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Carbohydrate Research 262 (1994) 127–135

CARBOHYDRATE RESEARCH

Chemical methylation of agaroid hydroxyl-groups

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Received 16 August, 1993; accepted in revised form 23 March, 1994

Abstract

Partially methylated derivatives of agar or agarose were prepared by methylating an agar or agarose with various levels of natural methylation in alkaline solution with dimethyl sulfate. The resultant derivatives were analysed for methylation pattern, gel setting temperature, and gel strength. The extent to which the gelling temperature was lowered was proportional to the level of methylation at the 2-position of the D-galactosyl residues. Since the 2-hydroxyl group on the D-galactose is the only hydroxyl group which has a binding function in the double helix model, this observation is strongly supportive of this model of agarose gels.

1. Introduction

Agarose is a linear polymer with agarobiose as a repeating unit, the polymer consisting of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl residues. There are four hydroxyl groups per repeating disaccharide unit available for substitution, namely O-2, O-4, and O-6 on D-galactose, and O-2 on the 3,6-anhydro-L-galactose. Agar is a natural polymer with the idealized agarose backbone, but with low levels of substitution by sulfate ester and pyruvic ketal, and often with more significant levels of methyl ether. The major use of agar is as a gelling agent. A 1% solution of agar or agarose, when cooled, will form a gel at temperatures between 34 and 43°C, depending on the origin of the agar [1]. Once gelation has occurred, a true solution can only be obtained by heating the gel to between 85 and 95°C.

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SSDI 0008-6215(94)00118-Y

The variation in gelling and melting temperatures appears to depend on the extent and nature of methyl substitution. Native agars, especially those from red seaweeds in the family Gracilariaceae, frequently contain *O*-methyl groups [2] and invariably the gel setting temperatures of these naturally methylated agars are higher than for gels of nonmethylated agars, e.g., those from the family Gelidiaceae. Thus, the gel setting temperature of a *Gelidium* agar is approximately 35°C while that of a *Gracilaria* agar is frequently greater than 40°C. There may also be a corresponding increase in the gel melting temperature, perhaps greatest in the agar from *Curdiea coriacea* [3], which gives gels that do not melt until above 100°C. This agar is almost completely methylated on O-2 of the 3,6-anhydro-L-galactose and on O-6 of the D-galactose. On the other hand, the alkylation of agar under laboratory conditions generally produces a product with both lower gel setting and gel melting temperatures [4,5]. An agarose derivative with such a lowered gel setting temperature is an item of commerce, although for convenience of manufacture the substituents are hydroxyethyl rather than methyl groups.

One question that remains is why naturally and chemically methylated agars have such different properties, when they appear to have essentially the same type of substitution. Of particular interest in this respect is that when a naturally methylated agar (1.9% methoxyl content) with a gel setting temperature of 40.5°C was alkylated [4], the gel setting temperature fell by about the same level per unit of added methylating agent as it did for a nonmethylated agar. Although it is clear that the chemically modified agars will have different patterns of alkylation, depending on the initial level of natural methylation of the agar, the effect of chemical methylation appeared to be approximately the same, after correcting for the different starting points. Thus, both the chemical and natural methylation appear to give separable and additive effects, but in opposite directions.

Guiseley [5] has hinted at two possible explanations for why the effect of the alkylation depended on whether it arose through chemical modification or from natural sources. Firstly, he suggested that laboratory methylation could be random, and secondly he proposed that another type of methylation was present. He identified 6-*O*-methylgalactose in the laboratory-methylated material, together with another unknown material which was not the 4-*O*-methyl derivative. An alternative site for methylation is not, in itself, an explanation, as the agar from *Curdiea coriacea* is almost fully methylated at the 6 position of the 3-linked galactose as well as at the 2 position of the 4-linked 3,6-anhydrogalactose [3], and the gelling temperature of this polymer is ~ 44°C while the remelting temperature is > 100°C. The gelling properties of *C. coriacea* agar also seem to rule out the possibility that the higher gelling temperature is due to a block copolymer of methylated polysaccharides alternating with blocks of nonmethylated units, as opposed to random methylation in the chemical case, since this polysaccharide is essentially fully methylated at the two positions [3].

It has been proposed that, in the gel state, the agarose chains exist as double helices [6–8] and that these helices then further self-associate to form aggregates. Arnott et al. [9] examined substituted agaroses by X-ray fibre diffraction techniques and studied the optical rotations of their solutions as a function of

temperature. They observed by polarimetry a transition to an ordered conformation as the temperature drops. Their conclusions were that their results best fitted a double helix model for the ordered conformation, and that the geometry of the helices was independent of the substitution, although substitution did affect the temperature at which gelling and/or aggregation occurred. Hydrogen bonding between the chains of the double helix is mediated by isolated water molecules located within the cavity of the double helix, which interact with two agarobiose units, one from each chain. These water molecules adopt a tetrahedral configuration and form hydrogen bonds with HO–2 groups on two D-galactosyl residues and, using the hydrogen atoms from the water, with O-5 atoms on two 3,6-anhydrogalactosyl residues. Apart from these interactions, there was no apparent hydrogen bonding between chains in a helix.

The double helix model has not been universally accepted. Foord and Atkins [10] considered that the evidence for the double helix is largely circumstantial, while Rochas [11] stated that the agarose gel may be a double helix, or single helices in pairs. Rochas found that the transition temperature as observed by polarimetry was independent of concentration, while the gel setting temperature depended on the rate of cooling, and on how it was measured. He claimed that the enthalpy change (ΔH) associated with the gelation process did not arise from a conformational transition, but rather was largely due to intermolecular interactions.

Braudo [12] proposed that double helix formation is not a prerequisite for gelation. For kappa-carrageenan, gelation and the transitions to and from a double helix, as observed by changes in optical rotation, take place at different temperatures. The same was not found for agarose gelation. While Braudo's results were obtained in 1:1 water–Me_2SO, and hence may not be directly transferable to the water situation, nevertheless it is apparent that gelation is not one simple transition. As a consequence, different methods of determining a gelling temperature may give different results through recording different parts of the gelling transition.

One approach towards resolving this problem is to examine the substitution patterns of chemically methylated agars with lowered gel setting temperatures, in order to determine whether there is any difference between them and naturally occurring agars, and to determine whether this difference is related in any way to the double helix model, which is the only model that is sufficiently specific to be used as a basis for relating chemical substitution to physical properties. While commercially available agarose having a low setting temperature is usually substituted with hydroxyethyl groups (for convenience of manufacture), we have elected to study the methylated derivatives, as the analytical methods and related data are fully available.

2. Materials and methods

Agars were obtained from the New Zealand red seaweeds *Pterocladia lucida*, *Gracilaria chilensis*, and *Curdiea codioides*, and were treated with alkali following

Table 1
Gelling temperatures for methylated agars

	Polysaccharide origin	Gel temp (°C)	Drop in gel temp (°C)	Gel strength (g/cm^2)	Me_2SO_4 (mL/g of agar)
	P. lucida	35	0	1100	0
(a)	*P. lucida*	27	8	440	0.7
(b)	*P. lucida*	22	13	82	1.5
(c)	*P. lucida*	~10	25	15	2.2
(d)	*G. chilensis*	42	0	950	0
(e)	*G. chilensis*	27	15	410	1.4
(f)	*C. codioides*	44	0	nd [a]	0
(g)	*C. codioides*	29	15	nd	1.3

[a] nd, Not determined.

methods previously published [13]. These agars were chosen because they have almost zero, moderate, and high natural levels of methyl ether substituents, respectively. Reagents used were laboratory grade without further purification.

Methylation of the agars reported in Tables 1 and 2 followed the procedures outlined by Guiseley [4,5], except that the resultant product was purified by freeze–thawing of the gel, followed by dialysis of the resultant solid against deionized water. To assist with reproducibility, exactly the same equipment was used for each reaction, with the same magnetic stirrer set at a constant rate of rotation. The addition of dimethyl sulfate was made from the same dropping funnel for each preparation, with the drop rate as nearly as identical as possible for each reaction. Finally, three additional reactions were carried out at much

Table 2
Composition of polymers determined by reductive hydrolysis

Substitution	Samples [a]						
	a	b	c	d	e	f	g
3,6-Anhydrogalactoses							
Nil	47	32	24	44	36	48	41
2-*O*-Methyl-	6	17	27	4	18	0	10
Total anhydrogalactoses	53	49	51	48	54	48	51
Galactoses							
Nil	38	29	19	30	18	6	5
2-*O*-Methyl-	3	10	12	0	4	0	1
4-*O*-Methyl-	0	0	1	0	0	0	0
6-*O*-Methyl-	5	10	8	22	18	46	32
2,4-Di-*O*-methyl-	0	0	2	0	0	0	0
2,6-Di-*O*-methyl-	1	2	6	0	5	0	7
4,6-Di-*O*-methyl-	0	0	1	0	1	0	2
2,4,6-Tri-*O*-methyl-	0	0	0	0	0	0	2
Total galactoses	47	51	49	52	46	52	49

[a] Samples as identified in Table 1; the data show mol% of component sugars. The original *P. lucida* agar is very closely 50% galactose, 50% anhydrogalactose.

Table 3
Composition of further polymers determined by reductive hydrolysis.

Substitution	Samples [a]		
	a	b	c
3,6-Anhydrogalactoses			
Nil	50	49	36
2-*O*-Methyl-	5	5	16
Total anhydrogalactoses	55	54	52
Galactoses			
Nil	36	40	28
2-*O*-Methyl-	4	2	5
4-*O*-Methyl-	1		2
6-*O*-Methyl-	3	3	7
2,4-Di-*O*-methyl-			1
2,6-Di-*O*-methyl-	1	1	3
4,6-Di-*O*-Methyl-			1
2,4,6-Tri-*O*-methyl-			1
Total galactoses	45	46	48

[a] Further samples made following the general procedures for samples listed in Tables 1 and 2. Gel strengths (in g/cm^2) and setting temperatures were: (a) 140, 28°C; (b) 490, 31°C; (c) 120, 24.5°C.

lower temperatures, where the agar was gelling or had gelled and was mechanically stirred. The compositions of the products of these reactions are reported in Table 3.

^{13}C NMR spectra of the polymers were recorded for 5% w/v solutions in D_2O–H_2O at 90°C with a Bruker AC 300 spectrometer (acquisition time, 0.8 s; delay, 0.5 s; pulse width, 90°). Sugar analyses were carried out following Furneaux and Stevenson [14], using the reductive hydrolysis procedure to produce alditol acetate derivatives which were then identified by GLC–MS, and quantified by GLC using authentic standards to obtain relative response factors under the same operating conditions. Gel strengths were measured at ambient temperature by penetrating the gel (cured overnight) with a circular plunger of facial area 1 cm^2. All gel strengths refer to a 1% w/v gel. Gel setting temperatures were obtained by cooling the solution in a stainless steel dish, and recording temperature (T) and time (t). The solution was cooled rapidly to 50°C, then more slowly but at as near to a constant rate as possible by means of regularly flowing water across the exterior of the dish. The gelling temperature was recorded as when dT/dt most closely approached zero, which also corresponded to the observation of small semisolid pieces on the side of the steel when a film of solution was drawn upwards. The value of the gel setting temperature is known to depend on how the experiment is done [11], and additionally there is a level of subjectivity about exactly when a solution gels, particularly when there are dramatic changes in gel strength. While the values given here are not absolute, we consider that the results are valid for comparative purposes, as all measurements were performed in a consistent manner, and should record a similar point in the cooling transition for each sample.

3. Results and discussion

Methylation of each agar proceeded smoothly, although in some cases a small amount of insoluble material was also formed, and this was discarded. The relationships between the proportion of methylating reagent used, and the gel strength and gelling temperature of the products are presented in Table 1. Addition of equivalent amounts of reagent depressed the melting temperature of each of the agar types by roughly the same amount. The results are similar to those reported by Guiseley [4,5] although, for the preparations reported here, the same amounts of reagent appear to be giving a greater effect. Because dimethyl sulfate is not immediately soluble in alkali, this difference may arise through higher stirring speeds or a different rate of addition and is not considered significant.

The drop in gel strength due to a given amount of reagent is quite marked for the *Pterocladia lucida* agar, although relatively less so for the *Gracilaria chilensis* agar. Gel strength data are not listed for the *Curdiea codioides* samples, since the gel from the methylated sample was too weak to measure.

The sugar compositions of the resultant polymers are given in Table 2. Since the polymers are composed of repeating units of substituted agarobiose, the sum of the anhydrogalactopyranosyl residues should equal the sum of the galactopyranosyl residues, and should be 50%. Within the range of experimental error, this is the case. All available hydroxyl groups appear to have the potential to be methylated, although reaction at the axial 4-hydroxyl group on the D-galactosyl residue occurred only on severe alkylation, and this site is essentially unreactive. The most reactive site appears to be the 2-hydroxyl group on the anhydrogalactosyl residue.

The ^{13}C NMR spectra of the highly methylated *Pterocladia* samples are consistent with the substitution patterns outlined in Table 2. Additionally, for samples b and c in Table 2, the signal at 98.6 ppm, which corresponds to C-1 of the anhydrogalactose where the O-2 position is methylated, shows a narrow but distinct splitting. Despite the significant differences in the level of methylated anhydrogalactose, the relative ratios of peak heights (1:1.3 and 1:1.22, respectively) are relatively constant. The corresponding signals for the non-methylated anhydrogalactose and the corresponding signal for the agar from *Curdiea coriacea* [3] do not show this splitting. The conclusion from this is that C-1 of the 2-*O*-methyl-anhydrogalactosyl residues in the chemically methylated agar occurs in two slightly different environments. There is some indication, therefore, that this additional signal arises from an interaction between a 2-*O*-methyl-3,6-anhydrogalactosyl residue and a methyl group on the adjacent galactose. The chemically methylated agars from *Gracilaria chilensis* and *Curdiea codioides* did not show this signal splitting because the signal resolution was not adequate.

To consider how gelation might be affected by temperature, we offer the following model. In a solution of hot agar, we suggest that a section of an agar chain can form an association with a similar section, which does not necessarily have to be from another molecule but could be from a distant part of the same molecule, if the chain is folded. This association is represented as an equilibrium

$$2(\mathrm{ag})_s \rightleftharpoons [(\mathrm{ag})\text{-}(\mathrm{ag})]_s \quad (1)$$

where the left-hand side represents free sections of agar chain in solution, and the right-hand side represents lengths of associated agar chain, in solution, in a structural relationship which could lead to gelation. There will be other interactions, but if they cannot lead to gelation they are not relevant to this argument. We consider that at equilibrium these associated sections behave as independent entities. By that, it is meant that the behaviour of a given section depends only on the nature of the interactions of the species, and not on what other sections of the same chain, but in separate associations, are doing.

The equilibrium constant K will be given by

$$\ln K = -\Delta H_s/(RT) \quad (2)$$

where ΔH_s is the enthalpy of association of sections of mean length for that value of K. The more the equilibrium lies towards association, the stronger will be the links between the chains, which in turn can only arise through the presence of more interactions, which requires greater lengths of chain to be associated. The constant K therefore can be considered to reflect what fraction of each chain, on average, is associated. As the temperature decreases, ln K increases, that is, more associations are formed. As a consequence, the solution becomes more viscous as the apparent molecular weight increases through more independent associations being formed. This increase in viscosity is observed, and it appears to be a true equilibrium inasmuch as the viscosity decreases again if the solution is reheated, although the time taken to reach true equilibrium on reheating may be quite long.

As associations continue to form on cooling, the molecules will become more tangled, and there will come a point where associations are no longer independent. As associations become closer together on a chain (or more frequent), the physical linkage between associations will reach the point where the formation of one linkage may more strongly inhibit another from dissociating, and the solution gels. If this model is correct, there will be a value of K just above the gelling point where the solution is in equilibrium, but only just. Since this point is determined by the mean distance between sections of aggregation on the average polymer chain, and not on any other structural or chemical feature, we assume that this value of K is the same for all polymers at the last point of equilibrium on the cooling curve prior to gelation. If this is so, then $\Delta H/T$ should be constant for each polymer. There is an important consideration within this model. For an agar solution to avoid gelation at a given temperature, it is not a requirement that a whole agar strand can become completely free in solution, but rather that any lengths of associated agar chain can dissociate. Gelation occurs when lengths of associated agar chains become frozen for geometric reasons, which removes from the equilibrium considerations some of what should be on the right-hand side of Eq. 1. The effect is conceptually similar to what would happen if it had precipitated.

The double helix model now makes an interesting prediction, because the major interactions between chains of the helix are hydrogen bonds to the water molecule between the strands. Methylation at O-2 of a D-galactosyl residue will remove one of these hydrogen-bond interactions with this water molecule in the helix cavity, and could replace it with a repulsive interaction. This will lead to a reduction in

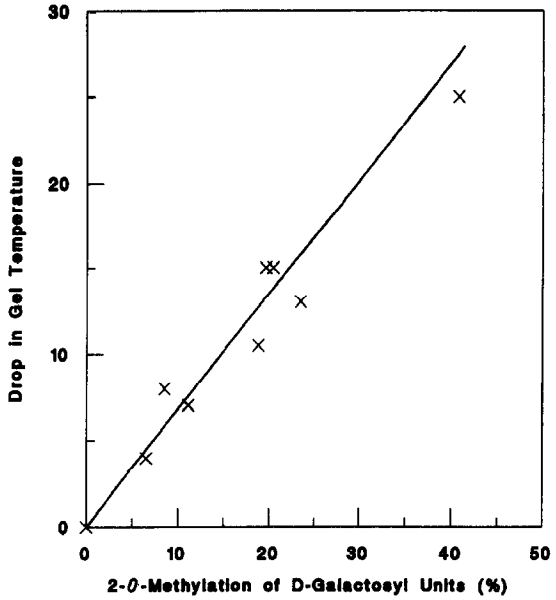

Fig. 1. Plot of change in gel setting temperature versus total 2-*O*-methylation on D-galactosyl units.

ΔH, and accordingly, in order to maintain the constant value of K pertaining at incipient gelation, a lowering of the temperature T. Thus the double helix model does at least offer a possible explanation for the variation of gelling temperature with substitution; in particular, the lowering should be proportional to the level of substitution at O-2 of the D-galactosyl residues. Furthermore, this is the only position where methylation can remove such a hydrogen bond directed internally to the helix. Alkylation at other positions should have no effect on interactions within the double helix; hence, the lowering of the gelling temperature as predicted above should depend only on the total O-2 substitution, including 2,4-disubstitution, 2,6-disubstitution, and 2,4,6-trisubstitution. By a corollary of this reasoning, natural methylation, which substitutes hydroxyl groups on the external surface of the double helix, must produce its effect largely through differences in interactions with the solvent, or other double helices at junction zones.

When the drop in gel setting temperature is plotted against the total level of methylation at O-2 of the D-galactosyl residues, an approximately straight-line correlation is found, as can be seen in Fig. 1. Data from three further samples of chemically methylated *Pterocladia lucida* agars, made at lower temperatures, are included here and their compositional analyses are shown in Table 3. No correlation appears possible with substitution at any other position; increased gelling temperatures are found with very high levels of methylation on O-6 of galactosyl and O-2 of the anhydrogalactosyl residues in natural polymers [3], and there is insufficient methylation on O-4 of most polymers for a significant effect.

To conclude, we find the drop in gel setting temperature for laboratory methylated agars to be directly proportional to the level of methylation at the

2-position of the D-galactosyl residues. This observation is consistent with what could be expected from the double helix model for agarose gelation, where it is the formation of the helices that provides the irreversible nature of the transitions, and hence the thermal hysteresis.

Acknowledgments

We thank H. Wong for recording the NMR spectra, a referee for helpful comments, and the New Zealand Foundation for Research, Science and Technology for financial assistance under contracts CRL 101, CRL 201, and CO8302.

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