Carbohydrate Research, 206 (1990) 367–372
Elsevier Science Publishers B.V., Amsterdam

Note

Characterization of carrageenan fractions from Norwegian *Furcellaria lumbricalis* (Huds.) Lamour. by ^{1}H-n.m.r. spectroscopy

Svein H. Knutsen*, David E. Myslabodski, and Hans Grasdalen
Division of Biotechnology, Laboratory for Marine Biochemistry, The Norwegian Institute of Technology, University of Trondheim, 7034 Trondheim-NTH (Norway)

(Received September 11th, 1989; accepted for publication, February 5th, 1990)

The sulphated galactan from *Furcellaria lumbricalis*, commercially known as furcellaran, has a repeating backbone of alternating 3-linked β-D-galactose and 4-linked α-D-galactose residues. Of the β-Gal units, ~50% are 4-sulphated (β-Gal-4S) and the α-Gal units are mainly in the 3,6-anhydro form (α-3,6AGal). The structural relationship of this polysaccharide to kappa-carrageenan has been shown by treatment with kappa-carrageenase from *Pseudomonas carrageenovora*[1]. However, the use of kappa-carrageenase to quantify the content of kappa-carrageenan[1] may be questioned since this enzyme possibly requires a particular minimum number of units in the kappa-carrageenan. Investigation and classification, based on the chemical structure, have confirmed the nature of kappa-carrageenan and that the β-Gal units are incompletely sulphated[2,3]. ^{13}C-N.m.r. measurements[4,5] have shown that, for the carrageenan from *F. lumbricalis*, the ratio of the integrated intensities of the signals for C-1 of α-3,6Gal in α-3,6AGal-β-Gal and α-3,6AGal-β-Gal-4S is close to unity after treatment with alkali.

Since it has not been possible to resolve this carrageenan into sulphated and neutral fractions, the β-Gal units and β-Gal-4S units are likely to occur in the same molecule. In addition, alkali-labile 6-sulphate in α-Gal units has been detected by n.m.r.[5] and chemical methods[6], and β-Gal-6S units (*Phyllophora* type carrageenan) have also been found[7,8]. The occurrence of α-3,6AGal-2S units has been reported in the carrageenans from Canadian[6] and Russian[7] *Furcellaria*.

Carrageenans and agars, extracted under non-alkaline conditions, generally have some of the α-Gal units 6-sulphated (α-Gal-6S). These units can be transformed into α-3,6AGal units by treatment with strong alkali or, enzymically, by sulphoeliminase[9,10] and are termed precursors[11]. However, little work has been done on the distribution of these types of residue along with the 4-linked 3,6AGal and 3-linked Gal units. Precursors are detected routinely with specific enzymes[12–14] combined with ^{13}C-n.m.r. spectroscopy, where the patterns of C-1 resonances before and after treatment with alkali indicate the type of precursor.

* Author for correspondence.

0008-6215/90/$03.50

Elucidation of the sequence of precursors and the patterns of substitution in agar from *Porphyra* sp. was achieved by the use of high-field ^{13}C-n.m.r. spectroscopy and specific enzymes[15], but it remains to be established fully for carrageenan systems.

Since the carrageenan from *F. lumbricalis* contains residues of both neocarrabiose and its 4-sulphate, the content and the distribution of the precursors and the sequences of 3,6AGal units, with and without sulphate, will give information about the microstructure. Therefore, some structurally extreme fractions of carrageenan from *F. lumbricalis* were prepared (see Experimental), based on solubility in water and aqueous KCl, and characterized by high-resolution ^{1}H- and ^{13}C-n.m.r. spectroscopy. The cold-water (FLC) and hot-water (FLH) extracts were each fractionated into components that were soluble (FLCS and FLHS) and insoluble (FLCI and FLHI) in 0.1M potassium chloride. Non-alkali-treated, precursor-rich carrageenan fractions normally give well-resolved peaks for C-1 of the 3-linked "precursor" unit (at ~ 104.9 p.p.m.) but, often, a less well-resolved signal or signals for C-1 of the 4-linked unit (96–98 p.p.m.) (unpublished data). The use of ^{1}H-n.m.r. spectroscopy is superior to the less-sensitive ^{13}C-n.m.r. variant in detecting and quantifying the minor contents of precursors in this class of polymers.

The 500-MHz ^{1}H-n.m.r. spectra of the model substances (Fig. 1) contain resolved signals at 5.09 and 5.11 p.p.m. for H-1 of α-3,6AGal of the residues of neocarrabiose and its 4-sulphate, respectively, and H-4 of the β-Gal-4S in the latter resonated at 4.83 p.p.m. with an intensity equal to that for H-1 at 5.11 p.p.m. Hence, integration of the intensities of the signals at 5.11 and 5.09 p.p.m. indicates directly the kappa-character as defined by Usov[16]. This result was achieved earlier and indirectly by measuring the ratio of the

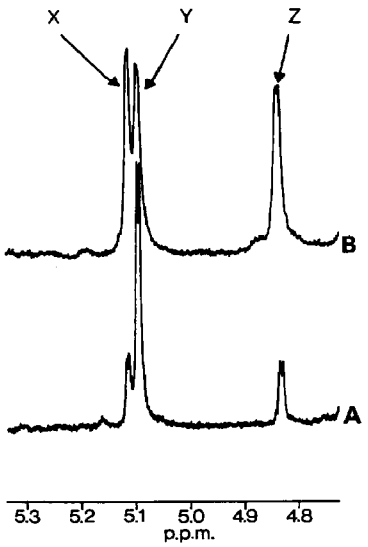

Fig. 1. Region for the H-1 signals in the 500-MHz ^{1}H-n.m.r. spectra of alkali-treated carrageenan from *F. lumbricalis*; A, partly desulphated (same as Fig. 3) and B, kappa-carrageenase treated; X, H-1′ of α-3,6AGal-β-Gal-4S; Y, H-1′ of α-3,6-AGal-β-Gal; Z, H-4′ of β-Gal-4S-α-3,6AGal; $J_{1,2}$ 2.4 Hz for α3,6AGal.

integrated intensities of the signals for H-4 of Gal-4S and H-1 of 3,6AGal, assuming a perfect alternating sequence of units[17].

Only the fraction FLHI gave a ^{1}H-n.m.r. spectrum characteristic for carrageenan from *F. lumbricalis*[5] with signals at 5.11 and 5.09 p.p.m. (Fig. 2). In the alkali-treated carrageenan from this alga, these two residues always co-occur and treatment of a solution of carrageenan from *F. lumbricalis* with a specific kappa-carrageenase reduced the viscosity (data not shown) but did not produce any enzyme-resistant fraction lacking the kappa-carrageenan structure (Fig. 1) as found for *Eucheuma gelatinae*[13]. The signals at 4.83 and 5.11 p.p.m., characteristic for α-3,6AGal-β-Gal-4S, were found for each of the 4 extracts. The ^{1}H-n.m.r. spectra of fractions FLCS (Fig. 2) and FLHS (spectrum not shown) did not contain detectable signals from sequences of the α-3,6AGal-β-Gal type (5.09 p.p.m.) before treatment with alkali, and had characteristic signals at ~5.3, ~5.2, and 4.87 p.p.m. Alkali treatment of fraction FLCS eliminated these broad signals and gave a spectrum similar to that from fraction FLHI with about equal intensities of the signals at 5.09 and 5.11 p.p.m.

The signals at 5.2 and 4.87 p.p.m. had similar intensities and were assigned tentatively to H-1 in α-Gal-6S and H-4 in Gal-4S, respectively, in the precursor O-(α-D-galactosyl 6-sulphate)-(1→3)-*O*-(β-D-galactosyl 4-sulphate). This assignment of the signal for H-1 is also compatible with a downfield displacement of 0.14 p.p.m.,

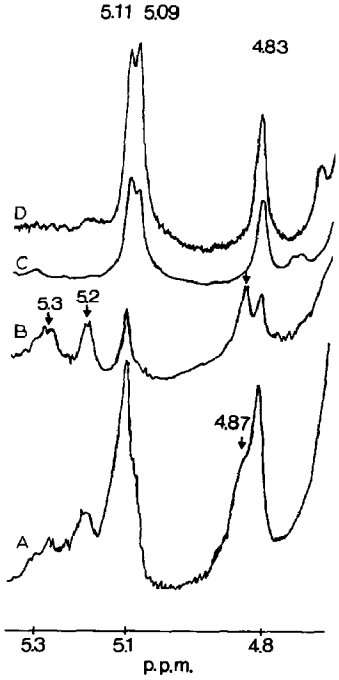

Fig. 2. Region for the H-1 signal in the 400-MHz ^{1}H-n.m.r. spectra of KCl-fractionated carrageenan from *F. lumbricalis* harvested March 28, 1986; A, FLCI; B, FLCS; C, alkali-treated FLCS; and D, FLHI. Alkali-sensitive signals ~5.3, ~5.2, and 4.87 p.p.m. are marked with arrows.

relative to that of H-1 of 3,6-AGal observed for α-L-galactose 6-sulphate in the corresponding precursor of agarose[18].

Desulphation of the β-Gal units in the carrageenan precursor will probably alter the chemical shift of the resonance of H-1 in a neighboring α-Gal-6S unit only slightly. A shift 0.1 p.p.m. towards lower field points to the broad doublet at 5.3 p.p.m. as the candidate. This tentative assignment leads to a consistent picture when relative intensities are compared before and after the treatment with alkali. Whether the β-Gal units towards the non-reducing end were or were not 4-sulphated was not evident from our data.

Thus, in fraction FLCS, the 3,6AGal residues were part of α-3,6AGal-β-Gal-4S sequences, and α-3,6AGal-β-Gal units were not detectable by n.m.r. spectroscopy in the precursor-rich, soluble fractions *before* treatment with alkali. This treatment converted α-Gal-6S units into 3,6AGal units, independently of the sulphation of neighboring units, and could produce 3,6AGal units in regions where the sulfoeliminase enzyme is ineffective. The 400-MHz ^{1}H-n.m.r. spectrum of the fraction FLCI (Fig. 2) has broad signals due to the viscosity of the solution. However, this fraction contains α-3,6AGal-β-Gal-4S and α-3,6AGal-β-Gal units together with their respective precursors, α-Gal-6S-β-Gal-4S and α-Gal-6S-β-Gal.

The biosynthesis pathway of the partly sulphated carrageenan from *F. lumbricalis* is not evident from the foregoing data. The formation of 3,6AGal *in vivo* could be more complicated than for the idealized pathway proposed from mu- to kappa-carrageenan and from nu- to iota-carrageenan[19]. Whether the carrageenan from *F. lumbricalis* is a "not-yet-sulphated" or a desulphated kappa-carrageenan is not known. The finding that all the neocarrabiose sequences in fraction FLCS before treatment with alkali are 4-sulphated could be explained by the requirement[10] of this sulphation for the enzymic formation of 3,6AGal.

The general biosynthesis scheme for carrageenans proposed by Craigie and co-workers[19] cannot be used, without modifications, for the carrageenans from *F. lumbricalis* and *E. gelatinae* with incomplete 4-sulphation of the 3-linked β-Gal units. There is also a conflict with the above general scheme with carrageenans from *Phyllophora* sp., *F. lumbricalis*[7], and *Rhizoella verruculosa*[20] where 6-sulphated 3-linked β-Gal units are present.

EXPERIMENTAL

Dried and milled *F. lumbricalis*, harvested in the Trondheimsfjord, was treated with 0.1M hydrochloric acid for 30 min at 4° and then washed for 4 h with running tap water at 8°. A cold-water extract (FLC) was obtained by stirring the residue with distilled water for 12 h after neutralizing with 0.01M sodium hydroxide. The residue was collected and suspended in distilled water, the pH was adjusted to 8 with NaOH, and the suspension was stirred for 1 h at 120°, to give the hot extract (FLH). Each extract was precipitated with 2-propanol, resolubilized, dialyzed against neutral distilled water at 25°, and lyophilized. The total yield of water-extractable polysaccharide was ~35% of

the initial dry weight, with a ratio for FLC and FLH of ~1:10. Each extract was fractionated on the basis of the solubility in 0.1M KCl by the leaching procedure[21] to give soluble (FLCS and FLHS) and insoluble fractions (FLCI and FLHI). FLC contained >80% of KCl-soluble material, whereas FLH contained <40%. The yields of the various fractions from *F. lumbricalis* depended on the time of harvesting.

The above four fractions were investigated by ^{1}H-n.m.r. spectroscopy. The assignments of the H-1 and C-1 resonances were based on 25-MHz ^{13}C- (Fig. 3) and 500-MHz ^{1}H-n.m.r. spectra (Fig. 1) of desulphated[22] or kappa-carrageenase treated, alkali-modified[23] carrageenan from *F. lumbricalis*. Parameters used for the 400-MHz ^{1}H- and 25-MHz ^{13}C-n.m.r. spectra have been published[5]. The 500-MHz ^{1}H-n.m.r. spectra were recorded at 80° with a Bruker WM-500 spectrometer, using 18k data points and a pulse recycling time of 2.7 s. Chemical shifts (p.p.m.) are referred to external Me_4Si *via* internal sodium 3-(trimethylsilyl)propionate-d_4 (TSP, δ 2.4 for ^{13}C and δ 0 for ^{1}H).

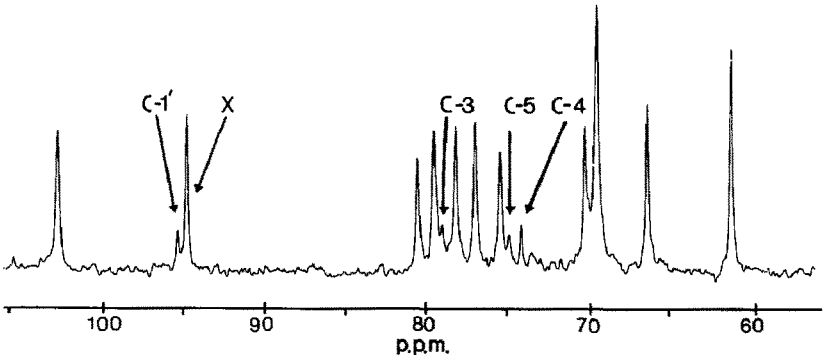

Fig. 3. Signals for C-1', 3,4,5 of α-3,6AGal-β-Gal-4S in the 25-MHz proton-decoupled ^{13}C-n.m.r. spectrum of partly desulphated carrageenan from *F. lumbricalis*; X is the resonance for C-1' in α-3,6-AGal-β-Gal. Assignments were made after comparison with data for kappa- and desulphated kappa-carrageenan[24].

ACKNOWLEDGMENTS

We thank Professor Bjørn Larsen for his assistance, and the Bioenergy Program of the Norwegian Agricultural Research Council for support (to S.H.K.). The 400-MHz ^{1}H-n.m.r. spectra were recorded at the University of Trondheim by Mrs. Astrid M. Schie Bergan and the 500-MHz spectra were recorded at the MR-Center in Trondheim under the supervision of Dr. J. Krane.

REFERENCES

1 W. Yaphe, *Can. J. Bot.*, 37 (1959) 751–757.
2 T. J. Painter, *Can. J. Chem.*, 38 (1960) 112–118.
3 S. V. Yarotsky, A. S. Shashkov, and A. I. Usov, *Bioorg. Khim.*, 4 (1978) 745–751.
4 S. S. Bhattacharjee, W. Yaphe, and G. K. Hamer, *Proc. Int. Seaweed Symp.*, 9 (1979) 379–385.
5 S. H. Knutsen and H. Grasdalen, *Bot. Mar.*, 30 (1987) 497–505.
6 T. J. Painter, *Proc. Int. Seaweed Symp.*, 5 (1966) 305–313.

7 A. I. Usov and V. S. Arkhipova, *Bioorg. Khim.*, 7 (1981) 385–390.
8 S. H. Knutsen, Presented at the 4th International Meeting of the Societe pour L' Algologie Appliquee, Lille, France, 1987.
9 D. A. Rees, *Biochem. J.*, 81 (1961) 347–352.
10 K. F. Wong and J. S. Craigie, *Plant Physiol.*, 61 (1978) 663–666.
11 C. J. Lawson and D. A. Rees, *Nature (London)*, 227 (1970) 392.
12 C. Bellion, G. Brigand, J. C. Prome, and D. W. S. Bociek, *Carbohydr. Res.*, 119 (1983) 31–48.
13 C. Greer and W. Yaphe, *Bot. Mar.*, 27 (1984) 473–484.
14 C. Bellion, W. Yaphe, and G. K. Hamer, *Proc. Int. Seaweed. Symp.*, 10 (1981) 379–384.
15 L. M. Morrice, M. W. McClean, W. F. Long, and F. B. Williamson, *Eur. J. Biochem.*, 133 (1983) 673–684.
16 A. I. Usov, *Bot. Mar.*, 27 (1985) 367–373.
17 D. Welti, *J. Chem. Res. (M)*, (1977) 3566–3587.
18 M. Lahaye, Ph.D. Thesis, McGill University, Montreal, Canada, 1986.
19 J. S. Craigie and K. F. Wong, *Proc. Int. Seaweed Symp.*, 9 (1979) 369–377.
20 J. Mollion, S. Moreau, and D. Christiaen, *Bot. Mar.*, 29 (1986) 549–552.
21 D. J. Stancioff and N. F. Stanley, *Proc. Int. Seaweed Symp.*, 6 (1969) 595–609.
22 T. C. S. Dolan and D. A. Rees, *J. Chem. Soc.*, (1965) 3534–3539.
23 J. S. Craigie and C. Leigh, in J. A. Hellebust and J. S. Craigie (Eds.), *Handbook of Phycological Methods: Physiological and Biochemical Methods*, Cambridge University Press, Cambridge, 1978, pp. 109–131.
24 A. I. Usov and A. S. Shashkov, *Bot. Mar.*, 27 (1984) 189–202.