acetylated (2-*O*-Ac-Glc*p*A; 3-*O*-Ac-Glc*p*A; 2,3-di-*O*-Ac-Glc*p*A) was expressed as a percentage of the molar ratio (specific glucuronic residue/glucuronic residue entirety) (Table 1). We noted the substitution degree of oligoglucuronans was lower those of the HMW glucuronan; more, the substitution degree and the degree of polymerization of oligoglucuronans decreased concomitantly.

No 2,3-di-*O*-acetylated residues were detected in oligoglucuronans dp < 5 obtained by degradation of a purified glucuronan with the pure glucuronan lyase. By comparison of ^{1}H NMR spectrum of unacetylated oligoglucuronans (Michaud et al., 1997) to those obtained here, we concluded the two terminal residues in oligoglucuronans obtained in this study were not acetylated. This result was significant of a cleavage site situated between two unacetylated residues. Oligoglucuronans (dp 2 to >9) obtained by degradation of the mainly 3-*O*-acetylated glucuronan with the specific lyase were collected (Table 2); we noted that 19% of degradation products were dp 4 oligoglucuronans. This result can be correlated to previous works where the dp 4

Table 1
Substitution degrees and molar ratios of residues species (2-*O*-Ac-Glc*p*A, 3-*O*-Ac-Glc*p*A, 2,3-di-*O*-Ac-Glc*p*A and unacetylated) of mainly 3-*O*-acetylated glucuronan Glya degradation products

	Substitution degree (%)	Unacetylated residues (%)	2-*O*-acetylated (%)	3-*O*-acetylated (%)	2,3-di-*O*-acetylated (%)
HMW native glucuronan	58.97	41.03	18.45	35.15	5.37
Glucuronan dp 11	38.61	61.39	8.25	25.65	2.35
Oligoglucuronan dp 5	34.60	65.40	13.84	20.76	0
Oligoglucuronan dp 4	26.59	73.41	7.93	18.66	0

Table 2
Massic ratios of oligoglucuronans purified after gel filtration chromatography on a Bio-Gel P-6 column, from 50 mg of mainly 3-*O*-acetylated glucuronan

Dp	2	3	4	5	6	7	8	9	<9
Massic ratio (%)	4.42	11.40	19.40	0.70	16.00	5.80	0.68	8.50	32.00

was identified as the ultimate enzyme substrate (Michaud et al., 1997).

We noted, the ratio of H-1 signals from unacetylated residues belonging to unacetylated sequence, to all H-1 signals decreased from 15.7 to 11.25 and 9.54 from dp 11 to 5 and 4, respectively. This result confirms the polymer cleavage occurs in unacetylated sequences; however, the lack of 2,3-di-*O*-Ac-Glc*p*A residue in oligoglucuronans dp < 5 obtained in the tested conditions indicates diacetylated residues localized near an unacetylated sequence may inhibit the cleavage.

These results differ from those obtained with oligoglucuronans purified from a *S. meliloti* M5N1CS fermentation medium (Pirlet et al., 1999) where it was described that oligoglucuronans mainly from dp 3 to 6 contained about 15.5% (molar ratio) of 2,3-di-*O*-Ac-Glc*p*A residues. This accumulation of di-*O*-acetylated oligoglucuronans with small dp only in fermentation medium during glucuronan production, may correspond to changes in the physical structure of the glucuronan molecule. Such differences were yet observed for succinoglycan degradation by ExoK and ExsH glycanases depolymerizing specifically nascent succinoglycan (York and Walker, 1998a). Probably random coil glucuronan structures are more susceptible to lyase and are inhibited by di-*O*-acetylated Glc*p*A residues neighbour to the cleavage site.

Acknowledgements

This work was supported by "Pôle Génie des Procédés" (Conseil Régional de Picardie).

References

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology*, *84*, 188–198.

Brown, B. J., & Preston, J. F. (1991). L-guluronan-specific alginate lyase from a marine bacterium associated with *Sargassum*. *Carbohydrate Research*, *211*, 91–102.

Burova, T. V., Golubeva, I. A., Grinberg, A. Y., Mashkevich, A. Y., Grinberg, V. Y., Usov, A. I., Navarini, L., & Cesáro, A. (1996). Calorimetric study of the order-disorder conformational transition in succinoglycan. *Biopolymers*, *39*, 517–529.

Courtois, B., Hornez, J. P., Courtois, J., & Derieux, J. C. (1983). Mise en évidence d'une propriété métabolique de *Rhizobium meliloti* utilisable pour sa classification. *Annales de Microbiologie*, *134*, 141–147.

Courtois, J., Courtois, B., Heyraud, A., Colin-Morel, P., & Rinaudo, M (1993). Composés polymères de l'acide glucuronique, procédé de préparation et d'utilisation notamment en tant que moyens gélifiants, épaississants, hydratants, stabilisants, chélatants ou floculants. French Patent, WO-A. 93/18174.

Courtois, J., Seguin, J. P., Roblot, C., Heyraud, A., Gey, C., Dantas, L., Barbotin, J. N., & Courtois, B. (1994). Exopolysaccharide production by the *Rhizobium meliloti* M5N1CS strain. Location and quantification of the sites of *O*-acetylation. *Carbohydrate Polymers*, *25*, 7–12.

Da Costa, A., Michaud, P., Petit, E., Heyraud, A., Colin-Morel, P., Courtois, B., & Courtois, J. (2001). Purifiation and properties of a glucuronan lyase from *Sinorhizobium meliloti* M5N1CS (NCIMB 40472). *Applied and Environmental Microbiology*, *67*, 5197–5203.

Dantas, L., Courtois, J., Courtois, B., Seguin, J. P., Gey, C., & Heyraud, A. (1994). NMR spectroscopic investigation of oligoglucuronates prepared by enzymic hydrolysis of a (1 → 4)-β-D-glucuronan. *Carbohydrate Research*, *265*, 303–310.

Giavasis, I., Harvey, L. M., & McNeil, B. (2000). Gellan gum. *Critical Review on Biotechnology*, *20*, 177–211.

Hashimoto, W., Miki, H., Tsuchiya, N., Nankai, H., & Murata, K. (1998). Xanthan lyase of *Bacillus* sp. strain GL1 liberates pyruvylated mannose from xanthan side chains. *Applied and Environmental Microbiology*, *64*, 3765–3768.

Hotchkiss, A. T., Jr., Revear, L. G., & Hicks, K. B. (1996). Substrate depolymerization pattern of *Pseudomonas viridiflava* SF-312 pectate lyase. *Physiological and Molecular Plant Pathology*, *48*, 1–9.

Kennedy, L., McDowell, K. R., & Sutherland, I. W. (1992). Alginases from *Azotobacter* species. *Journal of General Microbiology*, *138*, 2465–2471.

Michaud, P., Courtois, J., Courtois, B., Heyraud, A., Colin-Morel, P., Seguin, J. P., & Barbotin, J. N. (1994). Physicochemical properties of extracellular (1 → 4)-β-D-glucuronan produced by the *Rhizobium meliloti* M5N1CS strain during fermentation: evidence of degradation by an exoenzyme activated by Mg^{2+}. *International Journal of Biological Macromolecules*, *16*, 301–305.

Michaud, P., Pheulpin, P., Petit, E., Barbotin, J. N., Heyraud, A., Courtois, B., & Courtois, J. (1997). Identification of a glucuronan lyase from a mutant strain of *Rhizobium meliloti*. *International Journal of Biological Macromolecules*, *21*, 3–9.

Pirlet, A.-S., Guentas, L., Heyraud, A., Pheulpin, P., Vijalayakshmi, M. A., Barbotin, J. N., Courtois, B., & Courtois, J. (1999). Influence of acetyl substituent on oligoglucuronans separation by anion exchange chromatography. *Carbohydrate Polymers*, 155–160.

Pirlet, A. S., Pitiot, O., Guentas, L., Heyraud, A., Courtois, B., Courtois, J., & Vijalayakshmi, M. A. (1998). Separation of low-molecular-mass acetylated glucuronans on L-histidine immobilized onto poly(ethylene-vinyl alcohol) hollow-fiber membranes. *Journal of Chromatography A*, *826*, 157–166.

Schiller, N. L., Monday, S. R., Boyd, C., Keen, N. T., & Ohman, D. E. (1993). Characterization of the *Pseudomonas alginate* lyase gene (algL): Cloning, sequencing and expression in *Escherichia coli*. *Journal of Bacteriology*, *175*, 4780–4789.

Skjak-Braek, G., Grasdalen, H., & Larsen, B. (1986). Monomer sequence and acetylation pattern in some bacterial alginates. *Carbohydrate Research*, *154*, 239–250.

Sutherland, I. W. (1995). Polysaccharide lyases. *FEMS Microbiology Review*, *16*, 323–347.

Sutherland, I. W. (1999). Polysaccharases for microbial exopolysaccharides. *Carbohydrate Polymers*, *38*, 319–328.

York, G. M., & Walker, G. C. (1997). The *Rhizobium meliloti exoK* gene and prsD/prsE/exsH genes are components of independent degradative pathways which contribute to production of lowmolecular-weight succinoglycan. *Molecular Microbiology*, *25*, 117–134.

York, G. M., & Walker, G. C. (1998a). The *Rhizobium meliloti* Exok and ExsH glycanases specifically depolymerize nascent succinoglycan chains. *Proceedings of the National Academy of Sciences USA*, *95*, 4912–4917.

York, G. M., & Walker, G. C. (1998b). The succinyl and acetyl modifications of succinoglycan influence susceptibility of succinoglycan to cleavage by the *Rhizobium meliloti* glycanases ExoK and ExsH. *Journal of Bacteriology*, *180*, 4184–4191.

Yu, S., Kenne, L., & Pedersen, M. (1993). α-1,4-Glucan lyase, a new class of starch/glycogen debranching enzyme. I. Efficient purification and characterization from red seaweeds. *Biochimica et Biophysica Acta*, *1156*, 313–320.

Yu, S., Bojsen, K., Svensson, B., & Marcussen, J. (1999). α-1,4-glucan lyases producing 1,5-anhydro-D-fructose from starch and glycogen

have sequence similarity to α-glucosidases. *Biochimica et Biophysica Acta*, *1433*, 1–15.

Yu, S., Christensen, T. M. I. E., Kragh, K. M., Bojsen, K., & Marcussen, J. (1997). Efficient purification, characterization and partial amino acid sequencing of two α-1,4-glucan lyases from fungi. *Biochimica et Biophysica Acta*, *1339*, 311–320.