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Gel-Permeation Studies on Xanthan–Galactomannan Interactions

Norman W. H. Cheetham,* Barry V. McCleary,† Grace Teng,* Felicia Lum* and Maryanto*

*School of Chemistry, The University of New South Wales, PO Box 1, Kensington 2033, Sydney, Australia

†Biological & Chemical Research Institute, NSW Department of Agriculture, Rydalmere 2116, Australia

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SUMMARY

Interactions between samples of locust bean gum (LBG) and xanthan gum have been observed directly by means of gel-permeation chromatography. The degree of interaction depends on the molecular weight of the LBG sample.

The relative amounts of each polysaccharide type in the complex have been determined. Those LBG chains least substituted with galactose bind preferentially to the xanthan.

INTRODUCTION

The interaction between xanthan gum, the extracellular polysaccharide formed by *Xanthomonas campestris*, and certain D-galacto-D-mannans has been well documented (Dea *et al.*, 1977; McCleary, 1979; Rees *et al.*, 1982). Gel formation may occur at total polysaccharide concentrations as low as 0·2% in water (McCleary, 1979), though the capacity of the galactomannans to participate in the associations is dependent on primary structure. The β-1-4-linked D-mannan

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chains sparingly substituted at C_6 with α-D-galactosyl units show evidence of more extensive interaction than those more highly substituted. Thus locust bean gum (LBG) (23% galactose) interacts strongly, while guar (38%) interacts little. There appears to be a specific requirement for unsubstituted sequences or faces in the galactomannan (McCleary, 1979; Rees *et al.*, 1982). Enzymic studies have shown that galactomannans which contain a high proportion of regular alternating structure (leading to one unsubstituted and one fully substituted face) show stronger interactions with xanthan than do others with a less regular substitution, but with comparable galactose content (McCleary, 1979).

The interactions to form gels have been studied by various means. Optical rotation (Dea *et al.*, 1977) tends not to be reproducible because of gel strain. Viscosity measurements can be useful, but in some cases the viscosities are not directly related to the amount of galactomannan present (McCleary, 1979). The 'gel-islands' formed in the presence of salt (Dea *et al.*, 1977; McCleary, 1979) can be isolated by high-speed centrifugation, and the non-gelling polysaccharide may be recovered by washing the gels with 0·5 M potassium chloride solution. This technique was used to study gel formation between xanthan and a number of galactomannans, over a range of concentrations. In the case of xanthan and LBG, the percentage of carbohydrate recovered as a gel ranged from 54% (xanthan/LBG ratio of 2:5) to 90% (xanthan/LBG ratio of 5:2). Rheological studies of gels or solutions, e.g. dynamic viscosity and yield stress, provide valuable information about intermolecular networks (Rees *et al.*, 1982).

The chain length of the galactomannan is critical for the formation of a three-dimensional gel network. In order to study the interactions more closely, it was decided to employ LBG samples which had been partially depolymerized by the action of a β-mannanase. It was hoped that, at concentrations which would result in gel formation by the native polysaccharide, no gel would form, and thus permit study of the interaction by optical rotation and gel-permeation methods. Optical rotation and GPC studies using xanthan depolymerized by ultrasonication are being employed to extend the work, and will be reported elsewhere. Depolymerization by ultrasonication appears to affect the molecular weight only (Paradossi & Brant, 1982) leaving the primary structure virtually intact, and thus should provide a valid model for interaction studies.

EXPERIMENTAL

Materials

Xanthan (Ketrol) samples were obtained from Kelco Division of Merck & Co. Inc., USA. Cell debris and protein were removed by the method of Holzwarth (1976), which leaves the xanthan in the sodium form. The nitrogen content was lowered from 2% to 0·8% by this treatment. The hot-water soluble fraction of locust bean gum was extracted from the commercial locust bean flour 'indal' (industries de Alfarrova, Portugal) as described previously (McCleary *et al.*, 1983). Aliquots (150 ml) of galactomannan solution (1·0% w/v) in 0·1 M sodium acetate buffer (pH 4·5) were incubated with β-D-mannanase (0·20 nKat) (where a Katal is the basic unit of enzyme catalytic activity) at 40°C for periods up to 2 h. Reaction was terminated at appropriate times by incubation at 100°C for 10 min and the solution was added to two volumes of ethanol to precipitate the polysaccharide. This material was then washed with ethanol and acetone and dried *in vacuo*. Six fractions, A–F, of decreasing molecular weight were obtained. Fraction A was the original, undegraded hot-water soluble fraction.

HPLC analyses

The HPLC system consisted of an M6000 pump (Waters), a 7125 injector (Rheodyne), and an ERC-7510 refractive index detector (ERMA Optical Works Ltd, Tokyo). The molecular weight of each LBG fraction was determined by high-performance gel-permeation chromatography. A 1% w/v solution in 0·5% NaCl and 0·02% aqueous sodium azide was used, with a Shodex Ionpak KS-806 column, 500×8 mm, run at a flowrate of 0·7 ml min^{-1}. Shodex pullulan standards p-82 (Showa Denko K.K., Tokyo), for aqueous gel permeation, were used to calibrate the columns. Samples A–F had the following molecular weights, respectively: 350, 295, 270, 180, 144 and 95×10^3. They were found to have the following mannose/galactose ratios (determined by GLC), respectively: 5·49, 5·2, 5·1, 5·3, 5·2 and 5·6. For interaction studies, a TSK-Gel G6000 PW column (Toyo Soda Co. Ltd, Tokyo), $600 \times 7{\cdot}5$ mm, was used, at a flowrate of 0·6 ml min^{-1}, with water as solvent. Samples of xanthan were dissolved by wetting them

with ethanol, stirring in water overnight, and (a) autoclaving at 120°C/20 min or (b) stirring at 90°C/30 min. LBG was dissolved similarly to (b), but sonication (Sonicor sonifier, microprobe; Sonicor Instrument Corp., Copiague, New York) for 30 min was added before the stirring step.

All samples were filtered through a Millipore membrane (1·2 μm). Total carbohydrate in the filtered samples was determined by the phenol–sulphuric acid method (Dubois *et al.*, 1956).

Where necessary, solutions of xanthan and LBG in water were mixed thoroughly and injected into the TSK column.

Gas chromatographic analyses

Collected fractions were analysed for mannose, glucose and galactose as follows: polysaccharide (1–2 mg) was heated in 2M trifluoroacetic acid (0·5 ml) for 90 min at 120°C in a sealed tube. The acid was removed by warming in a stream of nitrogen and aldononitrile acetates were prepared according to Turner & Cherniak (1981). The aldononitrile acetates were preferred to the alditol acetates, as during the borohydride reduction step of the latter some of the glucuronic acid, present as its lactone, is reduced to glucitol (Lehrfeld, 1981). Gas chromatography was carried out on a Shimadzu GC-9A, fitted with a 3390-A integrator (Hewlett-Packard) — column, fused silica capillary (25 M), of OV1701; temperature programme, 200°C for 10 min, 2°C min^{-1} for 10 min, hold at 220°C for 20 min.

RESULTS AND DISCUSSION

Gel-permeation behaviour of xanthan

With water as the HPLC solvent, the GPC behaviour of purified xanthan gum (between 0·1 and 1% concentration) depends on the method used to dissolve it, and on storage time. When autoclaved, aggregates are dispersed and a profile such as that in Fig. 1(a) results. On standing in a refrigerator overnight, *or* on dissolution by the heat/stir method, the profile is essentially as shown in Fig. 1(d). The disaggregated xanthan emerges close to the void volume of the column

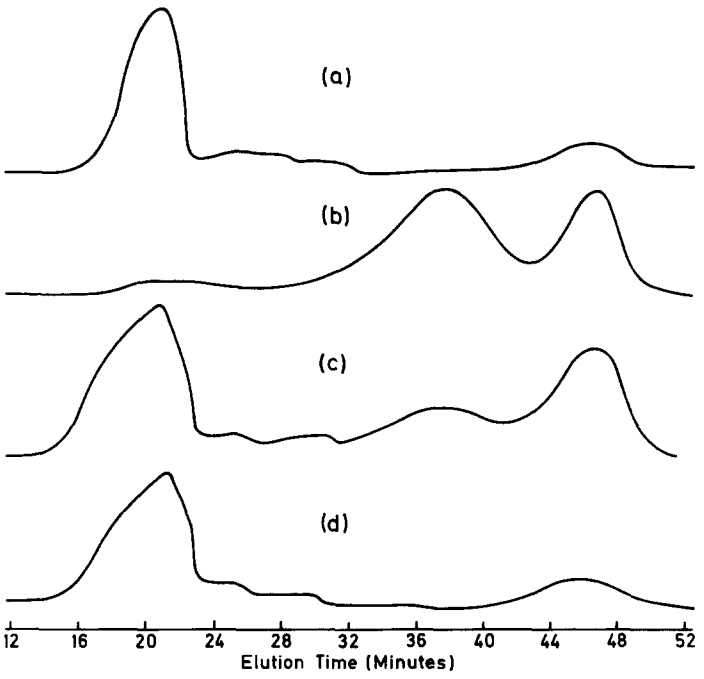

Fig. 1. Gel permeation chromatography profiles of: (a) Xanthan (1% w/v) dissolved in water by autoclaving; (b) locust bean gum fraction E (1% w/v); (c) a 1 : 1 mixture of (a) and (b); (d) a sample of (a) after refrigeration overnight.

(≈ 11 ml, Fig. 1(a)). However, solutions stored at 4°C overnight contain some aggregated material which elutes even closer to the void volume, shown in Fig. 1(d) as a hump on the leading edge of the peak. The column exclusion limit prevents resolution of the disaggregated and the aggregated fractions. The presence of xanthan aggregates (microgels) and their effect on the apparent molecular weight has been demonstrated by Morris *et al.* (1983). As far as the interaction with LBG and formation of a complex is concerned, as indicated by decrease in the size of the LBG peak and by monosaccharide analysis, there does not appear to be any significant difference between xanthan in true solution and that containing aggregates. For the interaction studies here reported, the autoclave method of dissolving was used, as the heat/stir method yielded aggregates, which were difficult to filter. However it has been shown (Cheetham & Punruckvong, 1985) that

autoclave conditions, especially in water alone, remove some of the pyruvate from xanthan. Thus, in the interpretation of the results obtained using autoclaved xanthan, this point should be taken into consideration.

As xanthan is a polyelectrolyte, when dissolved in pure water at relatively low concentrations, it experiences polyelectrolyte swelling. Its GPC peak has the elongated leading front and sharp trailing edge characteristic of such solutes (Belenkii & Vilenchik, 1983). This is just observable at 1% concentration (Fig. 1(a)). The GPC behaviour of xanthan in salt solutions is quite different. The molecular size decreases substantially, typical of most polyelectrolytes. This may also reflect the formation of the ordered conformation which has been proposed for xanthan in salt solutions. The shape of the GPC peak in salt solution is more symmetrical. More detailed discussions on the GPC behaviour of xanthan in salt solutions will be reported elsewhere.

Gel-permeation behaviour of locust bean gum

The LBG samples used here have elution times ranging from A(32 min, M_W 350×10^3) to F (38 min, M_W 95×10^3) at a flowrate of 0·6 ml min^{-1}. The peak at 46 min (total permeation) consists of buffer salts, etc. Some LBG samples also show evidence of aggregation on standing (peaks appear near V_0). As xanthan and LBG can both aggregate on standing, experiments involving mixtures of the two polymers were performed on the same day as the polymers were dissolved.

Mixtures of xanthan and LBG

Xanthan and LBG-A

A mixture of equal volumes of xanthan (0·1%) and LBG-A(0·1%) was heated to 60°C and then allowed to cool to room temperature. It was more viscous than either polysaccharide alone, but did not gel. With care, it could be drawn into a syringe, and injected into the HPLC column. Most of the peak (peak 2, Table 1) due to LBG disappeared, and a large hump at the void column formed. Increasing the ratio of xanthan to LBG to 2:1 resulted in a complete removal of the LBG peak. Total recovery of carbohydrate after mixing determined by the method of Dubois *et al.* (1956) was in the range 85–95% of that

TABLE 1
Molar Ratios (Determined by GLC of Peracetylated Aldononitriles) of Mannose, Glucose and Galactose in GPC Peaks of Various Xanthan/LBG Combinations

Sample	*Man:Glu:Gal*		*X:LBG in peak 1*[c]
	Peak 1[a]	*Peak 2*[b]	
Xanthan (X)	1·04:1:0		
1:1 X+LBG-A		Too small to analyse	
2:1 X+LBG-A		None	
1:2 X+LBG-A		Too small to analyse	
LBG-E		5·2:0:1	
1:1 X+LBG-E	11·9:4·9:1	3·9:0:1 (LBG-E_2)	1·25:1
2:1	12·0:6·3:1	4·2:0:1	2·3:1
LBG-F		5·6:0:1	
1:1 X+LBG-F	11·5:4·86:1	4·6:0:1	1·6:1

[a] Elutes at V_0. Includes X/LBG complex, plus any excess xanthan.
[b] Unbound LBG.
[c] w/w, from peak 1 values, assuming Man/Glu in xanthan = 1·04, and that Man:Glu:glucuronic acid is 2:2:1.

injected. When the ratio xanthan/LBG was 1:2, a slightly larger peak 2 remained, compared with that for the 1:1 ratio of xanthan/LBG. For a 1:3 xanthan/LBG ratio, the increase in LBG peak size over that for the 1:2 ratio was close to that expected by the presence of the additional LBG. It was thus assumed that at the 1:2 level virtually all xanthan binding sites for LBG had been saturated. The ratio of xanthan to locust bean galactomannan in this complex was thus ~1:2 (w/w). As the molecular weight of LBG-A is $3{\cdot}50 \times 10^5$, and if that of disaggregated xanthan is taken as $2{\cdot}5 \times 10^6$, then approximately 14 molecules of LBG-A bind to a molecule of xanthan. Molecular weights of non-aggregated xanthan have been reported from $\sim 2 \times 10^6$ (Dintzis *et al.*, 1970; Rinaudo & Milas, 1978) to $3{\cdot}6 \times 10^6$ (Milas & Rinaudo, 1979). Agreement on an M_W of about 2×10^6 Daltons seems to be emerging (Launay *et al.*, 1985). Tako *et al.* (1984) found that for an LBG sample of M_W 263×10^3 a w/w ratio of xanthan/LBG of 1:2 formed a gel of maximum dynamic modulus at a total polysaccharide concentration of 0·2%. They assumed that the junction sites on each polysaccharide were essentially saturated, and accordingly that, in the

sample studied, there were about twice the number of junction sites available on the xanthan relative to those on the LBG. However they did not allow for the molecular weight of each polysaccharide, and did not calculate the molar ratios in which xanthan and LBG combined. The methods used by Tako *et al.* could not distinguish between bound and unbound polysaccharide. The present GPC method allows one to do so, though only at concentration levels where gelation does not cause sample injection problems.

Xanthan plus LBG-E

LBG-E has a M_W of 144×10^3 and a Man/Gal ratio of 5·2:1. LBG-E and xanthan, both at 1% w/v, could be mixed without gelation occurring. A 1:1 mixture of such solutions had the profile shown in Fig. 1(c). The peak material emerging was collected, freeze-dried and hydrolysed with 2 M trifluoroacetic acid. The peracetylated aldononitriles were analysed by gas chromatography. Analysis of the molar ratios of monosaccharides in the combined xanthan/LBG peak allowed the ratio of xanthan to LBG in the complex to be determined (Table 1). Approximately 44% of the LBG-E peak area remained, and the hump on the leading edge of the first peak, indicating the presence of the xanthan/LBG complex, was typical of these experiments. In this experiment, in which a 1:1 mixture of xanthan/LBG was injected, the xanthan/LBG ratio in peak 1 was 1·25:1. Calculations based on this yield a value of 20% for LBG-E unbound (cf. 44% estimated by residual peak area). A 2:1 initial mixture gave a 2·3:1 ratio of xanthan/LBG-E in peak 1. This corresponds to only 13% of the LBG-E remaining unbound. However, the size of the residual LBG-E peak on the chromatogram was only slightly less than that for the 1:1 ratio. Thus in both cases estimation of the amount of LBG-E unbound by using peak areas is at variance with estimations using sugar ratios. We believe the most useful (and accurate) measurements to characterize peaks 1 and 2 is that of the sugar ratios. Measurement of the peak 2 areas in an analytical run, as in Fig. 1, led to larger residual areas than expected by sugar ratios. Part of the discrepancy lies in the fact that, when peak material was being collected for analysis, larger sample volumes were used. These gave chromatograms with much larger (and closer to overlapping) peaks than those in Fig. 1. To avoid problems of overlapping material in such peaks, a region between peaks 1 and 2 was not collected. It is probable that some kind of equilibrium is being

set up in the column, and that the composition of this inter-peak region, which included some of the trailing edge of peak 1 and leading edge to peak 2, may be of some significance. Refinements of the technique are in progress. The Man/Gal ratios in the residual LBG (peak 2, Table 1) are lower than those in the original LBG. This indicates that LBG less substituted by D-galactosyl residues is being preferentially bound to xanthan, consistent with previous reports on xanthan/galactomannan interactions. The monosaccharide ratios of the carbohydrate material in peak 1 are consistent with this (Table 1). Thus for peak 1 in the chromatogram of the sample mixture xanthan/LBG-E of 2:1 (Man/Glu/Gal ratio of 12·0:6·3:1), the relative amount of glucose and mannose from xanthan is the same (Man/Glu = 1·04 in xanthan). The remaining mannose (12·0 − 6·3 = 5·7) arises from the bound LBG, giving this a Man/Gal ratio of 5·7, a higher value than that (5·2) in the original LBG-E.

Xanthan plus LBG-F

LBG-F has a M_W of 95×10^3 and a Man/Gal ratio of 5·6:1. With this it was also possible to mix 1% solutions with 1% xanthan without gelation occurring. The elution profile for a 1:1 mixture of xanthan (1%) and LBG-F (1%) showed that approximately 55% of the LBG-F peak area remained, implying that this percentage remained unbound. Analysis of peak 1, however (Table 1), gives a ratio of xanthan to LBG-F of 1·6:1, which is equivalent to 37% LBG-F unbound. Again the GPC peak size is misleading. Increasing the xanthan/galactomannan ratio of 2:1 reduced the LBG-F area to 42% of the original. Further increase in xanthan apparently removed no more LBG-F as judged by peak areas of an analytical run. However, sugar ratios for the 2:1 and 3:1 cases were not determined. Further, and consistent with the results obtained for LBG-E, the Man/Gal ratio of the LBG-F subfraction in peak 2 is lower, and that in peak 1 is higher, than in the original sample (Table 1).

Xanthan plus unbound LBG-E

The results above indicate that the larger the molecular weight of LBG, the more is bound to xanthan, though the material bound in all cases is held firmly enough to form complexes separable by GPC. Enzymic depolymerization of LBG must reduce the size of regions on LBG chains which are suitable for interaction with xanthan. The

TABLE 2
Molar Ratios of Mannose, Glucose and Galactose in GPC Peaks 1 and 2 Arising from Mixtures of Xanthan and Unbound LBG-E_2

Sample	*Man:Glu:Gal*	
	Peak 1	*Peak 2*
1:1 X + LBG-E_2[a]	0·86:1:0	4·01:0:1

[a] Unbound LBG-E (Peak 2) isolated by GPC from an X + LBG-E (1:1) mixture.

unbound fragments have lower Man/Gal ratios than the original LBG. However, based on prior knowledge of the Man/Gal ratios required for interaction of galactomannan with xanthan, it might be expected that interaction would occur.

To further investigate this point, unbound LBG-E_2 (peak 2, Table 1) was collected, freeze dried, and remixed with fresh xanthan solution. Gel permeation peak areas showed that virtually none of the LBG-E_2 bound to the xanthan. After collection of fractions and hydrolysis, GLC showed that there was virtually no galactose in peak 1 of the 1:1 mixture, and that the residual LBG-E_2 had a similar Man/Gal ratio (4·01:1, Table 2) to that before mixing (3·9:1, Table 1). It would be expected that LBG with such a Man/Gal ratio would bind to xanthan. Further work is under way to clarify the situation. Use of a preparative GPC column is planned to overcome the problems of overlapping peaks observed with the analytical column.

CONCLUSIONS

The results presented here demonstrate the formation of a xanthan/LBG complex which can be studied by gel-permeation chromatography. A disadvantage is that the complex cannot be separated from excess xanthan, as the size exclusion limit of the column is not sufficiently large. It has been demonstrated that LBG samples as small as M_W 95×10^3 can enter into complex formation, but to a lesser extent than higher M_W LBG. There is evidence that the xanthan preferen-

tially complexes with those LBG chains which have a high mannose/galactose ratio. The distribution of galactosyl units along the mannan chain may also be important. Further work to confirm this, e.g. by treatment of unbound LBG with β-mannanase, and examination of the resulting oligosaccharide patterns, is under way.

Experiments using xanthan which has been depolymerized by ultrasonication are in progress. Preliminary work indicates the formation of a complex which is partly resolved from unbound xanthan. Interaction studies employing smaller-sized xanthan and LBG samples may form the basis of a valid model system, allowing study of the interaction process, and molecular conformations adopted therein.

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REFERENCES

Belenkii, B. G. & Vilenchik, L. Z. (1983). *Modern Liquid Chromatography of Macromolecules*, Elsevier, Amsterdam, p. 287.

Cheetham, N. W. H. & Punruckvong, A. (1985). *Carbohydr. Polym.* **5**, 399.

Dea, I. C. M., Morris, E. R., Rees, D. A., Welsh, E. J., Barnes, H. A. & Price, J. (1977). *Carbohydr. Res.* **57**, 249.

Dintzis, F. R., Babcock, G. E. & Tobin, R. (1970). *Carbohydr. Res.* **13**, 257.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *J. Amer. Chem. Soc.* **28**, 350.

Holzwarth, G. (1976). *Biochemistry* **15**, 4333.

Launay, B., Guvelier, G. & Martinez-Reyes, S. (1985). In *Gums & Stabilizers for the Food Industry 2*, eds G. O. Phillips, D. J. Wedlock & P. A. Williams, Pergamon, Oxford, p. 79.

Lehrfeld, J. (1981). *Anal. Biochem.* **115**, 410.

McCleary, B. V. (1979). *Carbohydr. Res.* **71**, 205.

McCleary, B. V., Nurthen, E., Taravel, F. R. & Joseleau, N.-P. (1983). *Carbohydr. Res.* **118**, 91.

Milas, M. & Rinaudo, M. (1979). *Carbohydr. Res.* **76**, 189.

Morris, E. R., Rees, D. A., Young, G., Walkinshaw, M. D. & Darke, A. (1977). *J. Mol. Biol.* **110**, 1.

Morris, V. J., Franklin, D. & I'Anson, K. (1983). *Carbohydr. Res.* **121**, 13.
Paradossi, G. & Brant, D. A. (1982). *Macromolecules* **15**, 874.
Rees, D. A., Morris, E. R., Thom, D. & Madden, J. K. (1982). In *The Polysaccharides*, Vol. 1, ed. G. O. Aspinall, p. 195.
Rinaudo, M. & Milas, M. (1978). *Biopolymers* **17**, 2663.
Tako, M., Asato, A. & Nakamura, S. (1984). *Agric. Biol. Chem.* **48**, 2995.
Turner, S. H. & Cherniak, R. (1981). *Carbohydr. Res.* **95**, 137.