Carbohydrate Research, 185 (1989) 119–129
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SELECTIVE ACETYLATION OF MANNURONIC ACID RESIDUES IN CALCIUM ALGINATE GELS

GUDMUND SKJÅK-BRÆK*,

Laboratory for Marine Biochemistry, Division of Biotechnology, The Norwegian Institute of Technology, 7034 Trondheim (Norway)

SERGIO PAOLETTI

Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Trieste (Italy)

AND TERESA GIANFERRARA†,

LBT-Technological Biopolymer Laboratory, POLY-biòs Research Center, Area di Ricerca, Trieste, (Italy)

(Received June 4th, 1988; accepted for publication, July 7th, 1988)

ABSTRACT

Treatment of non-aqueous spherical beads of calcium alginate, or alginic acid gel, with acetic anhydride in pyridine results in acetylation without degradation. The degree and pattern of substitution were investigated using n.m.r. spectroscopy and degradation with specific enzymes. At low degrees of acetylation, substitution occurred almost exclusively on the mannosyluronic acid residues. At high degrees of acetylation, only a minor proportion of the isolated guluronic acid residues was substituted. The presence of *O*-acetyl groups inhibited depolymerisation by alginate lyases.

INTRODUCTION

The exocellular glycuronans produced by *Azotobacter vinelandii*[1,2] and some strains of *Pseudomonas*[3] resemble the alginates found in brown seaweeds in being linear chains of (1→4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid residues (G). The most conspicuous structural differences are the presence of *O*-acetyl groups in bacterial alginates[4] and the absence of contiguous G-residues in *Pseudomonas* alginates[5,6]. The degree of acetylation (d.a.) ranges from 0.1 to >1.0 acetyl group per monomer unit, only the mannuronic acid residues are acetylated[5,7], and the ratio of distribution between O-2 and O-3 is 3:2. Di-*O*-acetylated residues have been detected[5], the proportion being somewhat lower than expected

*Author for correspondence.
†Present address: Institute of Pharmaceutical and Toxicological Chemistry, University of Trieste, Trieste, Italy.

0008-6215/89/$ 03.50

on the basis of a random distribution[5]. The acetylated M units in *A. vinelandii* glycuronan cannot be epimerised[4] at C-5. Since acetylation occurs intracellularly and epimerisation occurs exocellularly, the organism can control the properties of its exopolymer. The *O*-acetyl groups are reported to protect alginate in a strain of *P. aeruginosa* from enzyme depolymerisation by degrading enzymes[8].

Many theories have been advanced concerning the biological significance of bacterial alginates. In the presence of calcium ions, the exopolymer from *A. vinelandii* will form a gel, thereby providing the cells with hydrophilic capsules which may protect them from other micro-organisms and desiccation[9], or with a barrier against heavy metal ions, or a diffusion barrier towards oxygen[10]. The available range of bacterial alginates is too limited to allow a structure–activity study. In order to extend this range, we have sought to acetylate the M-residues of brown-algal alginates selectively, and the results are now reported.

EXPERIMENTAL

Alginates. — A sodium alginate from *Laminaria hyperborea* stipes (LF/10/60), obtained from Protan A/S (Norway), contained 68% of G units and had an average number of units in the G-blocks[11] ($\bar{N}_{G>1}$) = 14.0. A fragment containing >87% of G units and with d.p. ~30 has been prepared[12] from *L. digitata* alginate, and an M-rich alginate (M >90%) was prepared from *Ascophyllum nodosum* fruiting bodies[12]. An acetylated M-block was prepared from *Azotobacter vinelandii* alginates by degradation with a specific guluronic acid lyase followed by gel-permeation chromatography[4]. A mannuronan has been isolated from *P. aeruginosa*[5]. An alginate, derived from *Macrocystis pyrifera* and obtained from Sigma, contained 39% of G units and had ($\bar{N}_{G>1}$) = 6.5. An alginate rich in alternating sequences of M and G [41% of G units and a nearest neighbour frequency of heteroglycosidic linked residues (F_{MG}) = 0.21] was isolated from old tissues of *Ascophyllum nodosum*[12].

Enzymes. — A specific α-L-guluronate lyase, isolated from liquid cultures of *Klebsiella aerogenes* and purified as described previously[4,13], degrades alginate by a β-elimination reaction at G-residues, exposing a G-residue as the reducing terminus and an unsaturated uronic acid residue on the new non-reducing terminus. The activity of the enzyme was assayed by incubating alginate (10 mg/mL) with alginate lyase (0.01 mg/mg of alginate) in Tris/HCl buffer (25mM, pH 7.0) and monitoring the increase in absorption. The same assay was used for a specific M-ase[14] isolated from Abalone.

Acetylation. — (*a*) *Of beads of Ca-alginate gel.* The beads were prepared by allowing droplets of aqueous 2% sodium alginate to fall into 0.1M calcium chloride. The viscous solution was pressed through a syringe (1.2 mm) and the size of the droplets was controlled by applying a coaxial air-stream[15]. The same procedure was followed for production of Sr^{2+} and Ba^{2+} beads. The beads, which were kept in 0.1M calcium chloride for 24 h, were 3 mm in diameter and had a final polymer

content after correction for shrinkage of 3.4%[16]. The water in the beads was exchanged for pyridine by keeping them in this solvent for 5–24 h. The water content of the beads was determined by using a Mettler DL18 Karl Fischer titrator. The beads (50 mL) were suspended in pyridine–acetic anhydride (1:1, 200 mL) for 1–24 h at 38°, collected by filtration, and washed thoroughly with water, and a solution in 0.05M EDTA (pH 7.0) was dialysed against 0.1M NaCl and then against water. The pH of the dialysate was adjusted to 7.0 with 0.1M NaOH and the polymer was isolated by freeze-drying.

(*b*) *Of beads of alginic acid.* Ca-alginate beads were suspended in M acetic acid, and then in glacial acetic acid in order to remove water. The residual calcium in the gel was determined by dialysing the beads against 0.1M HCl and analysing the dialysate by atomic absorption spectroscopy with a Perkin–Elmer AA spectrophotometer. The resulting beads of alginic acid were then acetylated as described in (*a*).

(*c*) *Of alginic acid.* Alginates LF-10 60, "M-rich" alginates from *A. nodosum*, and G-blocks isolated from *L. digitata* were converted into their free acid form by adding M HCl dropwise, to pH 3.0, to an aqueous 1% solution of each sodium salt. The precipitates were isolated, washed, partly dried, and acetylated in suspension as described previously[5].

N.m.r. spectroscopy. — Samples were prepared as described by Grasdalen *et al.*[11]. The ^{1}H-n.m.r. spectra were recorded at 92° with Bruker WM 400 and WM 500 instruments, and the ^{13}C-n.m.r.-spectra (25 and 125 MHz) with Jeol FX-100 and Bruker WM 500 instruments. The chemical shifts are expressed in p.p.m. downfield from the signal for 3-(trimethylsilyl)propanesulfonate. The diad and triad frequencies, the average block-lengths, and the contents of acetyl groups were determined[17].

Enzyme degradation. — To a solution of alginate (80 mg) in 25mM Tris/HCl buffer (40 mL, pH 7.5) containing NaCl (0.4 g) was added purified α-L-guluronate lyase (5 mg). After incubation for 24 h at 30°, another portion (3 mg) of enzyme was added. When no further increase in A_{230} could be detected, the reaction was stopped by heating and the oligo-uronides were fractionated[4] on a double column of P-4 Biogel.

RESULTS

Acetylation of alginates. — In the acetylation of alginic acid as a suspension in acetic anhydride[18,19], the presence of water was essential for the reaction and, although a high degree of acetylation was achieved, some degradation occurred. In the present work, sodium alginate was transformed into calcium alginate gel beads, which are stable and do not shrink significantly in most organic solvents[20]; most of the water can be exchanged for pyridine without loss of accessibility, so that acetylation proceeds smoothly. Moreover, the alginate gel beads were much easier to handle than the bulk-precipitated alginic acid and their water content could be

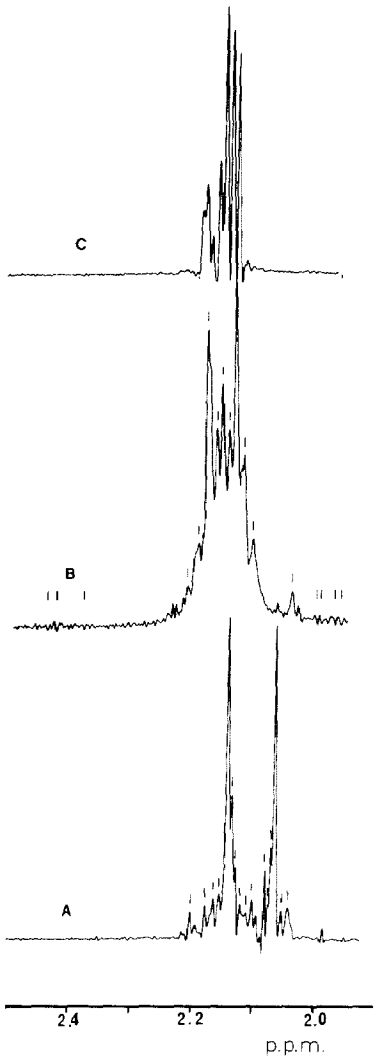

Fig. 1. Resolution-enhanced 500-MHz F.t.-^{1}H-n.m.r. spectra (AcO region) for acetylated alginates: A, chemically acetylated G-blocks isolated from *L. digitata* (F_G 0.87, d.a. = 0.60); B, chemically acetylated alginate enriched in mannuronic acid (F_M >0.90, d.a. = 0.38); C, D-mannuronan isolated from *P. aeruginosa* (d.a. = 0.63).

controlled precisely. Exchange of water for pyridine caused substantial shrinkage only when 10% of the original water was left, and the highest degree of acetylation (d.a.) at 35° was obtained at a water content of ~20%. Acetylation was rapid within the first 40 min, and proceeded slowly for the next 20 h. There were only small differences in the rate of reaction for different types of alginates or for beads on replacement of Ca^{2+} by Ba^{2+} or Sr^{2+}. Acetylation of alginic acid beads, however,

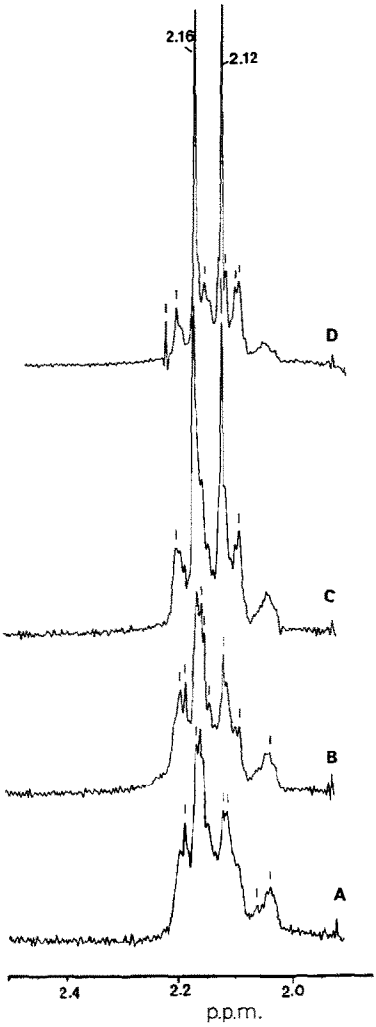

Fig. 2. 500-MHz F.t.-^{1}H-n.m.r. spectra (AcO region) for chemically acetylated alginates from *L. hyperborea* (F_G 0.68): d.a. values, A, 0.40; B, 0.56; C, 0.92; D, 1.53.

proceeded more rapidly than for calcium alginate beads with the same content of water, giving a final d.a. of >1.7.

The beads swelled significantly as the d.a. became high. Samples containing >65% of acetyl groups were soluble in aqueous 50% ethanol, and the acetylated polymers were collected by freeze-drying instead of precipitation.

Characterisation of products by ^{1}H-n.m.r. spectroscopy. — The substitution pattern was difficult to determine completely due to overlap of the resonances both in the region for anomeric protons and in the high-field region. However, by com-

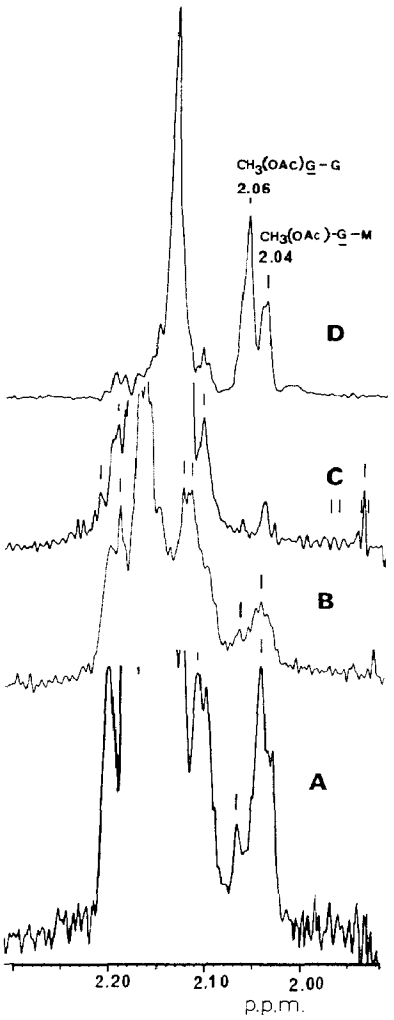

Fig. 3. 500-MHz F.t.-^{1}H-n.m.r. spectra (AcO region) for chemically acetylated alginates: A, from *A. nodosum* (F_G 0.40, d.a. = 0.40); B, from *L. hyperborea* (F_G 0.68, d.a. = 0.40); C, enriched in mannuronic acid (F_M >0.90, d.a. = 0.38); D, G-blocks isolated from *L. digitata* (F_G 0.87, d.a. = 0.60).

parison of the OAc resonances with those for acetylated M-blocks from bacterial alginates and chemically acetylated G-blocks, non-overlapped peaks at 2.06 and 2.04 p.p.m. could be assigned to (OAc)-G. The spectrum (Fig. 1A) of acetylated G-blocks displays one peak at 2.14 p.p.m. and two slightly smaller ones at 2.06–2.04 p.p.m. The largest peak overlaps with the (OAc)-M signals at 2.18–3.13 p.p.m. (Fig. 1B,C) and it probably also includes the signals from $(OAc)_2$-G. The bacterial alginate did not give the signals at 2.06–2.04 p.p.m., indicating that only (OAc)-M was present in native polymers. That the resonance at 2.06–2.04 p.p.m. was due to

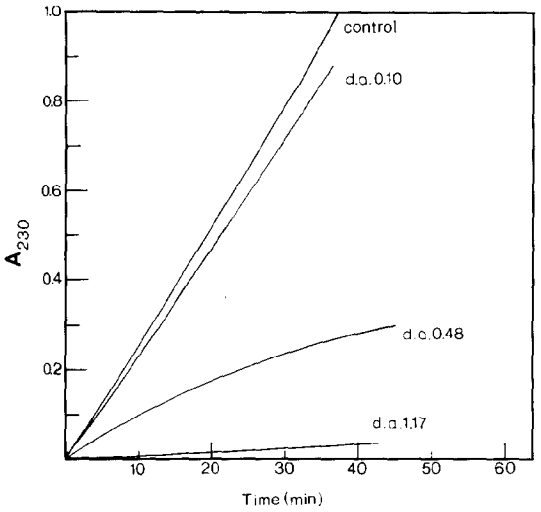

Fig. 4. Action of poly-α-L-guluronate lyase from *K. aerogenes* on acetylated alginate from *L. hyperborea*.

(OAc)-G was indicated by the decrease in intensity with increasing d.a. (Fig. 2 A–D). Likewise, the increase in the intensities of the resonances at 2.16 and 2.12 p.p.m. with increasing d.a. (Fig. 2) suggested that they were associated with diacetylated derivatives. The complex spectra for the OAc groups (Fig. 1), even from the acetylated mannuronan (Fig. 1B), were probably due to near-neighbour effects as demonstrated in Fig. 3, where the relative intensities of the resonances at 2.06 and 2.04 p.p.m. vary with the sequential structure on the alginate backbone.

In the spectrum of the acetylated G-blocks, the signal at 2.06 p.p.m. predominated, whereas the signal at 2.04 p.p.m. was predominant in that of acetylated alginate with 90% of M, suggesting that they originated from (AcO)-*GG* and (AcO)-*GM*, respectively. This inference was supported by the n.m.r. spectrum (Fig. 3) of an acetylated alginate from *A. nodosum*, which has a high proportion of alternating sequences. The intensity of the resonance at 2.04 p.p.m. was 17% of the total, whereas, in that of the *L. hyperborea* sample with the same d.a., the corresponding signal was <10%. From the spectra of acetylated samples of *L.*

TABLE I

INHIBITORY EFFECT OF ACETYL GROUPS ON ALGINATE LYASES[a]

Source of lyase	*D.a.*					
	0.05	*0.10*	*0.40*	*0.65*	*1.17*	*1.53*
K. aerogenes	94	92	53	44	2	
Abalone	82	57	44	23		3

[a]Activity given in % of the initial reaction rate with non-acetylated alginate as the substrate.

TABLE II

COMPOSITION OF OLIGOSACCHARIDES FROM A POLYGULURONATE-LYASE DIGEST OF ACETYLATED ALGINATE FROM *L. hyperborea* (d.a. = 0.10)

Fraction	*Yield (mg)*	*D.a.*	F_M	F_G
I–II	11.0	36	0.85	0.16
III	2.6	n.d.	n.d.	n.d.
IV	2.5	n.d.	n.d.	n.d.
V	9.0	4	0.11	0.89
VI	30.5	0	0.06	0.94
VII	15.5	0	0	1.00

hyperborea alginates in Figs. 2 and 3B, it is evident that the acetyl signal from non-contiguous G-units predominated over that of G-units occurring in the G-blocks. However, since the distribution of mono- and di-acetylated derivatives in the various samples is not known, determination of the d.a. on M and G could not be obtained from the ^{1}H-n.m.r. spectra.

Degradation with α-L-guluronate lyase. — The acetylated alginates were degraded with a specific poly-guluronate lyase, and the products were fractionated by gel-permeation chromatography and analysed by ^{1}H-n.m.r. spectroscopy. The activity of the enzyme on substrates with various d.a. (Fig. 4) shows that the presence of OAc groups inhibits depolymerisation. When the acetylated alginates were degraded with a lyase from Abalone, specific for the mannosyluronic acid residues[14], the expected stronger inhibition was observed (Table I).

When samples containing 10.5 and 47% of acetyl groups were treated with the lyase and the products were fractionated (Fig. 5), only the former sample has been degraded sufficiently to obtain oligomers for n.m.r. analysis. The results in Table II indicate that this polymer is selectively acetylated on the M units. The high-molecular-weight material (peak I), representing 16% of the total material and containing 84% of M, had an OAc content of 36%. In the lower oligomers,

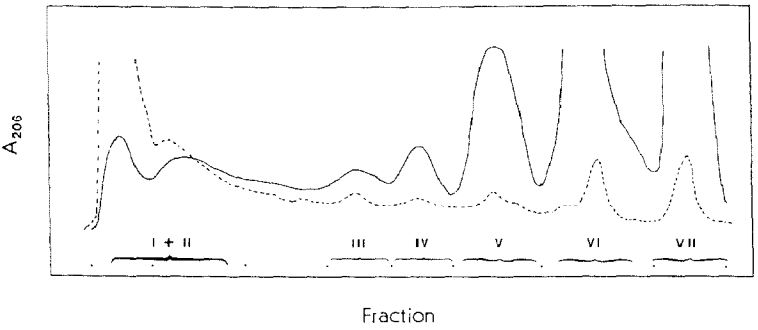

Fig. 5. Gel filtration of a poly-α-L-guluronate lyase digest of acetylated alginates from *L. hyperborea*: ——, d.a. = 0.10; ----, d.a. = 0.47.

TABLE III

ACETYLATION ON M AND G RESIDUES DETERMINED BY ^{1}H- AND ^{13}C-N.M.R. SPECTROSCOPY

Alginate	F_G	*D.a.*	*Acetylation (%)*	
			M	*G*
L. hyperborea	0.68	0.1	>90	<10
L. hyperborea	0.68	0.40	80	20
A. nodosum	0.40	0.40	63	36

there was a decrease in M and a low content of acetyl (d.a. ≤0.04). Thus, depolymerisation using the lyase could not be applied to polymers with a high d.a.

Characterisation of products by ^{13}C-n.m.r. spectroscopy[21]. — By comparing the intensities of the resonances for C-2 and C-3 in G with those for other carbons, the relative substitution on the G-residues could be determined. In samples with a

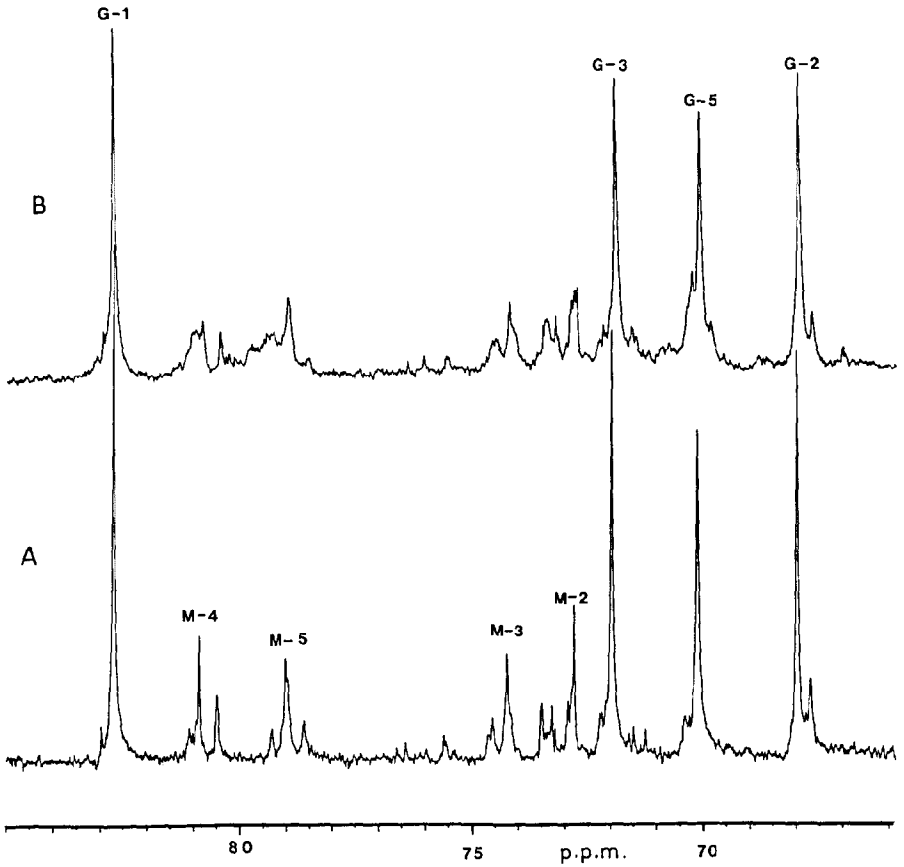

Fig. 6. 125-MHz ^{13}C-N.m.r. spectra (C-2/5 region) of A, chemically acetylated alginate from *L. hyperborea* (F_G 0.68, d.a. = 0.40), B, non-acetylated sample.

d.a. <40%, the substituents were almost equally distributed between positions 2 and 3 with the former preponderating slightly (Fig. 6). Given that (AcO)-G and $(AcO\text{-}G)_2$ were distributed randomly, the contribution to the ^{1}H-n.m.r. spectra from the OAc in the substituted G-residues could be calculated. The results given in Table III suggest that, even at higher d.a., substitution involves mainly the M-residues.

DISCUSSION

The d.a. of alginates depends strongly upon the water content[18], and is highest at a content of 20%. By taking advantage of the ordering of the G-residues in the calcium alginate gel, selective acetylation has been achieved. At low d.a., the M-residues and the heteroglycosidic-linked G residues were selectively substituted. The apparently lower reactivity of the contiguously linked G-residues must be attributed to their structural arrangement in the gel net-work. The G-blocks bind Ca^{2+} cooperatively in the junctions in the gel network[23]. At higher d.a., the G-residues are also substituted, causing[22] a gradual loss in affinity for Ca^{2+} and increasing the swelling of the beads. Lower reactivity of G-residues involved in the junctions is also indicated by the significantly higher proportions of (OAc)-G formed from an alginate with a high content of non-contiguous G-residues than with alginates with a high content of G-blocks.

The presence of OAc groups appears to protect the polymer from degradation by lyases. Alginate lyases are produced by several bacteria, including *A. vinelandii*, and some of the alginate-producing strains of *Pseudomonas*. The acetylation system in alginate-producing bacteria thus seems to function as a protection, both towards their own enzymes and towards degrading enzymes from other microorganisms. The effect of OAc groups on some physical properties of the alginates[22] is discussed in the following paper.

ACKNOWLEDGMENTS

We thank Ing. Wenche Strand and Siv. Ing. Arne Askildsen for technical assistance; Professors H. Grasdalen, T. J. Painter, and O. Smidsrød for helpful discussions; the Norwegian Research Council for Science and Technology (NTNF), the Italian National Research Council, and Protan A/S for financial support; and F.lli Janousek for a fellowship (to T.G.). The 500-MHz ^{1}H-n.m.r. spectra were recorded at the National N.M.R. Laboratory in Trondheim by Dr. J. Krane.

REFERENCES

1 A. LINKER AND L. R. JONES, *J. Biol. Chem.*, 291 (1966) 3845–3851.
2 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 44 (1966) 993–998.
3 B. LARSEN AND A. HAUG, *Carbohydr. Res.*, 17 (1971) 287–296.
4 G. SKJÅK-BRÆK, B. LARSEN, AND H. GRASDALEN, *Carbohydr. Res.*, 145 (1985) 169–174.
5 G. SKJÅK-BRÆK, H. GRASDALEN, AND B. LARSEN, *Carbohydr. Res.*, 154 (1986) 239–250.

6 V. SHERBROOK-COX, E. A. MILNE, AND P. GACESA, *Carbohydr. Res.*, 135 (1984) 147–154.
7 J. W. DAVIDSON, I. E. W. SUTHERLAND, AND C. I. LAWSON, *J. Gen. Microbiol.*, 98 (1977) 603–606.
8 A. LINKER AND L. R. EVANS, *J. Bacteriol.*, 159 (1984) 958–964.
9 H. L. SADOFF, *Bacteriol. Rev.*, (1976) 516–539.
10 I. W. SUTHERLAND, in I. W. SUTHERLAND (Ed.), *Surface Carbohydrates of the Prokariotic Cell*, Academic Press, London, 1977, pp. 22–96.
11 H. GRASDALEN, B. LARSEN, AND O. SMIDSRØD, *Carbohydr. Res.*, 68 (1979) 23–31.
12 A. HAUG, Report No. 30, The Norwegian Institute of Seaweed Research, 1964.
13 J. BOYD AND J. R. TURVEY, *Carbohydr. Res.*, 66 (1978) 187–194.
14 F. HAUGEN, personal communication.
15 J. KLEIN, K. D. VORLOP, H. M. KLUGE, AND P. WASHAUSEN, *DECHEMA-Monogr.*, 84 (1979) 274.
16 A. MARTINSEN, G. SKJÅK-BRÆK, AND O. SMIDSRØD, *Biotechnol. Bioeng.*, (1988) in press.
17 H. GRASDALEN, *Carbohydr. Res.*, 118 (1983) 255–260.
18 R. G. SCHWEIGER, *J. Org. Chem.*, 27 (1962) 1786–1789.
19 R. G. SCHWEIGER, *J. Org. Chem.*, 27 (1962) 1789–1791.
20 H. GRASDALEN, I. SVARE, AND O. SMIDSRØD, *Acta Chem. Scand.*, 28 (1974) 966–968.
21 H. GRASDALEN, B. LARSEN, AND O. SMIDSRØD, *Carbohydr. Res.*, 89 (1981) 179–191.
22 G. SKJÅK-BRÆK, F. ZANETTI, AND S. PAOLETTI, *Carbohydr. Res.*, 185 (1989) 131–138.
23 G. T. GRANT, E. R. MORRIS, D. A. REES, P. J. SMITH, AND D. THOM, *FEBS Lett.*, 32 (1973) 195–198.