ELSEVIER

PII: S0144-8617(96)00055-0

Carbohydrate Polymers **30** (1996) 31–35

0144-8617/96/$15.00 + .00

A comparison between reductive-cleavage and standard methylation analysis for determining structural features of galactomannans

Karen Kiwitt-Haschemie, Anja Renger & Hans Steinhart*

Institut für Biochemie und Lebensmittelchemie der Universität Hamburg Grindelallee, 117 20146 Hamburg, Germany

(Received 18 August 1995; revised version received 2 January 1996; accepted 13 April 1996)

Reductive cleavage with triethylsilane and either a mixture of trimethylsilyl methanesulfonate and boron trifluoride etherate or trimethylsilyl trifluoromethane-sulfonate as catalysts resulted for all the galactomannans (guar, carob and tara gum) in four partially methylated 1,5-anhydroalditol acetates of mannose and galactose as main products. In addition, the latter catalyst yielded small amounts of two further partially methylated 1,4-anhydroalditol acetates. The results accord the accepted structure for galactomannans i.e a backbone of 1,4-linked β-D-mannopyranose units with single α-D-galactopyranosyl residues substituted at C-6. Therefore, a mixture of trimethylsilyl methanesulfonate and boron trifluoride etherate as catalysts combined with a subsequent *in situ* acetylation is the preferred method for analysing structural features of galactomannans. The advantage of reductive cleavage in comparison to standard methylation analysis lies particularly in the fact that the time consuming optimisation of the hydrolysis step is avoided. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Methylation analysis (Lindberg *et al.*, 1973) is a widely used method for determining polysaccharide structure. However, according to Rolf and Gray (1982) reductive depolymerisation has several advantages which become particularly clear when the aim is the structural characterisation of complex carbohydrates which have different acid-labile groups or sugar residues. In particular reductive depolymerisation is an effective method for the structural characterisation of polysaccharides which contain acid-labile pentosyl residues (Heims *et al.*, 1989; Heims & Steinhart, 1991). Its application to agarose, which contains extremely acid-labile 3,6-anhydro-galactose, has recently been described (Kiwitt-Haschemie *et al.*, 1993). Other authors have described the application to polysaccharides with acyl substitutes (Mischnick, 1990; Sherman & Gray, 1992) or acetalic bound pyruvate (Fontaine *et al.*, 1991; Zeller & Gray, 1991).

The galactomannans of guar, carob and tara show the same basic structure: a linear mannan main chain of β-(1,4)-linked D-mannopyranosyl residues, on which D-galactopyranosyl residues are linked through a α-(1,6) glycosidic bond. The degree of galactose substitution depends on the galactomannan type (Whistler & BeMiller, 1973; Nittner, 1980). The higher lability of the 6-glycosidic linked galactopyranosyl residues in comparison to β-glycosidic linked main mannan chains often demands a time consuming optimisation of the hydrolysis conditions (Schuster, 1983) and therefore also of the hydrolytic step within the standard methylation analysis. With the aim of avoiding this optimisation step in the future, in this study the applicability of reductive depolymerisation to structurally characterise galactomannans is examined and the results compared with those from standard methylation analysis.

*Corresponding author.

EXPERIMENTAL

Highly purified galactomannans (gum content 99%) of guar, carob and tara were obtained from the SENN company (Switzerland). All further chemicals utilized were of the highest possible purity.

Methylation

Methylation of the galactomannans was performed using a slight modification of the method of Ciucanu and Kerek (1984).

Reductive cleavage

Reductive depolymerisation and acetylation of the permethylated galactomannans was performed using the process described by Jun and Gray (1987) and Kiwitt-Haschemie *et al.* (1993). Reductive depolymerisation was performed in the presence of triethylsilane (TES) (5 equivalent/glycosidic bond) and trimethylsilyl trifluoromethanesulfonate (TMS-O-triflate) (5 equivalent/glycosidic bond) or a mixture of trimethylsilyl methanesulfonate (TMS-O-mesylate) (5 equivalent/glycosidic bond) and boron trifluoride etherate (BF_3-etherate) (1 equivalent/glycosidic bond). Acetylation to partially methylated anhydroalditol acetates was carried out *in situ* in a trimethylsilyl trifluoromethanesulfonate catalysed reaction with acetic acid anhydride (Ac_2O) and N-methylimidazole. For the reaction which was catalysed with trimethylsilyl methanesulfonate/boron trifluoride etherate, acetylation was carried out *in situ* with a mixture of acetic acid anhydride and trifluoroacetic acid (TFA) (Kiwitt-Haschemie *et al.*, 1993).

Standard methylation analysis

Partially methylated alditol acetates were produced by a 4-hour hydrolysis of the permethylated galactomannans with 2 M trifluoroacetic acid at 120°C and subsequent acetylation with acetic acid anhydride and N-methylimidazole (Lindberg, 1972).

Gas-liquid chromatography/gas-liquid chromatography-mass spectrometry (GLC/GLC-MS)

The gas chromatographic analysis was performed with a Carlo Erba 5160 gas chromatograph, equipped with a flame ionisation detector, a Shimadzu CR-3A integrator and a J & W Scientific DB-5 fused silica capillary column (0.25 mm × 30 m; film thickness, 0.25 μm). The integrated area under the peak was corrected according to the effective-carbon-response (e.c.r.) method (Sweet *et al.*, 1975; Bowie *et al.*, 1984).

Mass spectrometric identification of the derivatives was carried out with a VG 70-250 mass spectrometer and a Hewlett-Packard Model 5890 gas chromatograph, equipped with a 30 m J & W DB-5 fused silica capillary column. Determination of the relative molecular masses of the derivatives was carried out by chemical ionisation (GLC-CIMS) with ammonia as the reactant gas. Characteristic $(M + H)^+$ and $(M + NH4)^+$ ions were detected. The substitution pattern was determined using electron impulse ionisation (GLC-EIMS).

RESULTS AND DISCUSSION

Following the reductive cleavage of the permethylated galactomannans (**Ib**) with TMS-O-mesylate/BF_3-etherate, four derivatives could be identified by means of GC and/or GC-MS (Fig. 1). The derivatives identified following reductive cleavage and acetylation confirm the accepted structure for the three galactomannans (**Ia**): From the 1,4-linked D-mannopyranosyl block of the main chain, 4-O-acetyl-1,5-anhydro-2,3,6-tri-O-methyl-D-mannitol (**1**) was produced. From the branching main chain links, 4,6-di-O-acetyl-1,5-anhydro-2,3-di-O-methyl-D-mannitol (**2**) was produced, and from the terminal D-galactopyranosyl elements of the side chains, 1,5-anhydro-2,3,4,6-tetra-O-methyl-D-galactitol was obtained (**3**). From the end-position D-mannopyranosyl residues of the mannan main chain, small amounts of 1,5-anhydro-2,3,4,6-tetra-O-methyl-D-mannitol were detected (**4**). Following reductive cleavage of the permethylated galactomannans with TMS-O-triflate, in addition to the expected derivatives **1**, **2**, **3** and **4**, it was possible to identify ~2–4 mol % of two further derivatives for each of the galactomannans examined. Mass spectrometric examination showed these derivatives to be 5-O-acetyl-1,4-anhydro-2,3,6-tri-O-methyl-D-mannitol (**1a**) and 5,6-O-acetyl-1,4-anhydro-2,3-di-O-methyl-D-mannitol (**2a**) (Fig. 2).

The fact that these derivatives did not occur for the reductive cleavage reaction catalysed with TMS-O-mesylate/BF_3-etherate suggests that these derivatives are isomerisation products of the 4- and/or the 4,6-linked mannopyranosyl residues. The formation of isomerisation products from the 4-linked hexopyranosyl residues in the presence of TMS-O-triflate had already been observed by Rolf *et al.* (1983) and had been exhaustively examined (Bennek *et al.*, 1983). This ring isomerisation occurs more frequently when traces of moisture are present in the reaction solution. Bennek *et al.* (1983) were able to extensively reduce the isomerisation by the addition of solid CaH_2 to the reaction solution. In this study it was found that the amount of isomerisation products could be reduced only slightly in this way, thus the addition of an appropriate admixture was dispensed with.

The results of the reductive cleavage of the galactomannans are presented in Table 1. Carob galactomannan gave the least galactitol derivative **3**. Guar galactomannan gave the greatest amount of this derivative. The almost equimolar ratio of **3**:(**2** + **2a**) confirms that the galactose in the native galactomannans is linked via a 1,6-bond to the mannan main chain. The degree of branching therefore decreases from the guar to the carob galactomannan.

Within standard methylation analysis, permethylated galactomannans were completely hydrolysed for 4 h at 120°C with TFA. The hydrolysis conditions which were selected were optimised (data not shown). The released monomers were reduced to the corresponding alditols and acetylated for gas chromatographic analysis.

The partially methylated anhydroalditol acetates analogic acyclic derivatives were then obtained. The results are summarized in Table 2. Broadly similar

Table 1. Molar ratios (mol %) of the partially methylated anhydroalditol acetates following reductive cleavage of permethylated guar, carob and tara gums

Compound[a] (Compound number)	GUAR		TARA		CAROB	
	TMS-O-triflate	TMS-O-mesylate/ BF_3-etherate	TMS-O-triflate	TMS-O-mesylate/ BF_3-etherate	TMS-O-triflate	TMS-O-mesylate/ BF_3-etherate
t-Gal_p (3)	32.3	34.3	18.8	22.2	17.6	18.9
t-Man_p (4)	0.6	0.7	0.4	0.4	0.9	1.6
4-Man_p (1)	31.0	32.1	59.3	59.8	61.2	61.9
5-Man_f (1a)	2.1	–	4.4	–	2.0	–
4,6-Man_p (2)	31.7	32.9	15.9	17.6	17.1	18.6
5,6-Man_f (2a)	2.3	–	1.2	–	1.2	–

[a]The numbers of the compounds abbreviated indicate the position of carbons bearing acetoxy groups. In addition "p" indicates 1,5-anhydroalditol derivatives, "f" 1,4-anhydroderivatives. For identification of compound numbers see text.

results were observed to those obtained by reductive cleavage: the molar mannose : galactose ratios increased from guar to carob galactomannan.

In Table 3 the molar mannose : galactose ratios obtained by both methods are summarized. For all the galactomannans the lowest mannose : galactose ratio was obtained from hydrolysis. But the results give no hint as to whether the mannose : galactose ratios obtained via the hydrolytic method are too high due to a premature destruction of the released galactose. This is a possibility since the α-(1,6)-glycosidic bonds of the mannan main chain are split more rapidly than are the

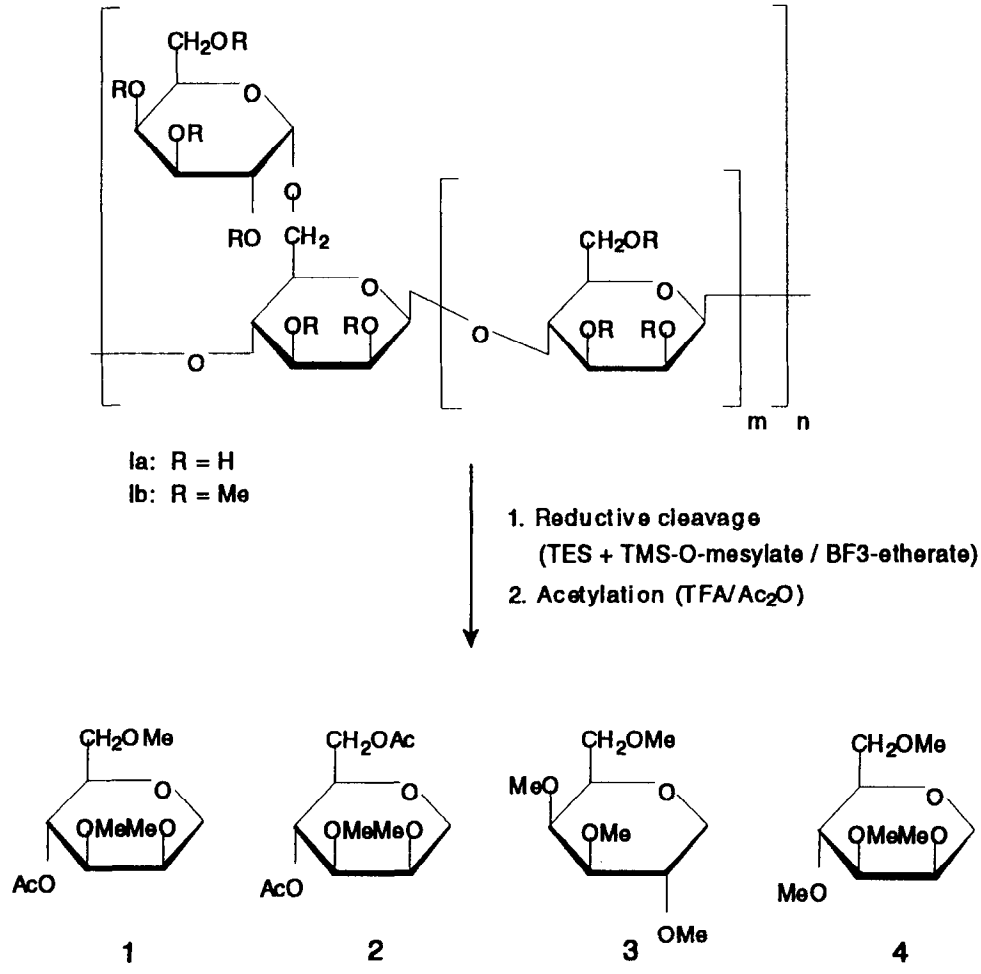
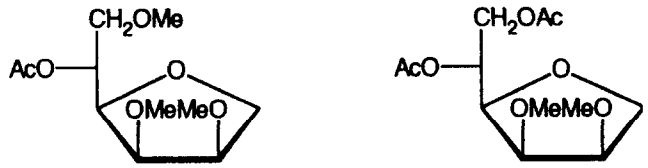


Fig. 1. Postulated structure of the galactomannans (**Ia**) and the partially methylated anhydroalditol acetates resulting from reductive cleavage of the permethylated galactomannans (**Ib**) with TMS-O-mesylate/BF_3-etherate. (For numbering of the derivatives, see text.)

Table 2. Molar ratios (mol %) of the partially methylated alditol acetates following hydrolysis of the galactomannans

Compound[a] (Compound number)	GUAR	TARA	CAROB
1,5-Gal (5)	36.4	23.6	19.5
1,5-Man (6)	1.0	0.4	0.9
1,4,5-Man (7)	26.4	50.3	60.2
1,4,5,6-Man (8)	36.2	25.7	19.4

[a]The numbers of the compounds abbreviated indicate the position of carbons bearing acetoxy groups.



1a 2a

Fig. 2. Structure of the isomerisation products from the reductive cleavage of the galactomannans with TMS-O-triflate as catalyst. (For numbering of the derivatives, see text.)

β-(1,4)-glycosidic bonds of the mannan main chain (Schuster, 1983). However, the equimolar ratio between derivatives **5** and **8** (Table 2) is not consistant with such an interpretation.

In the literature a galactose : mannose ratio of 2:1 for guar, 3:1 for tara, and 4:1 for carob has sometimes been quoted. Thus in Fig. 1 for guar m = 1, for tara m = 2, and for carob m = 3 (Whistler & BeMiller, 1973; Nittner, 1980).

For guar galactomannan, lower mannose : galactose ratios have been cited. Heyne and Whistler (1948), Hoffman and Svensson (1978) and Ahmed and Whistler (1950) determined a ratio of 1.8:1 hydrolytically. McCleary (1979), using an enzymatic process, obtained a ratio of 1.6:1. Preuss (1982) examined 21 guars using methanolysis and found a ratio of between 1.44–1.56:1. In addition, McCleary *et al.* (1985) were able to show some variation in the mannose : galactose ratios in between samples in the range 1.5-1.8:1. Whilst the mannose : galactose ratio of 1.7:1 from hydrolytic cleavage and of 1.9:1 from the reductive cleavage with TMS-O-mesylate/BF_3-etherate corresponds well with the values reported in the literature, the ratio from the reductive cleavage catalysed with TMS-O-triflate lies somewhat higher at 2:1 (see Table 3). It may reasonably be assumed that the cleavage was incomplete. The ratio is, however, not reduced when the reaction time is increased up to 16 h.

Little data is available on tara galactomannan (Pilnik *et al.*, 1980). In all cases the mannose : galactose ratio is given as 3:1. Here the results from the hydrolytic cleavage (3.2:1) and the mid-ratio from the reductive cleavage with TMS-O-mesylate/BF_3-etherate (3.5:1) agree well with the literature, whilst the reductive cleavage with TMS-O-triflate is, at 4.3:1, notably higher.

In addition, in the reductive cleavage of the permethylated carob galactomannan the molar mannose : galactose ratio following reductive cleavage with TMS-O-triflate is, at 4.7:1, which is higher when compared with that obtained by the TMS-O-mesylate/BF_3-etherate catalysed reaction. Again, the results of reductive cleavage with TMS-O-mesylate/BF_3-etherate fit better with results obtained by standard methylation analysis (4.1:1). In the literature, ratios for carob vary from 4:1 (Smith, 1948) through lower ratios of 3:1 (Preuss, 1982) and 3.4–3.5:1 (McCleary *et al.*, 1985) to higher ratios such as 4.3:1 (Lew & Gortner, 1943) or 5:1 (Hirst & Jones, 1948). These variations might be due to the anaytical method but could also reflect biological variations. Here also, the results obtained show good agreement with those reported in the literature.

It remains unclear why reductive cleavage with TMS-O-triflate produces a higher mannose : galactose ratio in all the galactomannans than does reduction with TMS-O-mesylate/BF_3-etherate. Notable is the higher amount of derivatives **3** and **2** and derivative **2a**, which represent the branching points of the mannan chain in relation to the unbranched chain links represented by the derivatives **1** and **1a**. However di- or oligosaccharides which would indicate an incomplete cleavage of branching points were not observed.

Since reductive depolymerisation with TMS-O-triflate gives isomerisation products and probably incomplete cleavage, reductive cleavage with triethylsilane and

Table 3. Molar mannose : galactose ratios (mol%) following reductive-cleavage method and standard methylation analysis of galactomannans

	Reductive-cleavage method[a]		Standard methylation analysis[b]
	TMS-O-triflate	TMS-O-mesylate/BF_3-etherate	
GUAR	2.1:1	1.9:1	1.7:1
TARA	4.3:1	3.5:1	3.2:1
CAROB	4.7:1	4.3:1	4.1:1

[a]Calculated by the molar ratios of derivatives **1**, **1a**, **2**, **2a** and **4** to derivative **3** (see table 1).
[b]Calculated by the molar ratios of derivatives **6**, **7** and **8** to derivative **5** (see table 2).

TMS-O-mesylate/BF_3-etherate as catalyst is the preferred method.

Both the results obtained from reductive cleavage and the results obtained from hydrolytic cleavage confirm the accepted structure of the galactomannans from guar, carob and tara seeds.

The results from reductive cleavage, particularly from reduction with TMS-O-mesylate/BF_3-etherate, correspond well with results of hydrolytic cleavage and literature data.

The results show that reductive depolymerisation - in particular with TMS-O-mesylate/BF_3-etherate as the catalyst – presents a good alternative to standard methylation analysis with regard to the structural characterisation of the galactomannans examined. The advantage of reductive cleavage lies particularly in the fact that the time consuming optimisation of the hydrolysis step may be dispensed with.

REFERENCES

Ahmed, Z.F. & Whistler, R.L. (1978). *J. Am. Chem. Soc.*, **72,** 2524–2525.

Baker, C.W.Whistler, R.L. (1975). *Carbohydr. Res.*, **45,** 237–243.

Bennek, J.A., Rolf, D. & Gray, G.R. (1983). *J. Carbohydr. Chem.*, **2,** 385–393.

Bowie, J.U., Trescony, P.V. & Gray, G.R. (1984). *Carbohydr. Res.*, **125,** 301–307.

Ciucanu, I. & Kerek, F. (1984). *Carbohydr. Res.*, **131,** 209–217.

Fontaine, T., Talmont, F., Dutton, G.G.S. & Fournet, B. (1991). *Anal. Biochem.*, **199,** 154–161.

Heims, H. & Steinhart, H. (1991). *Carbohydr. Polym.*, **15,** 207–214.

Heims, H., Steinhart, H. & Mischnick, P. (1989). *Carbohydr. Res.*, **191,** 343–350.

Heyne, E. & Whistler, R.L. (1948). *J. Am. Chem. Soc.*, **70,** 2249–2252.

Hirst, E.L. & Jones, J.K.N. (1948). *J. Chem. Soc.*, 1278–1282 .

Hoffman, J. & Svensson, S. (1978). *Carbohydr. Res.*, **65,** 65–71.

Jun, J.-G. & Gray, G.R. (1987). *Carbohydr. Res.*, **163,** 247–261.

Kiwitt-Haschemie, K., Heims, H., Steinhart, H. & Mischnick, P. (1993). *Carbohydr. Res.*, **248,** 267–275.

Lew, B.W. & Gortner, R.A. (1943). *Arch. Biochem.*, **1,** 325–337.

Lindberg, B. (1972). *Methods Enzym.*, **28,** 178–195.

Lindberg, B., Lönngren, J. & Thompson, J.L. (1973). *Carbohydr. Res.*, **28,** 351–357.

McCleary, B.V. (1979). *Carbohydr. Res.*, **71,** 205–230.

McCleary, B.V., Clark, A.H., Dea, I.C.M. & Rees, D.A. (1985). *Carbohydr. Res.*, **139,** 237–260.

Mischnick, P. (1990). *Minutes of the fifth intern. Symp. on Cyclodextrines*, Editions de Santé .

Neukon, U. & Pilnik, W. (1980). *Gelier- und Verdickungsmittel in Lebensmitteln*, Forster Verlag, Zurich.

Nittner, E. (1980). In *Gelier- und Verdickungsmittel*, eds. H. Neukom & W. Pilnik, Forster Verlag, Zürich.

Pilnik, W., Voragen, F., Neukom, H. & Nittner, E. (1980). In: *Ullmanns Encyclopädie der Technischen Chemie*, 4. Aufl., Bd. 4, Verlag Chemie, Weinheim .

Preuss, A. (1982), Dissertation, University of Münster (Germany) .

Rolf, D. & Gray, G.R. (1982). *J. Am. Chem. Soc.*, **104,** 3539–3541.

Rolf, D., Bennek, J.A. & Gray, G.R. (1983). *J. Carbohydr. Chem.*, **2,** 373–383.

Schuster, P.J. (1983) Dissertation, University of Münster (Germany) .

Sherman, J.S. & Gray, G.R. (1992). *Carbohydr. Res.*, **231,** 221–235.

Smith, F. (1948). *J. Am. Chem. Soc.*, **70,** 3249–3253.

Sweet, D.P., Shapiro, R.H. & Albersheim, P. (1975). *Carbohydr. Res.*, **40,** 217–225.

Whistler, R.L. & BeMiller, J.N. (eds) (1973). *Industrial Gums*, Academic Press, New York .

Zeller, S.G. & Gray, G.R. (1991). *Carbohydr. Res.*, **211,** 309–316.