Carbohydrate Research, 238 (1993) 27–38
Elsevier Science Publishers B.V., Amsterdam

Synergy of the agarose–carob galactomannan blend inferred from NMR and rheological studies

Tristan Turquois, François-R. Taravel and Cyrille Rochas
Centre de Recherches sur les Macromolécules Végétales, CNRS, B.P. 53 X, F-38041 Grenoble (France)

(Received October 18th, 1991; accepted in revised form May 15th, 1992)

ABSTRACT

Analysis of ^{13}C NMR spectra of carob–agarose binary gels shows evidence for interaction and intermolecular binding. The signals of C-1, C-4–C-5, and C-6 suggested the existence of three kinds of galactomannan chains. The first one, having a low content of galactose, was strongly bound and immobile. Chains having a high content of galactose could still be detected and were divided into two groups according to their mobility. Measurements of the elastic modulus and yield stress of different carob–agarose blends indicated that the gel structure of these mixed polysaccharides is defined by a coupled network with specific junction zones.

INTRODUCTION

The results presented here are part of our overall goal of interpreting the synergistic properties of mixed gels[1]. We have earlier examined the carob galactomannan–kappa carrageenan binary gel by means of ^{13}C NMR[2] and of rheological measurements[3].

Agarose is an essentially neutral polysaccharide, widely used as a gelling agent and stabiliser for the food industry, in which the disaccharide repeating unit is → 3)-β-D-Gal*p*-(1 → 4)-3,6-anhydro-α-L-Gal*p*-(1 →. Agarose forms gels by a coil-to-double helix transition[4,5] promoted by lowering the temperature (in the range 303–313 K).

Galactomannans are polysaccharides composed of linear chains of (1 → 4)-linked β-D-mannopyranosyl residues, to which are attached (1 → 6)-linked α-D-galactopyranosyl groups as single-unit side chains. For carob galactomannan, the proportion of α-D-galactopyranosyl groups is 23%. The distribution of D-galactose has been shown to be non-regular, with a high proportion of disubstituted disaccharide units, lesser amounts of trisubstituted trisaccharide units, and absence of blocks of substitution[6].

Correspondence to: Dr. F.-R. Taravel or to Dr. C. Rochas, CERMAV, CNRS, B.P. 53X, F-38041 Grenoble, France.

0008-6215/93/$06.00 © 1993 – Elsevier Science Publishers B.V. All rights reserved

The synergistic interaction of these mixed gels is well known[7] and their mechanical properties have been often studied[8]. However, we do not know if the mixed gel consists of interpenetrating networks, phase-separated networks, or coupled networks according to the nomenclature of Cairns et al.[1].

RESULTS AND DISCUSSION

^{13}C NMR of starting polysaccharides.—Spectra for agarose showed a progressive disappearance of all ^{13}C NMR signals observed on cooling the sample below the transition temperature. At 298 K, no further signals were detected. The establishment of local ordered and rigid structure, which restricts the motion of the chains, evidently induces this disappearance. This phenomenon has already been observed for carrageenan[9] and other polysaccharides[10].

The depolymerized carob samples did not show this disappearance. The spectra obtained, when the temperature was varied from 353 to 298 K, were consistent with the random-coil behaviour of this polysaccharide. The temperature decrease had no effect on the galactose–mannose ratio (Gal–Man). Also the temperature decrease was accompanied by a small broadening (× 1.8) of Lorentzian lines which characterized the ^{13}C NMR signals, and by enhanced resolution of the ^{13}C lines corresponding to diads[11].

^{13}C NMR of agarose-partially depolymerized carob mixed gel.—The polysaccharide blends at different ratios were studied under the same conditions at both sides of the transition temperature. At 353 K, at any ratio, both signals for components were visible. At 298 K the agarose signals disappeared completely, while those corresponding to the depolymerized carob galactomannan were always detected. This last spectrum was compared to a spectrum of depolymerized carob sample alone obtained at the same temperature and at the same concentration.

Gelation of agarose (40 mg/mL) mixed with carob (30 mg/mL) induced an intensity decrease of the signals relative to those of the carob alone. To quantify this disappearance, the spectra were recorded under conditions of quantitative analysis and in the absolute intensity mode. Comparison of the C-1 carob signals in the sample of carob alone with the C-1 carob signals in the mixture indicated that gelation of agarose led to the disappearance of 25% of the C-1 carob signals. This decrease was observed for all the carbon signals. A similar study involving a κ-carrageenan–carob mixed gel[2] at a similar ratio, led to a decrease of 40%. For an agarose concentration of 80 mg/mL, in the presence of carob (30 mg/mL), this signal disappearance was more pronounced (55%). This result showed that an almost stoichiometric relationship between agarose concentration and "missing" galactomannan signals could be established. However, for a lower agarose concentration, the signal disappearance was difficult to quantify precisely, the phenomenon being hidden by the high concentration of unperturbed galactomannan chains. Secondly, different local changes and a broadening of all the remaining ^{13}C signals appeared, independent of the agarose–carob ratio.

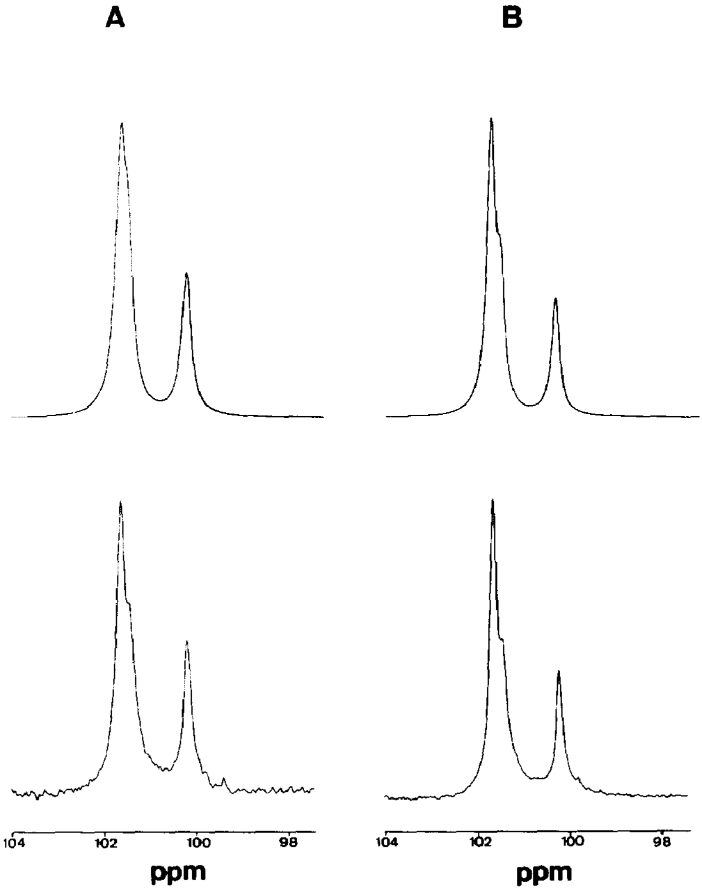

Fig. 1. Reconstructed (top) and experimental (bottom) C-1 signals at 298 K for: A, agarose (40 mg/mL)-depolymerized carob (30 mg/mL) blend in D_2O; B, depolymerized carob gum.

Taking into account the Gal–Man ratio and the linewidth, a reconstitution of the C-1, C-4–C-5, and C-6 signals was undertaken for the experimental spectra of both the carob–agarose mixture and the carob alone at 298 K. Figs. 1, 2, and 3 show the reconstruction of all carbon signals for a 4:3 agarose–carob mixture. For the signals of carob alone, the best fit was reached for a Gal–Man ratio of 23:77 and a linewidth of ~ 20 Hz for C-1, C-4, and C-5, 10 Hz for C-6 of the galactose unit, and 25 Hz for C-6 of unsubstituted mannose unit.

For the reconstruction of the signals of carob–agarose mixture, the Gal–Man ratio was 27:73 and two Lorentzian lines of equal occurrence were considered: one giving a narrow peak and the second one contributing to a broadened base at the same position. The linewidth of the former was equal to the linewidth of the carob alone at the same temperature. The linewidth of the latter was two times wider than the linewidth of the former.

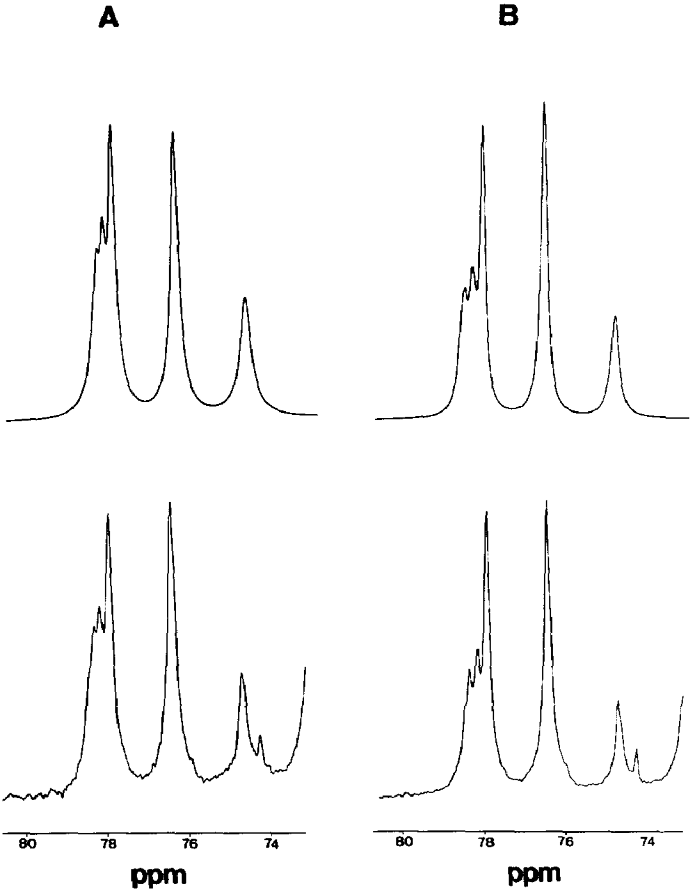

Fig. 2. Reconstructed (top) and experimental (bottom) C-4 and C-5 signals at 298 K for: A, agarose (40 mg/mL)-depolymerized carob (30 mg/mL) blend in D_2O; B, depolymerized carob gum.

Taking into account the Gal-Man ratios and the proportion of the signals that disappeared, it was possible to calculate the occurrence of unsubstituted mannose units that disappeared, and of galactose-substituted mannose units during gelation.

For the 4:3 agarose-carob mixture, these occurrences were 36 and 12% respectively, whereas they were 62 and 47% for the 8:3 agarose-carob mixture. These results supported the hypothesis of junction zones between both polysaccharides involving unsubstituted mannose residues. From these results, three different kinds of carob chains (or different regions of the same chain) may be defined: (*a*) Those corresponding to the "missing" signals. The corresponding chains become very rigid and their ^{13}C signals are broadened into the baseline. These chains (or part of the chains) have a low galactose content (Gal-Man is 11:89). Their mobility is completely lost. (*b*) and (*c*) These correspond to the remaining signals. Part (*b*) is not influenced by agarose gelation. These chains have a Gal-Man ratio of 27:73 and their mobility is not affected by the presence of the agarose network.

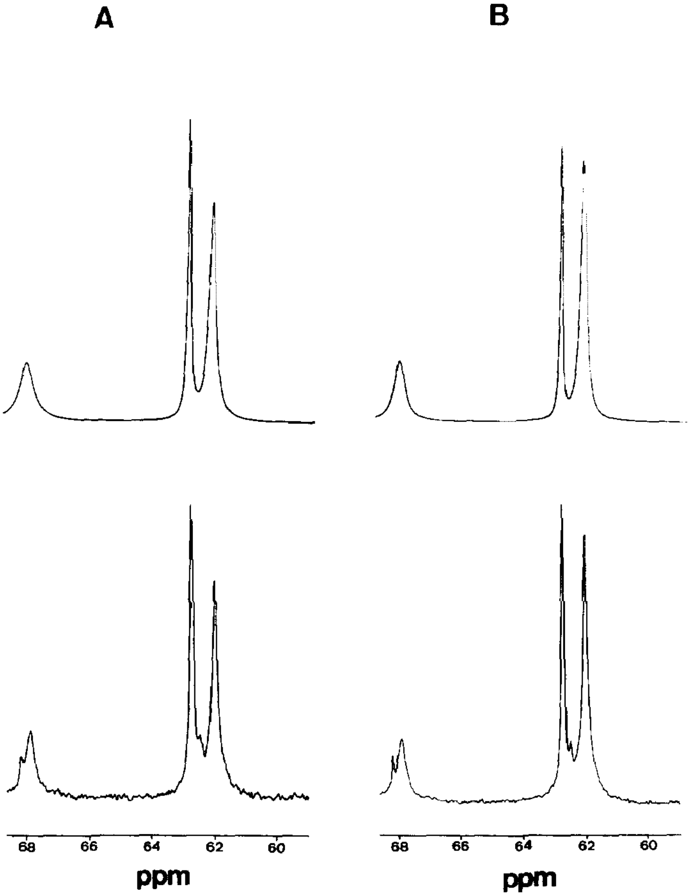

Fig. 3. Reconstructed (top) and experimental (bottom) C-6 signals at 298 K for: A, agarose (40 mg/mL)–depolymerized carob gum (30 mg/mL) blend in D_2O; B, depolymerized carob gum.

Part (c) of the chains shows a decreased mobility and a Gal–Man ratio of 27 : 73. Partial implication of unsubstituted mannose residues in the junction zones could explain this behaviour.

The occurrence of these contributions is closely related to the agarose–carob ratio of the blend. Furthermore, no significant variation (Table I) was observed for T_1 measurements of the narrow components for carob galactomannan alone and in a 4 : 3 agarose–carob mixture at 298 K. Similarly, it was not possible to distinguish the mobility of the different chains described previously: T_1 values corresponding to the broad component of the peak were not detected separately. These NMR experiments clearly show that the intermolecular binding occurring during gelation involves mainly unsubstituted D-mannose units.

Nevertheless, it was difficult to distinguish whether the resulting junction zones were between agarose and carob chains or between carob chains only, leading to an aggregation phenomenon promoted by agarose gelation.

TABLE I

^{13}C spin–lattice relaxation times of depolymerized carob galactomannan at 298 K [a]

Carbon atom [b]	T_1 (s)	
	alone	at the same concentration, but in mixture with agarose:
Man-1	0.274	0.258
Gal-1	0.305	0.300
	0.229	0.244
Man-4 [c]	0.267	0.237
	0.260	0.257
Man-5		
Gal → Man-5	0.271	0.265
Man-3	0.269	0.264
Gal-5	0.268	0.264
Man-2	0.249	0.251
Gal-3, Gal-4	0.279	0.262
Gal-2	0.256	0.257
Gal → Man-6	0.157	0.169 and 0.130
Gal-6	0.200	0.203
Man-6	0.144	0.126

[a] Measured at 75 MHz. [b] Carbon atom assignments according to their appearance from low to high field. [c] This splitting of lines is explained by assuming that the C-4 (Man) resonance is influenced by the presence or the absence of a branch on the preceding residue[11].

Mechanical properties of mixed gel as a function of galactomannan concentration at constant agarose concentration (4 g / L).—This study employed a set of samples having a constant agarose concentration (4 g/L), and the galactomannan concentration was varied from 0 to 15 g/L.

The rigidity of the gel increased and the syneresis phenomenon decreased when the concentration of galactomannan was increased. As described previously for κ-carrageenan–carob mixed gel[3], the curve of elastic modulus versus galactomannan concentration showed two zones (Fig. 4). Between 0 and 8–10 g/L, the elastic modulus was dependent on galactomannan concentration. The elastic modulus was the sum of the agarose modulus and of a complex modulus. However, it was not possible to distinguish if this complex was due to galactomannan self-association or to agarose–galactomannan interactions. Above 8–10 g/L, a stabilisation zone existed. All the possible regions of the galactomannan were complexed by intermolecular binding with all of the agarose chains. Also, the agarose content in the blend might not have been sufficient to permit greater self-association of galactomannan.

A comparison of the synergistic effect was also made for galactomannans of different origins. We found that the largest effect was observed (Fig. 4) for carob gum (with a Gal–Man ratio of 23 : 77) compared to guar gum (Gal–Man = 38 : 62). This was in agreement with previous findings concerning the degree of interaction of galactose-depleted guar galactomannan samples with xanthan and other polysaccharides[12].

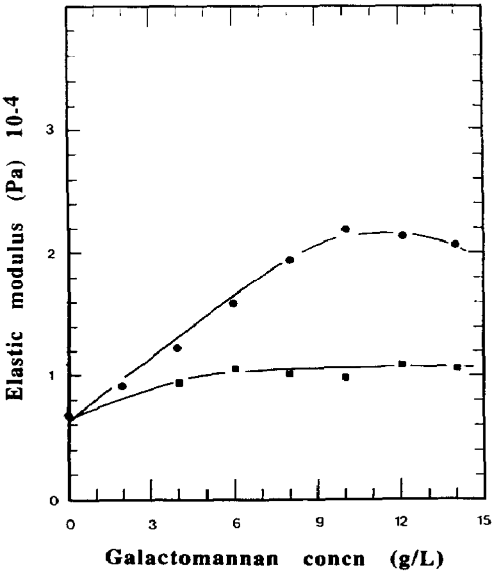

Fig. 4. Elastic modulus of agarose (4 g/L)–galactomannan blends as a function of galactomannan concentration: ●, carob gum; ■, guar gum.

The yield stress curves versus galactomannan concentration (Fig. 5) showed the same trend: an increase of the yield stress with the carob concentration. This effect is less significant for guar.

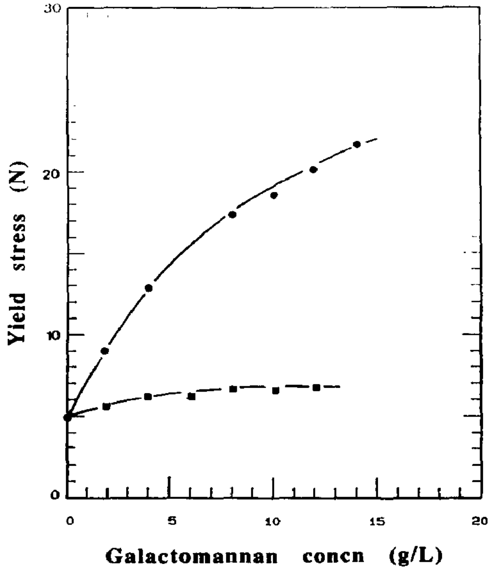

Fig. 5. Yield stress of agarose (4 g/L)–galactomannan blends as a function of galactomannan concentration: ●, carob gum; ■, guar gum.

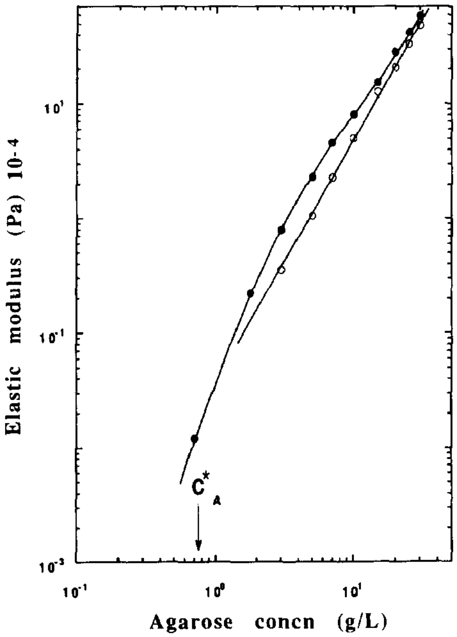

Fig. 6. Plot of elastic modulus versus agarose concentration for different conditions: ○, agarose alone; ●, agarose–carob (6 g/L) blend.

Mechanical properties of a mixed gel as a function of agarose concentration, the galactomannan concentration being constant.—The variation of the elastic modulus of the blend versus agarose concentration was studied as a function of galactomannan concentration (Fig. 6).

To understand properly the phenomena occurring, the agarose concentration was compared to C^*_A, the concentration for gelation. The actual value of C^* shown in the different figures was the concentration for which it was not possible to determine an elastic modulus with the Carri-Med rheometer (because of significant syneresis, very low modulus, etc.).

For agarose alone (Fig. 6), a gel was obtained only above C^*_A. Then the elastic moduli followed an exponential law in the form $E = kC^a$ with $a = 2.1$.

In the presence of galactomannan (6 g/L), a gel was obtained at agarose concentrations lower than C^*_A. Furthermore, the variation of elastic moduli did not follow an exponential law. It was observed that the elastic moduli of the blend tended towards the elastic modulus of agarose alone for a high agarose concentration. In the presence of 2 g/L galactomannan (results not shown) intermediate effects between the two preceding curves were obtained. These observations do not allow clear conclusion.

However, under similar conditions, with κ-carrageenan (Fig. 7) the same dependence was also observed, although with a more pronounced effect. Thus for carrageenan concentrations higher than C^*_K, the elastic moduli tended progres-

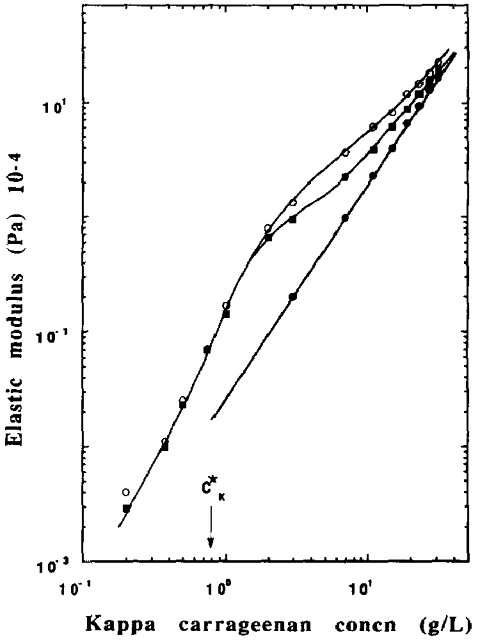

Fig. 7. Plot of elastic modulus versus κ-carrageenan concentration for different conditions: ●, κ-carrageenan alone; ■, κ-carrageenan–carob (2 g/L) blend; ○, κ-carrageenan–carob (6 g/L) blend.

sively towards the elastic modulus of carrageenan alone (this trend was even clearer for blends with 2 g/L galactomannan than for those having 6 g/L). For this range of carrageenan concentrations (above C_K^*), it was not possible to explain the synergy observed by either a galactomannan self-association or the existence of a coupled network only. Both phenomena may exist simultaneously.

Below C_K^*, the addition of galactomannan led to gelation of both binary systems and also to a strong variation of the elastic moduli. Considering the corresponding low concentration of κ-carrageenan, it was difficult to postulate how this polysaccharide was able to promote a self-association of the galactomannan chains. The fact that the elastic modulus was independent of the galactomannan concentration corroborated this assumption. Below C_K^*, the carob chains appeared as a binding agent, the number of junction zones being independent of the galactomannan concentration.

Above C_K^*, while increasing the concentration of κ-carrageenan, the gelling properties of the blend were similar to the properties of carrageenan alone. This appeared later in the presence of 6 g/L galactomannan rather than with 2 g/L. In these cases, it was not possible to reject the hypothesis of a self-association of the galactomannan chains promoted by the presence of the other polysaccharide, lowering the water activity, and stabilizing the network.

Thus, for the agarose–carob galactomannan system the same discussion involving C_A^* could be applied.

CONCLUSION

These experiments, and in particular the rheological studies of the binary gels (agarose or κ-carrageenan–carob galactomannan), show that the synergistic properties of these systems may be described mainly by a gel structure implying a coupled network defined by specific junction zones occurring via unsubstituted mannose residues of the galactomannan chains.

In addition, for high concentrations of mixed polysaccharides, a self-association of the galactomannan chains is also taking place, leading to some kind of separate network which interlace and form an interpenetrating network[1]. This phenomenon is, of course, dependent on the nature of the second polysaccharide (agarose or κ-carrageenan). In fact, if the phenomena observed for agarose and κ-carrageenan are the same, they are much more pronounced for κ-carrageenan. This could be related to the structural differences between these polysaccharides in terms of sulfate substitution, sugar conformation, and degree of polymerization.

EXPERIMENTAL

Materials.—Agarose and carob galactomannan were commercial samples obtained respectively from SOBIGEL and Elf Bio Sanofi (France). The former was extracted from *Gelidium sesquipedale* and the latter from the seeds of carob (*Ceratonia siliqua*). Galactomannan was purified before use according to methods already described[13]. The agarose sample was characterized previously[14,15]. According to Grasdalen and Painter[11], the key to successful NMR spectroscopy of galactomannan samples has proved to be an initial, limited depolymerization which decreases the viscosity of the solutions and improves their solubility. Partial acid hydrolysis[11,16], that is mild enough not to affect the structure of the polysaccharide, was used to obtain galactomannans of dp 50–60 (as estimated from ^{13}C NMR) and an intrinsic viscosity of 85 mL/g in water at 298 K (measured on a FICA automatic Ubbelhode viscometer). κ-Carrageenan samples were characterized previously[3].

NMR spectroscopy.—Samples of agarose (40 and 80 mg/mL), depolymerized carob gum (30 mg/mL) and their mixture at the same concentration, were prepared in D_2O in order to perform NMR experiments at different polysaccharide ratios. Experiments were performed at 75 MHz for ^{13}C on either an AM 300 or an AC 300 Bruker spectrometer, both equipped with a process controller, Aspect 3000 computer, and a variable temperature system.

^{13}C NMR spectra were obtained in the FT mode at several temperatures (353–298 K) with a spectral width of 7500 Hz and a resolution of 0.925 Hz per point. ^{13}C NMR was used to estimate the degree of polymerization of the partially hydrolyzed carob polysaccharide by comparing the relative intensities of the C-1 signals related to the reducing end of the chains versus the C-1 signals of all other units.

All ^{13}C NMR T_1 measurements were performed by use of the inversion-recovery method. T_1 Values were determined by use of the automatic T_1 calculation program provided by Bruker ($\pm 5\%$).

Spectrum simulation was made with the help of a band-shape simulation program available in our laboratory that can generate several Lorentzian lines, taking into account their chemical shift, intensity, and linewidth.

Rheological measurements.—Because of the amount of gum necessary for the experiments, the commercial gums were used without purification for preparation of the gels. Homogeneous gels of well defined geometry were prepared. Preparative procedures involved dispersion of each polymer in hot water, with stirring followed by mixing of the two hot polymer (95°C) solutions and introduction of the hot mixture into a cyclindrical mould (17 mm average diameter) and storage at room temperature. After 15 h the gel was cut into small cylinders (diameter, 17 mm and height, 17 mm).

For gels of high modulus (1000–200000 Pa), measurements of elastic modulus and yield stress were obtained by compression between parallel plates at room temperature on an Instron 4301 testing machine.

For gels of low modulus ($\leq$ 1300 Pa), storage moduli (G') were obtained as a function of time, at a frequency of 0.1 Hz and a temperature of 25°C on a Carri-Med rheometer. A parallel-plate geometry was used (3 cm diameter, 1 mm gap). The strain amplitude was fixed at 0.02. The hot samples (95°C) were placed onto the plate (maintained at 55°C) and then allowed to cool down to 25°C. Moduli were measured after standing for 15 h. Taking into account the relation $E = 3G'$ between the elastic modulus E and the shear modulus G', it was verified for one blend (carrageenan 2 g/L and carob 6 g/L) that the results obtained from the Instron testing machine and the Carri-Med rheometer, were the same.

ACKNOWLEDGMENTS

The authors thank Drs. M. Axelos, J.L. Doublier (INRA, Nantes) and J.M. Guenet (Université Louis Pasteur, Strasbourg) for helpful discussions.

REFERENCES

1 P. Cairns, M.J. Miles, V.J. Morris, and G.J. Brownsey, *Carbohydr. Res.*, 160 (1987) 411–423.
2 C. Rochas, F.R. Taravel, and T. Turquois, *Int. J. Biol. Macromol.*, 12 (1990) 353–358.
3 T. Turquois, C. Rochas, and F.R. Taravel, *Carbohydr. Polym.*, 17 (1992) 263–268.
4 I.C.M. Dea, A.A. McKinnon, and D.A. Rees, *J. Mol. Biol.*, 68 (1972) 153–172.
5 E.R. Morris and I.T. Norton, *Stud. Phys. Theor. Chem.*, 26 (1983) 549–593.
6 B.V. McCleary, A.H. Clark, I.C.M. Dea, and D.A. Rees, *Carbohydr. Res.*, 139 (1985) 237–260.
7 E.R. Morris, in P. Harris (Ed.), *Food Gels*, Elsevier Applied Science, London, 1990, pp 291–359.
8 M. Tako and S. Nakamura, *Agric. Biol. Chem.*, 52 (1988) 1071–1072.
9 C. Rochas, M. Rinaudo, and M. Vincendon, *Biopolymers*, 19 (1980) 2165–2175.
10 E.R. Morris, D.A. Rees, G. Young, M.D. Walkhinshaw, and A. Darke, *J. Mol. Biol.*, 110 (1977) 1–16.

11 H. Grasdalen and T. Painter, *Carbohydr. Res.*, 81 (1980) 59–66.
12 B.V. McCleary, I.C.M. Dea, J. Windust, and D. Cooke, *Carbohydr. Polym.*, 4 (1984) 253–270.
13 B.V. McCleary, E. Nurthen, F.R. Taravel, and J.P. Joseleau, *Carbohydr. Res.*, 118 (1983) 91–109.
14 F.M. Nicolaisen, I. Meyland, and K. Schaumburg, *Acta Chem. Scand., Ser. B*, 34 (1980) 579–583.
15 C. Rochas, M. Lahaye, W. Yaphe, and M.T. PhanViet, *Carbohydr. Res.*, 148 (1986) 199–207.
16 S.M. Bociek, M.J. Izzard, A. Morrison, and D. Welti, *Carbohydr. Res.*, 93 (1981) 279–283.