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## Preliminary communication

# Analysis of the enzymic hydrolysis products of agarose by $^{13}$C-n m r spectroscopy

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Agarose, the gelling component of agar, is composed of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose[1] The polysaccharide is degraded by hydrolytic enzymes extracted from marine bacteria These enzymes can be divided into two main groups, one cleaving the β-D-(1→4)-, and the other, the α-L-(1→3)-linkage Cleavage of the β-D-linkage of agarose yields oligosaccharides belonging to the neoagaro series, with a D-galactose residue at the reducing end and a 3,6-anhydro-L-galactosyl group at the nonreducing end [see Fig 1(a)] Cleavage of the α-L-linkage yields

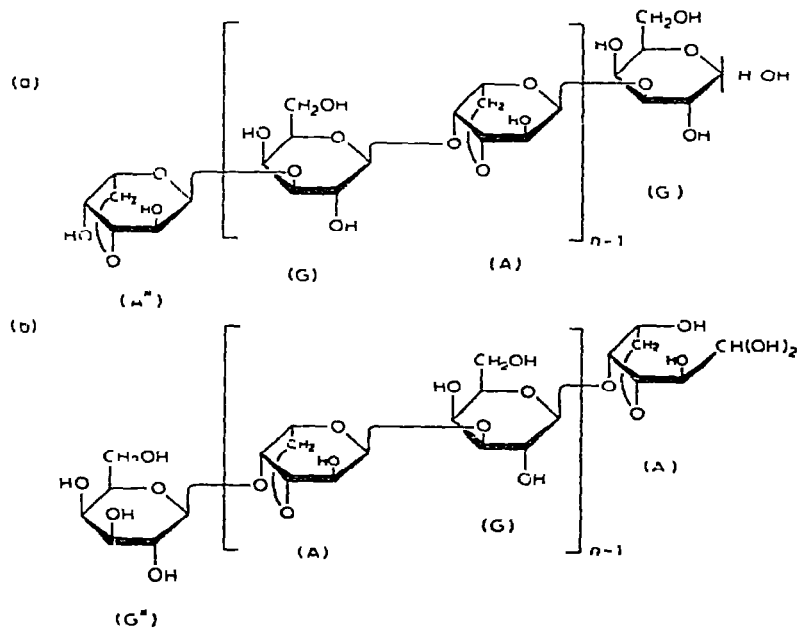


Fig 1 Oligosaccharides obtained by enzymic hydrolysis of agarose (a) neoagaro-oligosaccharides (A' [G-A]$_{n-1}$-G') produced by β agarase, (b) agaro-oligosaccharides (G"[A-G]$_{n-1}$-A') produced by α-agarase (G, D-galactose, A, 3,6 anhydro-L-galactose, *n*, number of biose units per oligosaccharide molecule, reducing end indicated by a single prime, nonreducing end by a double prime.)

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agaro-oligosaccharides having a 3,6-anhydro-L-galactose residue (in the open-chain, aldehydo form) at the reducing end and a D-galactosyl group at the nonreducing end [see Fig 1(b)]. Although β-agarases have been reported by several workers[1–5], the description of α-agarases is rare[4]

The oligosaccharides produced by agarolytic enzyme action have been analyzed by thin-layer chromatography on cellulose[6] However, on the basis of t l c alone, it is difficult to characterize these products with respect to their reducing and nonreducing end-groups, and the chemical methods required are rather tedious For this reason, we have investigated the possibility of using $^{13}$C-n m r spectroscopy for the analysis of agarose hydrolyzates In this communication, we report preliminary results for the substrate agarose, and for a series of known, purified neoagaro- and agaro-oligosaccharides[4] We have also examined crude, unfractionated digests obtained with purified β- and α-agarases[2,4]

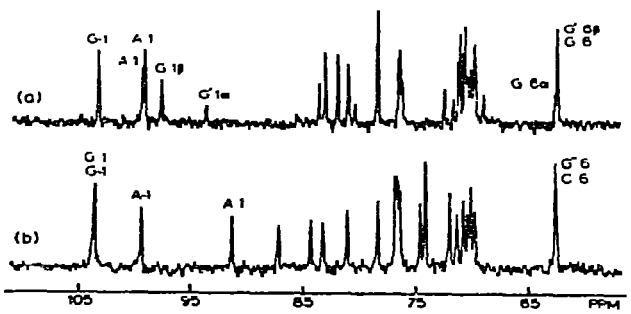


Fig 2 $^{1}$H-Decoupled $^{13}$C-n m r spectra ($D_2O$ solution, 35°) of oligosaccharides produced by enzymic hydrolysis of agarose (a) purified neoagarotetraose produced by β-agarase, (b) unfractionated mixture of agaro-oligosaccharides produced by α-agarase

Typical $^{13}$C-n m r spectra illustrating the effects of β and α cleavage are shown in Fig 2 The peak assignments are based on the chemical shifts of model compounds (see later) and on a comparison of relative intensities in the spectra of the neoagaro homologs For the purposes of the present discussion, attention is confined to the resonances of the anomeric carbon atoms (see Table I)

The chemical shifts of G-1 and A-1 of the agarose oligosaccharides differ only slightly from those of the undegraded substrate There is a small displacement of both resonances (0 5 p p m or less) for residues at the nonreducing end of the chain The shifts of G'-1α and G'-1β in the neoagaro series are in good agreement with C-1 shifts of the monosaccharide models α- and β-D-galactose (93 8 and 98 0 p p m , respectively) Similarly, there is a close correspondence between the shifts of A'-1 in the agaro-oligosaccharides and the C-1 shift of 3,6-anhydro-L-galactose (91 4 p p m )* or, in the case of agarotetraitol, 3,6-anhydro-L-galactitol (64 3 p p m ) It is clear from these results that $^{13}$C-n m r spectro-

*The chemical shifts of A' 1 and C 1 of 3,6-anhydro-L galactose are characteristic of hydrated aldehydes[8,9]

## TABLE I

$^{13}$C CHEMICAL SHIFTS[a] OF ANOMERIC CARBON ATOMS [p p m (±0 04) relative to external $Me_4Si$]

| | *G-1* | *G'-1* | *G" 1* | *A-1* | *A' 1* | *A"-1* |
|---|---|---|---|---|---|---|
| Agarose[b] | 103 53 | – | – | 99 30 | – | – |
| Neoagarobiose[c] (A"-G') | – | 93 80[h]<br>97 79[i] | – | – | – | 99 29 |
| Neoagarotetraose[c] (A"-G-A-G ) | 103 42 | 93 78[h]<br>97 79[i] | – | 99 49 | – | 99 29 |
| Neoagarohexaose[c] (A"-G-A-G-A-G') | 103 41 | 93 80[h]<br>97 79[i] | – | 99 47 | – | 99 29 |
| Neoagaro-oligomers[d] | 103 42 | 93 83[h]<br>97 83[i] | – | 99 50 | – | 99 31 |
| Agarobiose[e] (G"-A') | – | – | 103 96 | – | 91 45 | – |
| Agarotetraitol[c,f] (G"-A-G-A') | 103 63[j] | – | 103 54[j] | 99 47 | 64 32[k] | – |
| Agaro-oligomers[g] | 103 63 | – | 103 63 | 99 45 | 91 42 | – |

[a]22 628 MHz, $^1$H-decoupled, $^{13}$C spectra recorded on a Bruker WH-90 FT spectrometer in $D_2O$ solution (20–40 mg/ml) at 35° (spectral width, 1 5 kHz, pulse width, 70° (14 μs), repetition time, 1 363 s, 20,000–65,000 transients, 4K real data points) [b]Spectrum recorded at 95° [c]Isolated, and purified, by Yaphe and co-workers[4] [d]Unfractionated mixture of oligosaccharides produced by *Pseudomonas atlantica* β-agarase[2] [e]Prepared by mild, acid hydrolysis[7] [f]Characterized as a tetramer by $^{13}$C-n.m r spectroscopy (see Table II) [g]Unfractionated mixture of oligosaccharides produced by a Gram negative, marine bacterium α-agarase[3] [h]α Anomer [i]β Anomer [j]Assignments may be reversed [k]Reduced C 1 of A

scopy provides unequivocal evidence regarding the mode of action of agarolytic enzymes Cleavage of the β-D-(1→4)-linkage of agarose is indicated by the appearance of peaks at 93 8 and 97 8 p p m , having intensities in the ratio of 1 2 [see Fig 2(a)] In contrast, α-L-(1→3) cleavage is indicated by the appearance of a peak at 91 4 p p m [see Fig 2(b)] The spectra of the crude digests confirmed that the action of the purified enzymes is specific[2,4] , there is no evidence of α cleavage by β-agarase, or of β cleavage by α-agarase

Provided that the spectrum is fully relaxed[10,11], the integrated intensities of the anomeric carbon resonances can be used to calculate $n$, the number of biose units per oligosaccharide molecule For crude hydrolyzates, this calculation gives an estimate of the average chain-length of the oligomers As a check on the condition of complete relaxation the integrals were normalized to the total intensity of C-6 of the D-galactose residues** As the spin–lattice relaxation-time ($T_1$) of C-1 is expected to be approximately[10,11] twice that of C-6, incomplete relaxation of the anomeric carbon atoms should be detected by this procedure The accuracy of the calculation is also dependent on the (reasonable) assumption that the nuclear Overhauser enhancement (nOe) is the same for each of the integrated

**The chemical shifts of G-6, G'-6α, G'-6β, and G"-6 all occur in the narrow range 62 4–62.7 p p m (see Fig 2)

†For the relevant experimental conditions, see footnote *a* to Table I

TABLE II

INTEGRATED INTENSITIES OF ANOMERIC $^{13}C$ RESONANCES[a]

| *Oligosaccharide*[b] | *G-1* | *G'-1* | *A-1 + A''-1* | n[c] |
|---|---|---|---|---|
| Neoagarobiose | – | 0 99(1 00) | 0 98(1 00) | 0 99(1 00) |
| Neoagarotetraose | 0.51(0 50) | 0 50(0 50) | 1 02(1 00) | 2.00(2 00) |
| Neoagarohexaose | 0 68(0 67) | 0 29(0 33) | 0 98(1 00) | 3 27(3 00) |
| Neoagaro-oligomers | 0 76 | 0 29 | 1 02(1 00) | 3 92[d] |
| | *G-1 + G -1* | *A-1* | *A'-1* | n[c] |
| Agarobiose | 1 03(1 00) | – | 0 97(1 00) | 1 06(1 00) |
| Agarotetraitol | 1 00(1 00) | 0.51(0 50) | 0 51(0 50) | 2 04(2 00) |
| Agaro-oligomers | 1 03(1 00) | 0 61 | 0 40 | 2 45[d] |

[a]Normalized to the intensity of G-6 + G'-6 (neoagaro series) or G-6 + G''-6 (agaro series), estimated error, ±0 04, theoretical values in parentheses. [b]See footnotes *c–g* of Table I [c]Number of biose units per oligosaccharide molecule [d]Average value

resonances[10,11] The generally good agreement between the experimental and the theoretical values (see Table II) shows that the spectra are, in fact, fully relaxed† and that the nOe factors are equivalent

The results of this investigation demonstrate that $^{13}C$-n m r spectroscopy can provide valuable qualitative and quantitative information about enzyme reactions We are currently using these methods to study the degradation products of ι- and κ-carrageenans and other agar-type polysaccharides

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