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Water-Soluble (1→3), (1→4)-β-D-Glucans from Barley (*Hordeum vulgare*) Endosperm. I. Physicochemical Properties

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SUMMARY

Physicochemical methods have been used to define molecular weight, molecular weight distribution, solution behaviour and shape of (1→3),(1→4)-β-D-glucans purified from the 40°C water-extract of barley endosperm by precipitation with 30% saturated ammonium sulphate. The molecular weight and solution properties of a (1→3),(1→4)-β-D-glucan from Australian grown barley (cv. Clipper) are compared with a commercially available preparation. Weight and number average molecular weights are 290 000 and 210 000 respectively for the Clipper (1→3),(1→4)-β-D-glucan and 160 000 and 150 000 respectively for the commercial preparation. The degree of polydispersity is small, but this probably results from the selection of a specific population of (1→3),(1→4)-β-D-glucan molecules during isolation. The higher molecular weight of the Clipper (1→3),(1→4)-β-D-glucan is reflected in higher sedimentation coefficient and intrinsic viscosity values. Viscosity and sedimentation data indicate that the molecules are highly asymmetric, with axial ratios of approximately 100 and 80 for the Clipper (1→3),(1→4)-β-D-glucan and the commercial preparation, respectively. Both polysaccharides appear to exist in solution as extended, worm-like chains.

1. INTRODUCTION

Mixed-linkage (1→3),(1→4)-β-D-glucans (hereafter referred to as β-glucans) are major matrix components of cereal endosperm cell walls

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(Fincher & Stone, 1981) and in barley endosperm constitute up to 75% of the cell wall (Fincher, 1975). Barley β-glucans are also important in commercial practice. During the malting and mashing phases of the brewing process, failure to degrade the β-glucans adequately can lead to filtration difficulties, decreased extraction of fermentable sugars and may contribute to the formation of precipitates in beer (Luchsinger, 1967; Bathgate & Dalgliesh, 1975). Barley β-glucans are responsible for decreased growth rates in poultry due to their adverse effect on digestion and absorption of other dietary components (Gohl *et al.*, 1978). In addition, β-glucans are constituents of 'dietary fibre' (Bailey *et al.*, 1978) and have potential applications as thickening agents in the food industry. It is apparent therefore that knowledge of the solution properties of the polysaccharide is of fundamental importance.

Comprehensive information on the physical properties, molecular shape and solution behaviour of β-glucans is not available, although the high viscosities of aqueous solutions of the polysaccharide are well known and some isolated reports of other physical properties have been published. Values for the molecular weight of water-soluble barley β-glucan differ by orders of magnitude. A molecular weight of ~ 20 000 was reported for permethylated barley β-glucan as determined by osmotic pressure measurements (Aspinall & Telfer, 1954). Molecular weights calculated from sedimentation and diffusion coefficients range from 55 800 (Podradsky, 1964) to 220 000 (Igarashi & Sakurai, 1965). Fleet & Manners (1975) determined a degree of polymerisation equivalent to a molecular weight of ~46 000 by endgroup analysis, but noted the discrepancy between this value and those obtained by gel filtration chromatography, which may be as high as 400 000 (Bathgate *et al.*, 1974).

In the present study we have used physicochemical procedures to define the molecular weight distribution, solution behaviour and molecular shape of two preparations of water-soluble barley β-glucan. We have compared a sample purified by ammonium sulphate and acetone precipitation from water extracts of an Australian-grown barley with a commercially available β-glucan extracted by essentially the same procedure from barley harvested in the United Kingdom. The 40°C water-soluble β-glucan represents up to 20% (w/w) of total β-glucan in barley endosperm cell walls (Fincher, 1975), although lower values have been reported (Ballance & Manners, 1978). This fraction was chosen

for the present work because most physical and chemical information on barley β-glucans has been obtained using the same fraction, and because it is often used as a substrate in assays for (1→3),(1→4)-β-D-glucan endohydrolases. Detailed structures and compositional analyses of the polysaccharides are described in a forthcoming paper (Woodward *et al.*, 1983).

2. EXPERIMENTAL

2.1 Materials

Barley (*Hordeum vulgare* L. cv. Clipper) was kindly supplied by Mr K. Mander, Victorian Department of Agriculture and flour was prepared by Dr A. Bacic as described by Bacic & Stone (1981). After refluxing in 80% ethanol (Preece & MacKenzie, 1952) the flour was extracted three times with water at 40°C and the extract precipitated with 30% saturated ammonium sulphate (no polysaccharide was precipitated with 20% saturated ammonium sulphate). The precipitate was further purified by alternate precipitations (two each) with ammonium sulphate and 50% (v/v) acetone (Clarke & Stone, 1966). The final acetone precipitate was redissolved in water, dialysed, and made 10mM with respect to sodium maleate buffer, pH 6·8 (containing 10 mM $CaCl_2$, 10 mM sodium azide). Porcine pancreatic α-amylase (Type IV-A, Sigma Chemical Co., St. Louis, Missouri, USA) was added to give an enzyme to substrate ratio of approx. 1 : 33 (w/w). After 3 h at 40°C no further reducing sugars (Lever, 1972) were released, but the incubation was continued for 16 h. The α-amylase was precipitated by heating the solution at 100°C for 10 min and the precipitate removed by centrifugation. The solution was dialysed exhaustively against water in the presence of toluene and chloroform, and freeze dried. The resulting β-glucan was free of starch as judged by the iodine test and by additional incubations with α-amylase.

The commercial barley β-glucan (batch number 80147) was generously donated by Mr C. J. Dowzer, Biocon (Australia) Pty Ltd. The preparation had been extracted with water from flaked barley at 40°C, and purified from the 0–30% saturated ammonium sulphate precipitate by successive fractionation with ammonium sulphate and acetone (C. J. Dowzer, personal communication).

2.2 Preparation of β-glucan solutions

Freeze dried β-glucan was dissolved in high purity water at approx. 70°C. Solutions were dialysed against high purity water for 24 h at 4°C and filtered through a membrane filter (pore size 3·0 μm, Millipore Corp., Bedford, Massachusetts, USA) to remove undissolved material. During this procedure less than 5% (w/w) of the polysaccharide was lost. Dialysis water was retained as reference solvent when required. Concentrations were determined from the specific refractive increment.

High purity water was obtained from a 4-bowl Milli-Q water filtration system (Millipore Corp.), which yielded Type 1 water (ASTM Annual Book of Standards, part 31, Water, Method D-1193) of specific resistance greater than 10 MΩ cm at 20°C and organic material less than 0·1 ppm.

2.3 Specific refractive increment

Standard solutions of β-glucan (approx. 0·5 g dl^{-1}) were prepared as described and their absolute concentration determined by dry weight analysis. The stock solution was diluted by weight with water and the refractive index increment measured at each concentration using a Brice–Phoenix differential refractometer (Virtis Co., Gardiner, New York, USA).

2.4 Partial specific volume

Solution densities were determined electronically with a Digital Precision Density Meter DMA 02C (Anton Paar, K.G. Austria), using solutions of approx. 0·7 g dl^{-1}. The partial specific volume of the polysaccharide solutions was calculated from equation 12 of Kupke (1973).

2.5 Sedimentation velocity

Sedimentation experiments were performed at 42 000 rpm in a Beckman Model E analytical ultracentrifuge (Beckman Instruments Inc., Palo Alto, California, USA) at 20°C. Because low concentrations (0·4–2·0 mg ml^{-1}) were required, interference optics were used. The sedimentation coefficients were extrapolated to infinite dilution to yield $s^0_{20,w}$ and the concentration dependence of the sedimentation

coefficient (K_s) defined by the relationship (Creeth & Knight, 1965)

$$s = s^0(1 - K_s c) \tag{1}$$

where c is the concentration (g dl^{-1}).

2.6 Viscosity

The relative viscosities (η_r) of a series of dilute β-glucan solutions (0·01–0·04 g dl^{-1}) were determined at 25°C (±0·02°C) in a Cannon-Ubbelohde Four Bulb Shear Dilution Viscometer (Cannon Instrument Co., State College, Pa., USA) by adding increments of stock β-glucan solutions (0·04 g dl^{-1}) to 5·0 ml of dialysis water. The data were extrapolated to infinite dilution to yield intrinsic viscosity [η] as defined by the equations of Huggins (2) and Kraemer (3) (Tanford, 1961).

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \tag{2}$$

$$\frac{\ln \eta_r}{c} = [\eta] + k''[\eta]^2 c \tag{3}$$

2.7 Degree of hydration

The low temperature NMR method of Kuntz *et al.* (1969) was used to measure the degree of hydration of the β-glucans. Solutions of polysaccharides (2–4 mg ml^{-1}) and bovine serum albumin (approx. 1 mg ml^{-1}, Sigma Chemical Co.) were prepared in distilled water. The concentration of the bovine serum albumin was determined spectrophotometrically using an extinction coefficient at 280 nm ($E^{1\%}_{1cm}$) of 6·65.

NMR measurements were performed at −30°C in a Jeol PFT 100 Fourier-transform spectrometer equipped with a JNM-VT-3C temperature control. The hydration of 0·40 g g^{-1} for bovine serum albumin (White *et al.*, 1972) was used as a standard to calculate hydration. The operating conditions were: pulse width 19 μs, 20 000 accumulations, 50 ms repeat time, and a 10 kHz spectral width.

2.8 Molecular weight

All solutions were dialysed for 16 h against 0·15 M NaCl in high purity water and the dialysis solution used as a reference solvent. The weight

average molecular weight of the solution ($\bar{M}_w$) was determined by conventional sedimentation equilibrium ultracentrifugation at 15°C in a Beckman Model E analytical ultracentrifuge. The Clipper and commercial β-glucan solutions (0·8 mg ml^{-1}) were centrifuged at 7200 rpm and 8000 rpm respectively. Interference optics were used and data were recorded on Kodak spectroscopic plates. Oil was not used at the bottom of centrifuge cells because it appeared to cause aggregation of the polymer which resulted in a large area of blurred fringes at the base of the cell. Equilibration was attained after 72 h using the overspeeding technique of Chervenka (1969) and confirmed by invariance in the fringe pattern after a further 24 h. The $\bar{M}_w$ was calculated from the number of fringes crossed at equilibrium, using equation 40 of Coates (1970). Concentrations were determined in the synthetic boundary cell and agreed to within 1% of that calculated from the specific refractive increment.

The meniscus-depletion sedimentation-equilibrium procedure of Yphantis (1964) was performed at 15°C at 9000 rpm and 15 000 rpm for the Clipper and commercial β-glucans (0·2 mg ml^{-1}) respectively. Equilibration was reached within 24 h and confirmed by identical interference patterns after a further 5 h. The computer program of Roark & Yphantis (1969) was used to calculate number and weight average molecular weights, $M_n(r)$ and $M_w(r)$, at regular radial distances (r). The

TABLE 1
Physicochemical Properties of Barley β-Glucans

Property	*Clipper*	*Commercial*
Specific refractive increment k	0·143 ml g^{-1}	0·144 ml g^{-1}
Partial specific volume $\bar{v}$	0·622 ml g^{-1}	0·618 ml g^{-1}
Intrinsic viscosity $[\eta]$	6·90 dl g^{-1}	4·26 dl g^{-1}
Degree of hydration δ	0·42 g g^{-1}	0·45 g g^{-1}
Sedimentation coefficient $s^0_{20,w}$	4·5 S	3·5 S
Weight average molecular weight $\bar{M}_w$	290 000	160 000
Number average molecular weight $\bar{M}_n$	~210 000	~150 000
$\bar{M}_w/\bar{M}_n$	~1·4	~1·1
$\bar{M}_z/\bar{M}_w$ (Yphantis)	1·2	1·3
Diffusion coefficient D	$1{\cdot}1 \times 10^{-7}$ $cm^2 s^{-1}$	$1{\cdot}4 \times 10^{-7}$ $cm^2 s^{-1}$

weight average and z-average molecular weights $\bar{M}_w$ and $\bar{M}_z$ were determined by extrapolation of $M_n(r)$ and $M_w(r)$ respectively to the base of the solution column.

The number average molecular weight of the solution ($\bar{M}_n$) was determined at 23°C using a Melabs CSM-2 recording membrane osmometer (Wescan Instruments Inc., Santa Clara, California, USA) fitted with a Sartorius SM 11739 membrane. Concentration ranges of 1·5–2·5 mg ml^{-1} (Clipper) and 1·3–5·1 mg ml^{-1} (commercial) were used. Values were calculated from the equation

$$\frac{\pi}{c} = RT\left(\frac{1}{\bar{M}_n} + Bc\right) \tag{4}$$

where R is the gas constant, T the absolute temperature, B the second virial coefficient and c the concentration (g dl^{-1}).

3. RESULTS

3.1 Composition of the β-glucans

Compositional data for the two polysaccharide preparations are compared in a forthcoming paper (Woodward *et al.*, 1983). Glucosyl residues accounted for ~98% of recovered sugars and are linked by (1 → 3)- and (1 → 4)-linkages in ratios of 28 : 72 for the Clipper preparation and 30 : 70 for the commercial preparation. Small amounts of associated nitrogen and acetyl groups are also present.

3.2 Physical parameters

The physical properties of the preparations are shown in Table 1. It is apparent that the molecular weight of the Clipper β-glucan is considerably higher than that of the commercial preparation and this is reflected in a higher sedimentation coefficient and intrinsic viscosity. Because of the limited solubility of the β-glucans and the requirement for relatively high concentrations to obtain measurable osmotic pressures (i.e. greater than 1 cm of solvent) only narrow concentration ranges could be studied (see Experimental section). Within these ranges no significant differences in π/c were observed and values for the apparent $\bar{M}_n$ in Table 1 therefore represent averages of measurements taken at three different concentrations.

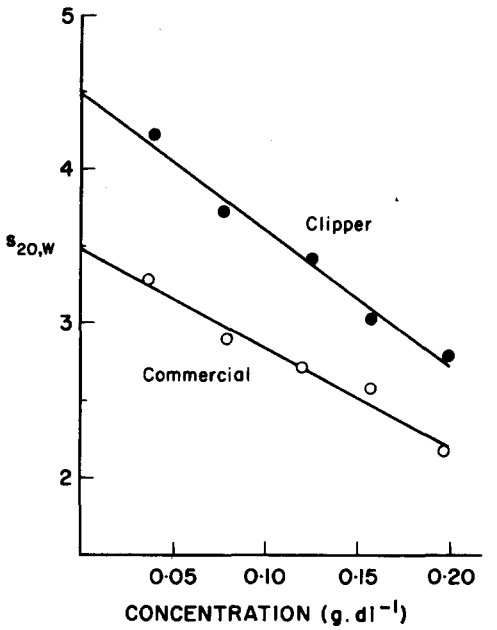

Fig. 1. Sedimentation coefficients of Clipper (●) and commercial (○) barley β-glucans in 0·15 M NaCl.

Sedimentation coefficients were linear in the concentration range of 0·4–2·0 mg ml^{-1} (Fig. 1). Sedimentation coefficients were identical with either 0·15 M NaCl or distilled water as solvent. Since no primary salt effect was evident, it appears there is little, if any, net charge associated with these β-glucans.

Combined Huggins and Kraemer extrapolations to the intrinsic viscosity are shown in Fig. 2. The data were linear over the concentration range studied and the values of $(k'-k'')$ were equal to the theoretical value of 0·5 (Bradbury, 1970) with an experimental error of ±10%. The relative viscosities of the β-glucan solutions were measured at two shear rates differing by a factor of three, but no shear rate dependence was detected. However, at the shearing stresses normally used in capillary viscometers, a shear rate dependence of the viscosity is not expected for asymmetric macromolecules if their molecular weight is less than 400 000 (Bradbury, 1970).

The $\bar{M}_w$ values determined by conventional equilibrium sedimentation (Table 1) are considered accurate to within ±15%. Values obtained

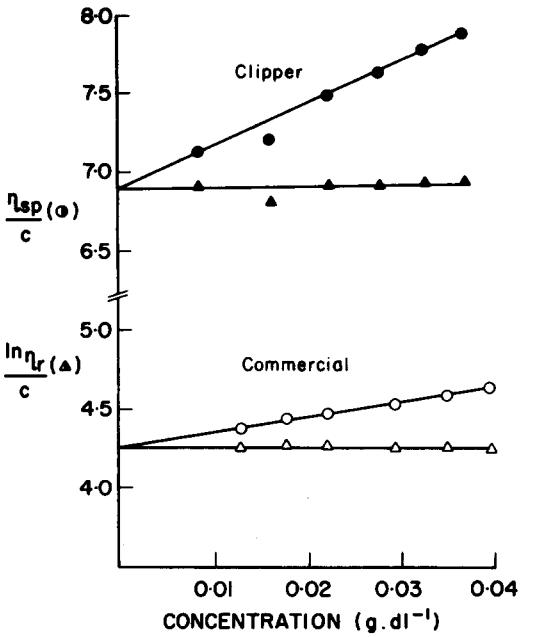

Fig. 2. Huggins (circles) and Kraemer (triangles) viscometric data for Clipper (closed symbols) and commercial (open symbols) β-glucans in 0·15 M NaCl.

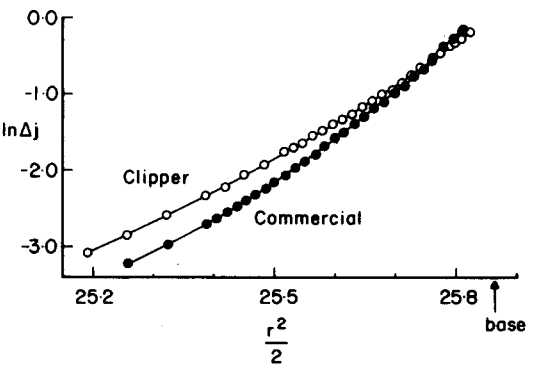

Fig. 3. Yphantis equilibrium sedimentation of Clipper (○) and commercial (●) barley β-glucans in 0·15 M NaCl. Δj is the interference fringe displacement and r is the radial distance.

with more dilute solutions, using Yphantis equilibrium sedimentation conditions and assuming ideal solution behaviour, were 360 000 and 180 000 for the Clipper and commercial β-glucans respectively. Since these are 10–25% higher than $\bar{M}_w$ values shown in Table 1, some non-ideality appears likely. Detailed examination of non-ideal behaviour was beyond the scope of this work and the effect was not investigated further. In addition there are a number of experimental errors which might account for such a difference (Yphantis, 1964). The $\bar{M}_w$ values shown in Table 1 have been used in all subsequent calculations.

The Yphantis sedimentation data is shown in Fig. 3. The curves are non-linear and indicate a small but clearly detectable degree of polydispersity (Yphantis, 1964), which has been defined by the $\bar{M}_z/\bar{M}_w$ ratio (Table 1). These values were calculated from data obtained from a single dilute solution for each β-glucan and are considered more reliable than the $\bar{M}_w/\bar{M}_n$ ratios which were derived using different solutions and by independent techniques.

4. DISCUSSION

The apparent $\bar{M}_w$ values of 290 000 and 160 000 respectively for the Clipper and commercial β-glucans are comparable with values of 210 000 (Djurtoft & Rasmussen, 1955) and 220 000 (Igarashi & Sakurai, 1965) calculated from sedimentation and diffusion coefficients for other preparations. We have applied the universal calibration parameter (Grubisc *et al.*, 1967) to the gel filtration and viscosity data of Bathgate *et al.* (1974). The recalculated molecular weight for their barley β-glucan is approx. 130 000.

Forrest & Wainwright (1977) also allowed for shape effects in estimating, from gel filtration data, a molecular weight of 40×10^6 for a β-glucan extracted with water from barley endosperm cell walls. This value is two orders of magnitude higher than those obtained in the present study and in previous work. The apparent discrepancy probably results from different extraction temperatures. The cell wall extract was prepared at 65°C (Forrest & Wainwright, 1977) compared with 40°C used in the present study for the isolation of the β-glucans from whole barley endosperm (see Experimental section), and additional polysaccharide of much higher molecular weight may have been extracted at the higher temperature. Thus the amount of β-glucan extracted from

barley flour (Fleming & Kawakami, 1977) or from isolated endosperm cell walls (Ballance & Manners, 1978) increases with the extraction temperature. It seems likely therefore that β-glucans of much higher molecular weight than the 40°C water-extract are present in barley endosperm cell walls (Fincher, 1975; Forrest & Wainwright, 1977).

The molecular weight ratios ($\bar{M}_z/\bar{M}_w$) of 1·2 and 1·3 for the Clipper and commercial β-glucans (Table 1) indicate that the polysaccharides exhibit a relatively narrow range of molecular weight dispersity. It is likely that these results arise from the selection of a specific population of β-glucan molecules during isolation, since water at 40°C extracts only a small proportion of total β-glucan (Fleming & Kawakami, 1977) and a further selection is made by ammonium sulphate and acetone precipitation.

From the physicochemical data summarised in Table 1, information on the tertiary structure of β-glucans can be obtained (Table 2). The frictional ratio (f/f_{min}) was calculated from the relationship

$$f = \left(\frac{f}{f_0}\right) 6\pi\eta \left[\frac{3M(\bar{v}_2 + \delta_1 v_1^0)}{4\pi N}\right]^{1/3} \quad (5)$$

where the terms are defined by Tanford (1961). The minimum value of the frictional coefficient (f_{min}) was calculated assuming a minimum frictional coefficient ratio (i.e. $f/f_0 = 1$) and minimum degree of hydration (i.e. $\delta = 0$). The f/f_{min} values of 4–5 indicate that the β-glucans are extensively solvated or asymmetric, or both (Tanford, 1961). Since the degree of hydration of both polysaccharides is not high compared with other macromolecules, including polysaccharides such as dextran, arabinoxylan, galactomannan and arabinogalactan-peptide (Andrewartha *et al.*, 1978, 1979) it is clear that the β-glucans are asymmetric.

Asymmetry has been determined using Eqn. (5) to calculate the Perrin shape factor f/f_0 and hence (assuming a prolate ellipsoid) the axial ratio (Tanford, 1961). The axial ratios have also been calculated (Table 2) from the relationship (Tanford, 1961)

$$[\eta] = \nu(\bar{v}_2 + \delta_1 v_1^0) \quad (6)$$

by solving for the Simha shape factor ν (Tanford, 1961), again assuming a prolate ellipsoid. Axial ratios derived by these two independent procedures are similar (Table 2) and clearly demonstrate the extreme asymmetry of the molecules. The axial ratio of the Clipper β-glucan is

TABLE 2
Tertiary Structure Parameters of Barley β-Glucans

Parameter	*Clipper*	*Commercial*
Frictional ratio f/f_{min}	5·2	4·5
Scheraga-Mandelkern beta function $\beta(a/b)$	$3{\cdot}1 \times 10^6$	$3{\cdot}0 \times 10^6$
Axial ratio a/b		
(a) sedimentation	110	95
(b) viscosity	100	80
(c) Scheraga-Mandelkern	~80	~60
Stokes radius a^0	22 nm	15 nm
Wales-van Holde ratio $K_s/[\eta]$	0·29	0·45

significantly higher than that of the commercial preparation and this is presumably related to its higher molecular weight (Table 1).

The Wales-van Holde ratio $K_s/[\eta]$ has been correlated with the tertiary structure of a variety of polymers in solution (Creeth & Knight, 1965). If the polymer is spherical the ratio is 1·6, while asymmetric polymers exhibit much lower values. The values determined experimentally for the β-glucans (Table 2) are comparable to those obtained for extremely asymmetric proteins such as fibrinogen and collagen (Creeth & Knight, 1965).

The asymmetry of the β-glucans has also been calculated from the Scheraga-Mandelkern beta function (Scheraga & Mandelkern, 1953)

$$\beta\left(\frac{a}{b}\right) = \frac{s^0[\eta]^{1/3}\eta_0}{100^{1/3}M^{2/3}(1-\bar{v}\rho)} \tag{7}$$

Although the errors associated with this determination are necessarily large because of the number of experimentally determined parameters involved, the values of $\sim 3 \times 10^6$ confirm that the β-glucans are prolate ellipsoids (Tanford, 1961). Furthermore, the axial ratios calculated from the beta function (Cantor & Schimmel, 1980) are consistent with the asymmetry evaluated from sedimentation or viscosity data (Table 2).

The maximum possible asymmetry for the linear β-glucans would be observed if they existed in solution as perfect rigid rods. Assuming both dimensions of the monomeric glucosyl residue to be 0·5 nm, the maximum axial ratios can be calculated from the degree of polymerization

as approx. 1300 and 900 for the Clipper and commercial preparations, respectively. Since the observed axial ratios are less than 10% of these (Table 2) the β-glucans do not exist as fully extended molecules, but exhibit considerable flexibility. They can therefore be described as assuming a worm-like chain conformation in solution (Bloomfield *et al.*, 1974; Holzwarth, 1981). The flexibility is presumably imparted by the insertion of irregularly-spaced 1e,3e-linkages (e = equatorial) in a glucan containing predominantly 1e,4e-linkages (Woodward *et al.*, 1983); this results in interruption or 'loosening' of the rigid, ribbon-like chain characteristic of (1 → 4)-β-glucans (Rees & Scott, 1971).

Indeed, a worm-like conformation is observed in space-filling models constructed in accordance with the major structural features of the polysaccharides (Woodward *et al.*, 1983). The biological implications of the β-glucan shape in the cell wall matrix are discussed in relation to their chemical structure in a forthcoming paper (Woodward *et al.*, 1983).

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