

Structure of a galactoarabinan-rich pectic polysaccharide of native and fermented blackgram (*Phaseolus mungo*)

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Two homogeneous galactoarabinans containing ~15% D-galacturonic acid were isolated and characterized from native and fermented blackgram. Linkage analysis indicated highly branched structures consisting of a (1→5)-linked L-arabinan backbone additionally being substituted with L-arabinofuranose, D-galactopyranose, D-galactopyranosyluronic acid and L-rhamnopyranose residues; a structure reminiscent of pectic polysaccharides. Analysis of carboxyl-reduced polysaccharides showed that the galacturonic acid residues are all essentially (1→4)-linked. The fermented polysaccharide had a relatively higher degree of branching which was manifested in its overall higher viscosity and as a result better product making qualities. The uronic acid carboxyls were at least partly involved in maintaining the solution viscosity and functional property attributes, because the carboxyl-reduced polysaccharide was devoid of both these characteristics.

INTRODUCTION

Blackgram (*Phaseolus mungo*) is a pulse traditionally used in the preparation of South Indian breakfast foods, such as *idli*, which is relished for its soft and spongy texture (Susheelamma & Rao, 1979). The components responsible for these properties are the surface-active proteins that generate a foam and as a result impart a porous structure to the food, and the viscogenic mucilaginous polysaccharide (~6%) that stabilizes the porous structure against thermal disruption during steaming. The overall carbohydrate composition (Ramadas Bhat & Tharanathan, 1986a) and the structure-function characteristics of the total polysaccharides of blackgram have been reported (Ramadas Bhat *et al.*, 1987). During fermentation of blackgram, for the preparation of leavened foods, it was found that the mucilaginous polysaccharide undergoes compositional and rheological changes (Changala Reddy *et al.*,

1989, 1990). Here, the fermentation is due to the activities of endogenous microflora in blackgram, in particular *Leuconostoc mesenteroides* but also yeasts, lactic acid bacteria and coliforms. In this paper we report on a comparative study of the chemistry and physical properties of a homogeneous polysaccharide obtained from comparable fractions of native and fermented blackgram.

MATERIALS AND METHODS

Isolation and purification of the polysaccharide

The mucilaginous polysaccharides were extracted from native and fermented (14 h at ambient temperature, with 4% NaCl) blackgram batter with aqueous 10% trichloroacetic acid (TCA) at 4°C (3 × 4 h each) (Ramadas Bhat *et al.*, 1987). The polysaccharides were

precipitated with acetone (3 vol.) and the precipitate dissolved in water, dialysed and freeze dried.

Fractionation

(a) Fractional precipitation with ethanol

An aqueous solution (0.2%, 1 g in 500 ml water) of the polysaccharide was brought to 72% ethanol concentration with good stirring and the precipitate (Fr.1, i.e. N1) formed collected by a brief centrifugation. Further additions of ethanol (to 78% followed by 81% conc.) to the clear supernatant yielded two more fractions (N2 and N3).

Similar treatment of the polysaccharide from fermented blackgram gave three fractions, viz. F1, F2 and F3 at ethanol concentrations of 79, 81 and 83%, respectively.

(b) Cetavlon complexing

Aqueous Cetavlon (2%) was added dropwise to incipient turbidity to aqueous solutions (0.3–0.5%) of fraction 2 (N2 and F2) from (a) above. After 1 h at 4°C the precipitated quaternary complex was collected by centrifugation, and the pellet dissociated by dispersion in NaCl (4 M), and the polysaccharide recovered by alcohol precipitation. This was repeated twice and the final precipitate dissolved in water, dialysed and freeze dried (N2-CP and F2-CP). The non-precipitated fractions (N2-CNP and F2-CNP) were recovered from the supernatant after acidification to pH 4 with dilute acetic acid, dialysis and freeze drying.

(c) Chromatography on DEAE-cellulose

The major CNP fractions were applied to a DEAE-cellulose (CO_3^-) column (3.7 × 29 cm) eluted successively with water, 0.1–0.5 M ammonium carbonate (AC) and 0.1–0.3 M NaOH. The carbohydrate in the fractions (13 ml) was monitored by the phenol- H_2SO_4 method (Pragna Rao & Pattabiraman, 1989), and pooled appropriately.

Homogeneity criteria

(a) Gel filtration

A solution of N2-CNP-0.1 M AC and F2-CNP-0.1 M AC (5 mg) in 0.05 M NaCl (1 ml) was loaded onto a precalibrated (with dextrans of known molecular weight) column of Sephacryl S-400 and eluted with 0.1 M NaCl (15 ml h⁻¹). Fractions (1.5 ml) were analysed for total carbohydrate.

(b) Sedimentation behaviour

The sedimentation behaviour of solutions of N2-CNP-0.1 M AC and F2-CNP-0.1 M AC (0.75% in 0.05 M NaCl) at 59 780 rev/min was determined in a Beckman Model E Spinco analytical ultracentrifuge.

(c) Electrophoresis

Microzone electrophoresis of the dyed (Procion Brilliant Red 2BS) 0.1 M AC derived polysaccharides (Anderson *et al.*, 1971) on cellulose acetate membranes was performed in a Beckman microzone cell in ammonium carbonate–NaCl buffer (0.05 M, pH 9.3) at an applied voltage of ~150 V and 7 mA current for 40 min.

(d) High performance size exclusion chromatography

HPSEC on E-Linear and E-1000 columns (Waters Associates, Milford, USA; ss, 3.9 mm × 30 cm) was performed on a Shimadzu HIC-6A chromatograph equipped with RID-6A refractive index detector, SCL-6A system controller and CR4A Chromatopac integrator. A 0.2% solution of the polysaccharide (10 µl) was injected into the system and eluted with water at a flow rate of 1.2 ml min⁻¹.

Carboxyl-reduction of polysaccharide

This was performed three times, according to the method of Taylor & Conrad (1972) by adding 1-cyclohexyl-2(4-methylmorpholino) ethyl-carbodiimide *p*-toluene-sulphonate and sodium borohydride (2 M). The reduced products were dialysed and freeze dried.

Methylation analysis

The polysaccharides were methylated by the Hakomori (1964) method and the products purified by passing through SEP-PAK C 18 cartridges. After hydrolysis with formic acid- H_2SO_4 the partially methylated alditol acetates were prepared (using NaBH_4 in $^2\text{H}_2\text{O}$) and analysed by GLC-MS (Jansson *et al.*, 1976).

Periodate oxidation

A solution of the polysaccharide (14 mg in 5 ml water) was oxidized with 10 mM sodium metaperiodate for ~72 h in the dark at 4°C. The excess periodate was reduced with 0.1 M ethylene glycol (2 ml) and the products reduced with NaBH_4 (30 mg) overnight. The solution of the reduced polyalcohol was then dialysed and freeze dried. The polyalcohol was hydrolysed with 0.5 M H_2SO_4 for 48 h at room temperature. Acetate derivatives were prepared and separated by GLC.

Chromium trioxide oxidation

The polysaccharide (10 mg) dissolved in formamide (2 ml) was acetylated with acetic anhydride–pyridine (1:1, v/v, 2 ml) at room temperature for 16 h. A portion of the acetylated polysaccharide in glacial HOAc (1 ml) was oxidized with CrO_3 (50 mg) with stirring at 60°C for 4 h. Both oxidized and unoxidized (control) materials were acid hydrolysed (1 N H_2SO_4 , 100°C, 6 h), alditol acetates prepared and analysed by GLC (Hoffman *et al.*, 1972).

Viscosity determinations

Aqueous solutions of the polysaccharides (0.1–0.6% were prepared and their viscosities determined in a multibulb capillary viscometer at $25 \pm 0.5^\circ\text{C}$. The intrinsic viscosity (η) was determined by extrapolation to zero concentration from a plot of reduced viscosity versus concentration (Greenwood, 1964).

General methods

The general and analytical methods used were those previously described (Salimath & Tharanathan, 1982; Ramadas Bhat & Tharanathan, 1986b). GLC-MS was performed with a Hewlett Packard HP 5985 GC-MS system fitted with a SP-2300 capillary column (0.25 μm film thickness, 30 m \times 0.25 mm i.d.). Helium was the carrier gas used and the temperature programme was $70 \rightarrow 200^\circ\text{C}$ at a ramp rate of 2°C min^{-1} .

RESULTS AND DISCUSSION

Fermentation of blackgram gave a total mucilaginous polysaccharide with considerably altered sugar composition and viscosity profiles, as compared with the native polysaccharide (Changala Reddy *et al.*, 1990). In

the former the content of arabinose decreased by $\sim 5\%$ whereas that of galactose increased by an order of magnitude. Consequently the Ara/Gal ratio of native and fermented polysaccharides was 8.9 and 3.9, respectively. The content of galacturonic acid did not change much. The η_r of fermented polysaccharide was $\sim 35\%$ higher than that of native polysaccharide, probably due to a much greater association between the polymer molecules. These gross changes were attributable to polysaccharide depolymerization by carbohydrate degrading enzymes which were being activated-released during fermentation (Changala Reddy *et al.*, 1990). Since the TCA-extracted blackgram polysaccharides, in the present study were obtained by acetone precipitation followed by solvent exchange drying and were easily soluble in water, detailed fractionation-structural studies could be performed on them.

Fractionation

The polysaccharides from native and fermented blackgram were fractionated by the scheme shown in Fig. 1. Fractional precipitation with alcohol (72–81%) afforded three fractions with Fr.2 being the most abundant in each case. The corresponding fractions N2 and F2 from native and fermented blackgram showed considerable differences in their specific viscosity and sugar

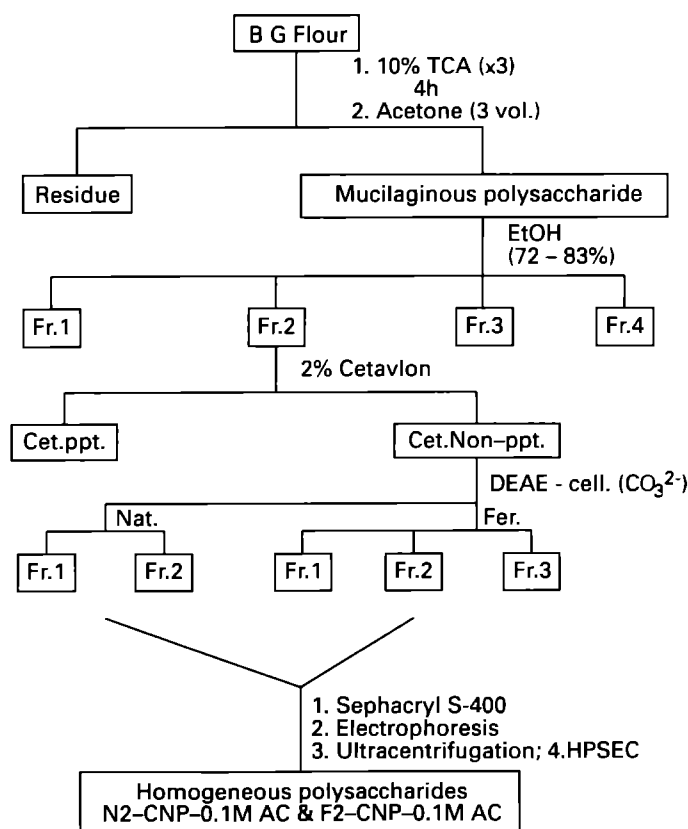


Fig. 1. Fractionation scheme and homogeneity tests performed to isolate pure polysaccharides from native and fermented blackgram.

Table 1. Fractional precipitation of native and fermented blackgram mucilaginous polysaccharides with ethanol

Fra. No.	Alcohol (%)	Yield (%)	(η°) (dl/g)	Rha	Ara	Gal	Ara/Gal	Gal A (%)
				_____ (%)				
Native								
1	72	9.2	133.4	1.9	70.4	8.2	8.6	18.4
2	78	83.8	182.0	0.9	70.5	11.9	5.9	16.2
3	81	7.0	213.6	1.2	60.8	16.9	3.6	11.7
Fermented								
1	79	34.8	135.0	0.6	74.5	11.6	6.4	12.5
2	81	50.7	221.3	1.4	75.3	10.2	7.4	13.2
3	83	14.6	237.1	0.8	74.8	13.2	5.7	11.0

composition (Table 1), particularly the Ara/Gal ratios (5.9 and 7.4). The $(\eta)_o$ value of F2 was $\sim 21\%$ greater than of N2. Cetavlon fractionation of N2 and F2 gave a higher yield of the non-precipitable fractions (N2-CNP and F2-CNP, see Table 2). However, both these fractions contained $\sim 15\%$ galacturonic acid indicating an incomplete separation of neutral and acidic polysaccharides. It is possible that the uronic acid carboxyls are partially esterified, like those in pectic polysaccharides, and thus are not available for complexing with Cetavlon. Interestingly, both N2-CNP and F2-CNP had comparable specific viscosities and neutral sugar profiles (Table 2). The specific viscosities of F2-CNP were 20% lower than that of N2-CP, probably due to the poor solubility of the former in water. The content of rhamnose was low in all these fractions.

The non-precipitated fractions were further fractionated on DEAE-cellulose (CO_3^{2-} form) and the fractions eluted with water, 0.1–0.3 M ammonium carbonate and 0.3 M NaOH were recovered (Fig. 2). In both cases the major fractions, N2-CNP-0.1 M AC and F2-CNP-0.1 M AC were eluted with 0.1 M ammonium carbonate solution. The fractions differed considerably in monosaccharide content (Table 3), that from the former was low in Rha but high in Ara content, and had an Ara/Gal ratio of 7.4; whereas the F2-derived fraction had slightly more of Rha, less of Ara and double the quantity of Gal (22%) and as a result its Ara/Gal ratio was almost half (3.4) that of the N2-derived fraction. The F2-derived fraction showed a significant decrease in the (η) value over that of the N2-derived fraction which

corroborated well with the concomitant fall in the Ara/Gal ratio. Both fractions had a sugar composition typical of that of pectic polysaccharides. Nevertheless, they were found to be homogeneous, as they showed a single peak/band by a variety of criteria, such as cellulose acetate membrane electrophoresis, sedimentation in an ultracentrifuge, gel permeation and high performance size exclusion chromatographic methods. The average molecular weights of the fractions were $\sim 1.18 \times 10^5$ and 0.9×10^5 , respectively for the N2- and F2-derived fractions.

Structure

The linkage patterns of the polysaccharide fractions were determined by permethylation with methyl iodide according to the Hakomori (1964) method, the products were hydrolysed and converted to their partially methylated alditol ($1\text{-}^2\text{H}$) acetate derivatives. Analysis by GLC-MS (Table 4) indicated a highly branched structure composed of variety of linkages. Thus, in the N2-CNP-0.1 M AC fraction for every 35 monosaccharide residues (37 residues for carboxyl-reduced polysaccharide) 11 are terminal non-reducing end residues consisting of nine L-arabinose and two D-galactose. There are 14 residues of arabinose and one residue of L-rhamnose involved in branching, of which seven arabinose residues are involved in branching through both 0-2 and 0-3. Since the only L-rhamnose derivative detected in the hydrolysate was 3-mono-O-methyl-L-rhamnose this residue must occupy solely internal posi-

Table 2. Analysis of Cetavlon fractionated polysaccharides derived from N2 and F2

Fraction	Yield (%)	(η°) (dl/g)	Rha	Ara	Gal	Ara/Gal	Gal A ^a (%)
			_____ (%)				
N2-CP	39.1	48.0	1.2	60.1	9.2	6.5	16.4
N2-CNP	60.9	58.0	1.1	59.8	8.1	7.4	14.8
F2-CP	28.4	38.0	1.2	53.9	11.9	4.5	18.4
F2-CNP	71.6	59.2	1.0	56.6	7.7	7.4	15.1

^aBy *m*-dihydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973).

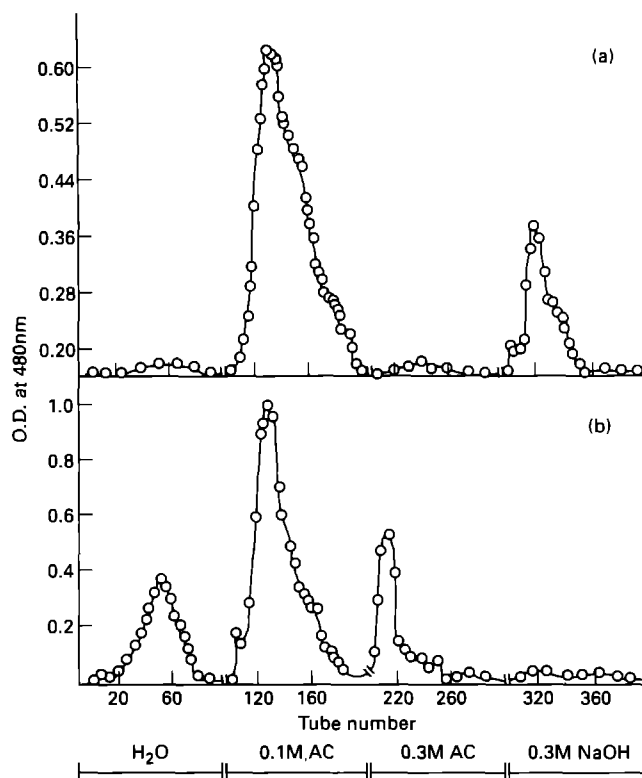


Fig. 2. Fractionation profile on DEAE-cellulose of (a) N2-CNP and (b) F2-CNP.

tions. 2,3,6-Tri-O-methyl-D-galactose, found only in trace amounts in the native fraction, increased by 2 mole proportions in the carboxyl-reduced polysaccharide, indicating that the D-galacturonic acid residues are essentially all (1→4)-linked. The Ara/Gal ratio of the methylated polysaccharide (7.5) was in close agreement with that of unmethylated fraction (7.4) and consistent with the structural features deduced.

The GLC-MS data of the permethylated F2-CNP-0.1 M AC fraction showed it to be considerably different from the N2-CNP-0.1 M AC fraction in qualitative and quantitative sugar composition (Table 4). Its slightly higher content of unmethylated arabinose indicated a relatively higher degree of branching, but the content of non-reducing terminal and intra-chain arabinose residues decreased by about a 1 mole proportion. The

content of (1→3)-linked galactose residues was twice that of the corresponding native unfermented fraction (see above) and the content of (1→4)-linked galactose residues increased from 2 moles in the original fraction to 4 moles in the carboxyl-reduced polysaccharide. Otherwise, the remaining structural features were comparable with that of N2-CNP-0.1 M AC polysaccharide. The Ara/Gal ratios of the methylated (3.6) and unmethylated (3.4) fractions were comparable.

On periodate oxidation the N2-derived polysaccharide consumed 0.77 mole of periodate per mole of 'anhydrosugar' with the liberation of 0.06 mole of formic acid, whereas the corresponding values for the F2-derived polysaccharide were 0.75 and 0.064 moles, respectively. Smith degradation of the reduced oxopolysaccharide gave glycerol and threitol together with

Table 3. Monosaccharide composition of fractions from DEAE-cellulose chromatography

Fraction	Yield (%)	(η°) (dl/g)	Rha	Ara	Gal	Ara/Gal	Gal A (%)
			(%)				
N2-CNP							
0.1 M AC	75.2	18.2	0.6	80.4	10.9	7.4	18.2
0.3 M NaOH	24.0	4.9	0.6	69.2	18.8	3.7	4.9
F2-CNP							
H ₂ O	29.0	10.0	0.4	83.3	15.0	5.5	15.4
0.1 M AC	58.3	8.9	0.8	74.7	22.2	3.4	17.0
0.3 M AC	12.7	10.2	2.7	77.4	18.7	4.1	20.0

Table 4. GLC-MS analysis of partially methylated alditol (1-²H) acetates derived from N2-CNP-0.1 M AC and F2-CNP-0.1 M AC polysaccharides

Glycosyl residue	Position of -OMe	Mode of linkage ^a	Mole proportions			
			N2-CNP-0.1 M AC		F2-CNP-0.1 M AC	
			Native	Carboxyl-reduced	Native	Carboxyl-reduced
Arabinose	2,3,5-	Araf-(1→	9.2	8.9	7.7	8.1
	2,3-	→5)-Araf-(1→	7.0	7.3	5.8	6.3
	2-	→3,5)-Araf-(1→	5.1	4.8	3.8	4.1
	3-	→2,5)-Araf-(1→	2.3	2.1	2.2	2.4
	-	→2,3,5)-Araf-(1→	6.7	7.0	8.1	7.8
Galactose	2,3,4,6-	Galp-(1→	2.3	2.1	1.7	2.4
	2,4,6-	→3)-Galp-(1→	1.8	2.0	4.1	3.8
	2,3,6-	→4)-Galp-(1→	0.2	2.2	2.2	3.9
Rhamnose	3-	→2,4)-Rhap-(1→	1.1	1.3	1.2	1.0

^aAraf, L-arabinofuranose; Galp, D-galactopyranose; Rhap, L-rhamnopyranose.

arabinose and galactose. The first two glycitols arose from oxidized arabinose and (1→4)-linked galactose residues, respectively; whereas the presence of unoxidized sugar residues is consistent with a high degree of branching and/or (1→3)-linked residues in the polysaccharides.

The highly negative (α)_D value (−64.2°, c. 0.67% in 0.5 N NaOH) for the unmethylated N2-derived polysaccharide suggested that the galactosidic bonds are predominantly of β -D-type and the arabinosidic bonds are of α -L-type. This agrees well with the chromium trioxide oxidation data of the fully acetylated polysaccharides, which showed over 90% destruction of galactose, total survival of rhamnose and only partial survival of arabinose. Taken together these results indicate that the destroyed sugar residues are of β -D-type and surviving residues are of α -L-type. The partial recovery of arabinose can be attributed to its furanosidic nature (Hoffman *et al.*, 1972).

Mild acid hydrolysis of the polysaccharide showed a considerable release of free arabinose and only traces of galactose and rhamnose, suggesting that the majority of the arabinose residues are present in the labile furanoid form. The acid-insoluble residue was composed of galactose and galacturonic acid.

The structure proposed for the major non-precipitable by Cetavlon fraction of blackgram bears a close resemblance to pectic polysaccharides (Stephen, 1983) and suggests that it possesses a L-rhamno-D-galacturonan core substituted by highly branched side chain appendages of L-arabinofuranose and D-galactose. In the previous study on total blackgram mucilage (Ramadas Bhat *et al.*, 1987), it was shown that contiguous L-rhamnose residues were present, as has been reported in an acidic polysaccharide complex from soybean cotyledon meal (Aspinall *et al.*, 1967) and white willow bark (Toman *et al.*, 1975). Arabinose side chains are also reported in the pectic polysaccharides of rape-

seed (Stoddart *et al.*, 1967; Siddiqui & Wood, 1976) and mustard seed (Rees & Wight, 1969).

The fully carboxyl-reduced polysaccharide is easily soluble in water but the solution has no appreciable viscosity, suggesting that the galacturonic acid carboxyls might be responsible for the viscosity of the mucilaginous polysaccharide, whereas the native polysaccharide gives a thick viscous dispersion in water. The viscosity and the foam stabilizing properties of the native polysaccharide are of special functional value in leavened food preparations from blackgram.

REFERENCES

- Anderson, D.M.W., Hendrie, A., Miller, J.R.A. & Munro, A.C. (1971). *Analyst*, **96**, 870.
- Aspinall, G.O., Cottrell, I.W., Egan, S.V., Morrison, I.M. & Whyte, J.N. (1967). *J. Chem. Soc. (C)*, 1071.
- Blumenkrantz, N. & Hansen, G.A. (1973). *Anal. Biochem.*, **54**, 484.
- Changala Reddy, G., Susheelamma, N.S. & Tharanathan, R.N. (1989). *Staerke*, **41**, 84.
- Changala Reddy, G., Susheelamma, N.S. & Tharanathan, R.N. (1990). *Carbohydr. Polym.*, **12**, 189.
- Greenwood, C.T. (1964). *Meth. Carbohydr. Chem.*, **3**, 179.
- Hakomori, S.I. (1964). *J. Biochem. (Tokyo)*, **55**, 205.
- Hoffman, J., Lindberg, B. & Svensson, S. (1972). *Acta Chem. Scand.*, **26**, 661.
- Jansson, P.E., Kenne, L., Liedgren, H., Lindberg, B. & Lonngrén, J. (1976). *Chem. Commun. Univ. Stockholm*, **8**, 1.
- Pragna Rao & Pattabiraman, T.N. (1989). *Anal. Biochem.*, **181**, 18.
- Ramadas Bhat, U. & Tharanathan, R.N. (1986a). *Cereal Chem.*, **63**, 376.
- Ramadas Bhat, U. & Tharanathan, R.N. (1986b). *Carbohydr. Res.*, **148**, 143.
- Ramadas Bhat, U., Salimath, P.V. & Tharanathan, R.N. (1987). *Carbohydr. Res.*, **161**, 161.
- Rees, D.A. & Wight, N.J. (1969). *Biochem. J.*, **115**, 431.

- Salimath, P.V. & Tharanathan, R.N. (1982). *Carbohydr. Res.*, **106**, 251.
- Siddiqui, I.R. & Wood, P.J. (1976). *Carbohydr. Res.*, **50**, 97.
- Stephen, A.M. (1983). In *The Polysaccharides—Vol. 2*, Academic Press, New York, pp. 154–61; and references therein.
- Stoddart, R.W., Barrett, A.J. & Northcote, D.H. (1967). *Biochem. J.*, **102**, 194.
- Susheelamma, N.S. & Rao, M.V.L. (1978). *J. Agric. Food Chem.*, **26**, 1434.
- Susheelamma, N.S. & Rao, M.V.L. (1979). *J. Food Sci.*, **44**, 1309.
- Taylor, R.L. & Conrad, H.E. (1972). *Biochemistry*, **11**, 1383.
- Toman, R., Karacsonyi, S. & Kubackova, M. (1975). *Carbohydr. Res.*, **43**, 111.