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Water-Soluble (1 → 3,1 → 4)-β-D-Glucans from Barley (*Hordeum vulgare*) Endosperm. IV. Comparison of 40°C and 65°C Soluble Fractions

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SUMMARY

A (1→3,1→4)-β-glucan preparation extracted from barley flour with water in the temperature range 40–65°C, followed by precipitation with 30% saturated ammonium sulphate, has a weight average molecular weight of 150000, an intrinsic viscosity of 4·04 dl g^{-1}, an axial ratio of approximately 70 and is polydisperse with respect to its molecular size. Structural analysis of the polysaccharide preparation by methylation and enzymic procedures shows that it consists of 69% (1→4)-linkages and 31% (1→3)-linkages and has relatively fewer blocks of three or more adjacent (1→4)-linkages than the water-soluble (1→3,1→4)-β-glucan extracted from barley flour at 40°C. It is concluded that the solubility of these polysaccharides is determined not so much by their degree of polymerization or overall asymmetrical conformation, but rather by small differences in fine structure which alter the ability of chains to align into relatively stable molecular aggregates.

INTRODUCTION

Cell walls of the starchy endosperm of barley are composed mainly of (1→3,1→4)-β-glucan (approx. 70%) and arabinoxylan (approx. 20%) (Fincher, 1975). Detailed structural analyses of two 40°C water-soluble (1→3,1→4)-β-D-glucans (referred to here simply as β-glucans) extracted from barley flour revealed that cellotriosyl and cellotetraosyl residues separated by single (1→3)-linkages constitute approximately 90% (w/w) of each polysaccharide, and that the remaining 10% consists of blocks of

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5–11 (1→4)-linked glucosyl residues (Woodward *et al.*, 1983*b*). Further analysis of the β-glucan preparations showed that the cellotriosyl and cellotetraosyl residues in the β-glucan preparations are arranged randomly within the polysaccharide chain (Staudte *et al.*, 1983).

Physicochemical methods have been used to define the molecular weight, solution behaviour and shape of these barley β-glucans. The polysaccharides consist of up to 1200 glucosyl residues in an extended, highly asymmetrical chain with an axial ratio of 80–110, and form aqueous solutions of high viscosity (Woodward *et al.*, 1983*a*). The conformations of the polysaccharides in aqueous solutions have been reconciled with their structural and linkage sequence characteristics by theoretical conformational analysis and the statistical mechanical theory of polymer chain conformation (Buliga *et al.*, 1986). A correct prediction of the observed molecular shape can be achieved only when the longer blocks of adjacent (1→4)-linkages are included in the modelling procedure, and it is apparent that chain flexibility arises principally from the isolated (1→3)-β-linkages (Buliga *et al.*, 1986).

Despite the comprehensive information on the physical, chemical and solution properties of the 40°C water-soluble barley β-glucan, the organization of the polysaccharide in the matrix of the cell wall and its interactions with other wall polymers have not been defined (Woodward & Fincher, 1983; Fincher & Stone, 1986). The 40°C water-soluble β-glucan accounts for up to 20% of total β-glucan in barley endosperm cell walls (Fincher, 1975), whereas approximately 50–70% of endosperm cell wall β-glucan is extracted with hot (65°C) water (Fleming & Kawakami, 1977; Ballance & Manners, 1978; Ahluwalia & Ellis, 1985) and it has been suggested that the occurrence of even longer blocks of adjacent (1→4)-linkages, in greater abundance, might explain the relatively tight binding of the 65°C water-soluble fraction in the cell wall (Woodward & Fincher, 1983). Furthermore, 65°C is the temperature traditionally used for the extraction of malt in the mashing phase of many brewing protocols and Anderson *et al.* (1978) have suggested that 65°C water-soluble β-glucan levels in barley flour might be useful indicators of malting quality. For these reasons, a 65°C water-soluble β-glucan has now been isolated from barley flour and we report here its physical, chemical and solution characteristics.

EXPERIMENTAL

Purification of β-glucan

Barley (*Hordeum vulgare* cv. Clipper) flour was refluxed in 80% ethanol and extracted at 40°C and the 0–30% saturated ammonium sulphate

precipitable β-glucan purified as previously described (Woodward *et al.*, 1983*a*). This fraction is referred to as the 40°C water-soluble β-glucan. The residual flour was dried by solvent exchange through ethanol and acetone prior to re-extraction for 30 min at 65°C with approximately 10 volumes water containing 0·1% (w/v) sodium azide. The flour was sedimented by centrifugation and extracted again at 65°C. The combined extracts were digested exhaustively at 40°C with 4 mg ml^{-1} porcine pancreatic α-amylase (type IV-A, Sigma Chemical Co.) in the presence of toluene and chloroform (Woodward *et al.*, 1983*a*). Control incubations showed that the α-amylase preparation had no activity against the 40°C water-soluble barley β-glucan or against carboxymethylpachyman. The α-amylase was inactivated at 100°C for 30 min and denatured protein removed by centrifugation. The extract was precipitated with 30% saturated ammonium sulphate followed by 50% (v/v) acetone and an additional precipitation with ammonium sulphate, dialysed and freeze dried (Woodward *et al.*, 1983*a*). The yield of this fraction was 0·26% (w/w) of the flour, and was shown to be free of starch by further incubation with α-amylase.

It should be emphasized that the purified fraction investigated here and referred to as the 65°C water-soluble barley β-glucan, is in fact the 0–30% saturated ammonium sulphate fraction of material extracted from barley flour with water in the temperature range 40–65°C.

Chemical analyses

The monosaccharide composition of the β-glucan was determined by gas–liquid chromatography of the alditol acetate derivatives (Blakeney *et al.*, 1983). Linkage positions were defined by the methylation procedure of Hakomori (1964) as described by Harris *et al.* (1984).

Nitrogen, ash, methoxy, acetoxy and uronic acid contents were determined by the Australian Microanalytical Service, Australian Mineral Development Laboratories, Fishermen's Bend, Victoria, 3207, Australia.

Fine structure

The 65°C water-soluble β-glucan (2 mg ml^{-1}) was dissolved in 10 ml 50 mM sodium acetate buffer, pH 5·0, containing 5 mM sodium azide and 400 μg ml^{-1} bovine serum albumin, and hydrolysed at 40°C for 20 h with (1→3,1→4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73) isoenzyme II purified from germinating barley (Woodward & Fincher, 1982*a*). Insoluble oligosaccharides which precipitated during hydrolysis were collected by centrifugation, washed thoroughly with water and

dried. Soluble oligosaccharides in the hydrolysates were fractionated by high-resolution gel-filtration chromatography on Bio-Gel P-2 calibrated with cellodextrin standards (Woodward *et al.*, 1983*b*). As a further measure of higher oligosaccharides released by the enzyme, the soluble fractions of the hydrolysates were precipitated with 4 volumes ethanol, the precipitates washed thoroughly with 80% ethanol, dried and weighed. For comparative purposes the hydrolysates of the 40°C water-soluble β-glucan from barley flour (Woodward *et al.*, 1983*a*) were precipitated with ethanol in the same way.

Physical properties

The intrinsic viscosity (η) was determined at 25°C as defined by the equations of Huggins and Kraemer (Tanford, 1961). The weight average molecular weight (M_w) of a 0·8 mg ml^{-1} polysaccharide solution was estimated by conventional sedimentation equilibrium ultracentrifugation at 15°C, 8000 rpm (Woodward *et al.*, 1983*a*) and the degree of polydispersity by the meniscus-depletion sedimentation equilibrium procedure of Yphantis (1964) at 15°C, 13000 rpm (cf. Woodward *et al.*, 1983*a*).

RESULTS

Composition

The composition of the 65°C water-soluble β-glucan is compared with that of the 40°C water-soluble β-glucan (Woodward *et al.*, 1983*b*) in Table 1. Small differences between the two polysaccharide fractions are evident. Unlike the 40°C water-soluble β-glucan, the 65°C water-soluble polysaccharide contains no detectable arabinose or xylose. The 65°C water-soluble β-glucan has much higher levels of uronic acids (Table 1). Methylation analyses showed that the 65°C water-soluble β-glucan contains relatively more (1→3)-β-glucosyl residues than the 40°C water-soluble preparation. It also has a higher nitrogen content (Table 1).

Fine structure

The Bio-Gel P-2 gel-filtration profiles of (1→3,1→4)-β-glucanase hydrolysates of the 40°C and 65°C water-soluble barley β-glucans are shown in Fig. 1 and the areas under each peak compared in Table 2. Again, small but significant differences are apparent. The product with

TABLE 1
Composition of Barley β-Glucans Extracted from Flour of the Variety Clipper

Composition	*Extraction conditions*	
	40°C[a]	*65°C*
Monosaccharide (% w/w)		
glucose	98·3	100
arabinose	1·1	0
xylose	0·6	0
mannose	trace	0
galactose	trace	0
ribose	0	trace
Linkage positions (% mol mol^{-1})		
(1 → 3)-glucosyl	28	31
(1 → 4)-glucosyl	72	69
terminal glucosyl	trace	trace
Nitrogen (% w/w)	0·18	0·4
Protein (% nitrogen × 6·25)	1·2	2·6
Ash (% w/w)	1·0	1·2
Methoxy (% w/w)	0·2	0·7
Acetoxy (% w/w)	1·5	1·9
Uronic acid (% w/w)	< 0·1	3·3

[a]From Woodward *et al.* (1983*b*).

an apparent DP of 1 presumably represents glucose released from polysaccharide chain termini by the action of the enzyme, although the levels of this component are anomalously high (Table 2). The hydrolysate of the 65°C water-soluble β-glucan contains relatively high levels of this component (Fig. 1, Table 2). However, the major hydrolysis products of both preparations are the tri- and tetrasaccharides (Fig. 1), which have been identified as 3-*O*-β-D-cellobiosyl-D-glucose and 3-*O*-β-D-cellotriosyl-D-glucose, respectively (Woodward & Fincher, 1982*b*; Woodward *et al.*, 1983*b*). Longer oligomeric products, which represent blocks of more than three adjacent (1 → 4)-β-glucosyl residues in the polysaccharide (Woodward *et al.*, 1983*b*), appear to be more abundant in the hydrolysate of the 40°C water-soluble β-glucan (Fig. 1). Similar small differences were observed in the levels of insoluble oligosaccharides which precipitated during hydrolysis and those precipitable by 80% ethanol; in both cases levels of these longer oligomeric products are lower in the hydrolysate of the 65°C water-soluble β-glucan (Table 2). In the experiments described here, the amount of insoluble oligo-

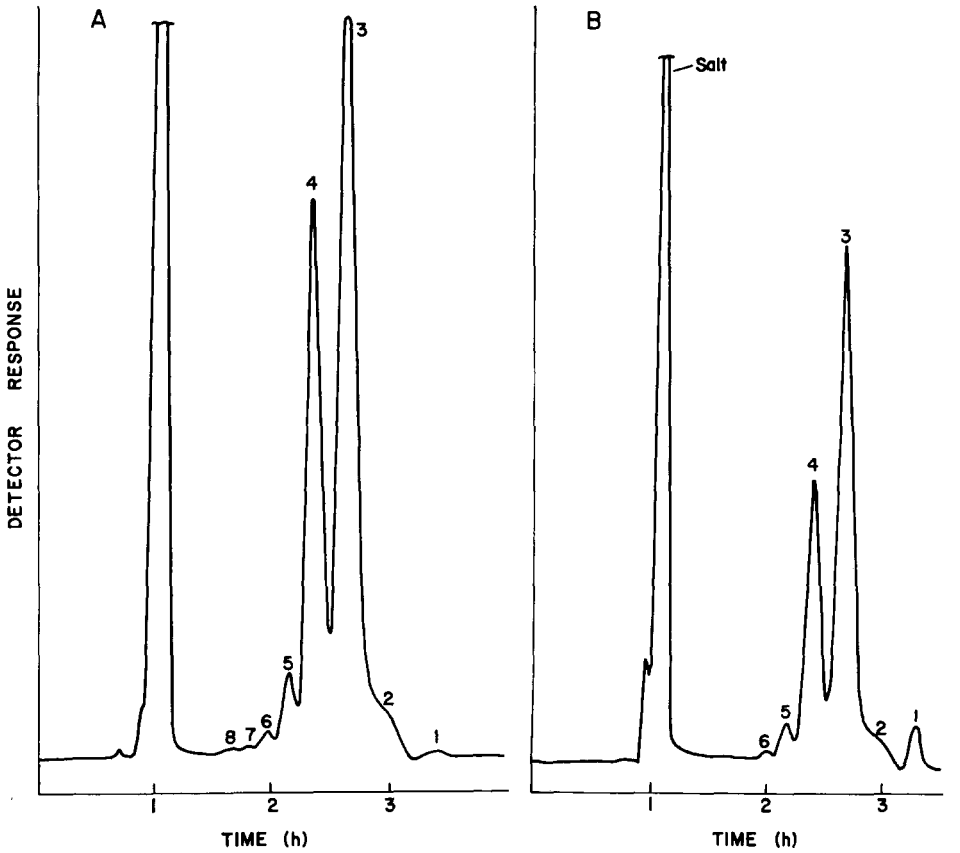

Fig. 1. Bio-Gel P-2 elution profile of soluble oligosaccharides released from 40°C water-soluble (A) and 65°C water-soluble (B) barley β-glucans by barley (1→3,1→4)-β-glucanase isoenzyme II. Numbers correspond to the DP of the oligosaccharides.

saccharide precipitated during hydrolysis of the 40°C water-soluble β-glucan (Table 2) is slightly higher than the amount (5% w/w) reported for the same polysaccharide in earlier experiments (Woodward *et al.*, 1983*b*).

Physical properties

Combined Huggins and Kraemer extrapolations of the viscosity data for the 65°C water-soluble β-glucan are shown in Fig. 2. The data are linear over the concentration range studied and indicate an intrinsic viscosity of 4·04 dl g^{-1} (Fig. 2). From this value for the intrinsic viscosity, and approximate values of 0·62 ml g^{-1} for the partial specific volume ($\bar{v}_2$) and 0·5 g g^{-1} for the degree of hydration (δ) (based on values for the 40°C

TABLE 2
Composition of Enzymic Hydrolysates of Barley β-Glucan Preparations

Component	*DP*	*40°C (% w/w)*	*65°C soluble (% w/w)*
(a) Water-soluble	1	1	4
	2	4	4
	3	56	58
	4	32	29
	5	5	5
	6	1	1
	7–8	< 1	0
(b) Water-insoluble	9 (average)[a]	7·4	7·0
(c) 80% ethanol precipitate of water-soluble products	not known	4·4	3·9

[a]Value determined for the 40°C water-soluble fraction by Woodward *et al.* (1983*b*). Values represent the mean of at least two determinations; errors are estimated at approximately 1%.

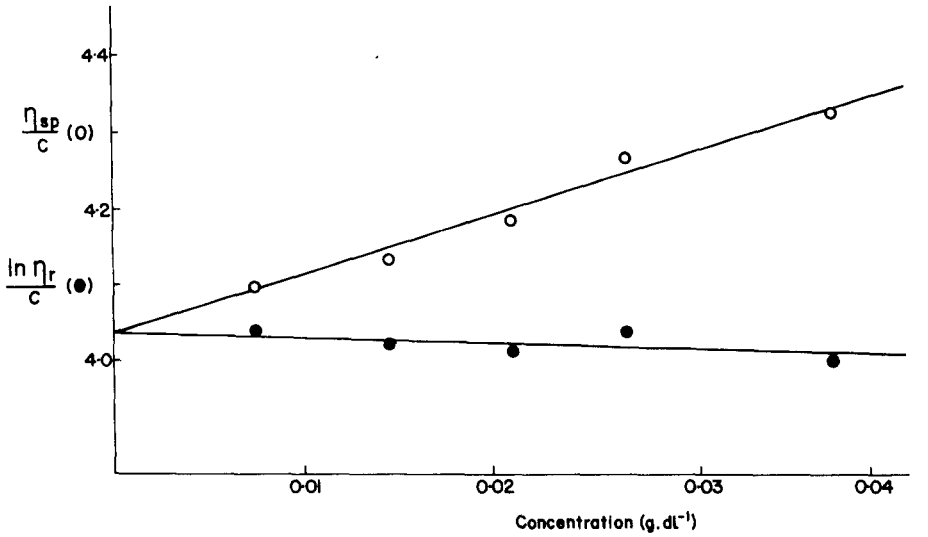

Fig. 2. Huggins (○) and Kraemer (●) viscometric data for 65°C water-soluble barley β-glucan.

β-glucans), the axial ratio of the 65°C β-glucan has been calculated as approximately 70. A value of this order of magnitude indicates that the β-glucan is essentially rod-like (Cantor & Schimmel, 1980). This conclusion is independent of the accuracy of the values used for $\bar{v}_2$ and δ, since

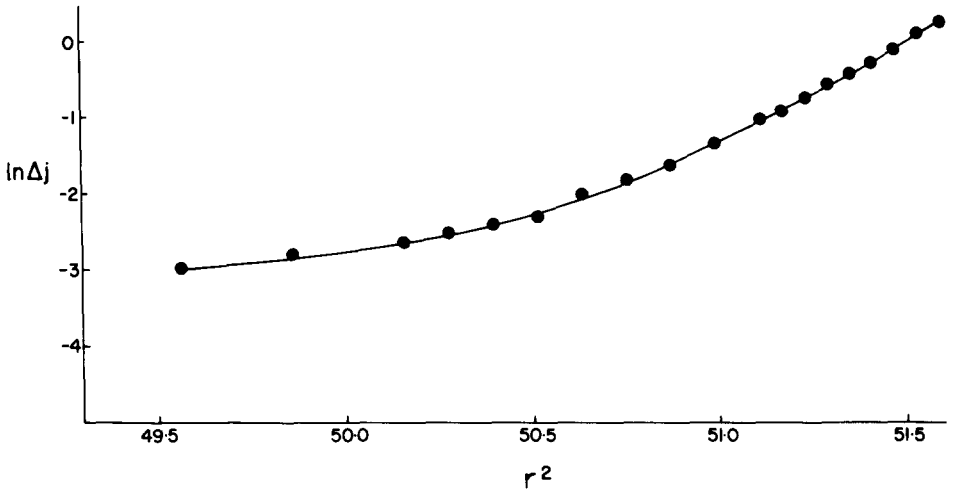

Fig. 3. Yphantis equilibrium sedimentation of 65°C water-soluble barley β-glucan. Δj is the interference fringe displacement and r is the radial distance.

both could vary by 100% and not affect the fact that the axial ratio of the β-glucan is very large.

The $\overline{M}_w$ value determined for the 65°C water-soluble barley β-glucan by conventional equilibrium sedimentation was 150000 and is accurate to within ±10%. The Yphantis equilibrium sedimentation data are shown in Fig. 3, where the non-linear nature of the curve indicates significant polydispersity of molecular size in the polysaccharide chains of the 65°C water-soluble β-glucan. The sedimentation data have been replotted in Fig. 4 to show the extent of this polydispersity. It is clear from Fig. 4 that there is an apparent limiting value of $\overline{M}_w$ of approximately 220000 for the 65°C water-soluble β-glucan. The polydispersity has been characterized further in Fig. 5, where the linear plot of $\Delta j^{-1/3}$ against reduced radius (where Δj is proportional to the β-glucan concentration) indicates a random molecular weight distribution of the 65°C β-glucan, as described by the most probable Schulz distribution (i.e. $M_n:M_w:M_z$ is 1:2:3) (Gibbons *et al.*, 1973). Similar linear plots were also observed for the 40°C β-glucan, indicating that both the 40°C and 65°C sources of β-glucan exhibit similar molecular weight polydispersity.

DISCUSSION

The (1→3,1→4)-β-glucan extracted with water from ethanol-inactivated barley flour in the temperature range 40–65°C and subsequently

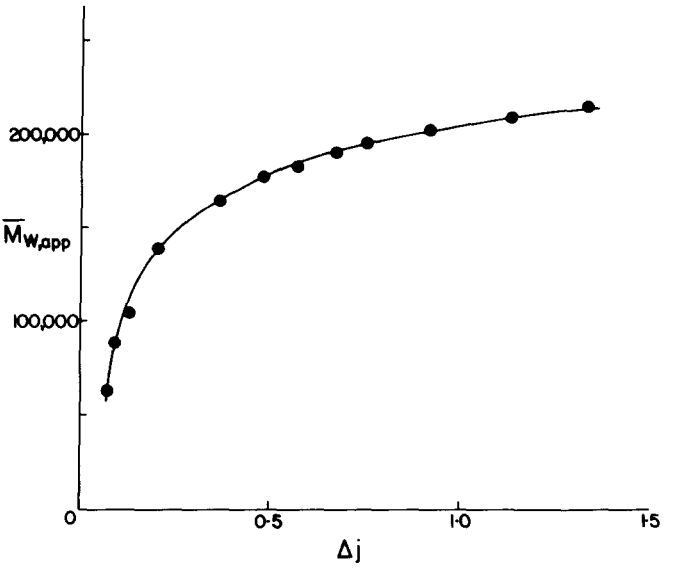

Fig. 4. Polydispersity of apparent weight average molecular weight ($\overline{M}_{w,app}$) for the 65°C water-soluble barley β-glucan. Δj is the interference fringe displacement.

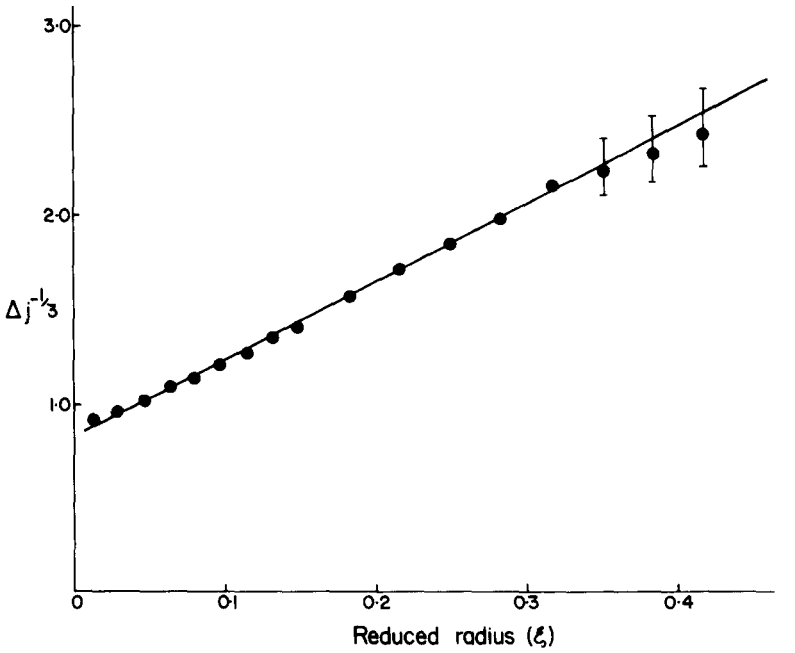

Fig. 5. Equilibrium distribution of 65°C water-soluble barley β-glucan under meniscus-depletion conditions. Δj is the interference fringe displacement and is proportional to β-glucan concentration (Gibbons *et al.*, 1973).

purified by fractional precipitation with ammonium sulphate, exhibits structural features and solution behaviour which differ slightly from the corresponding β-glucan extracted with water at 40°C. The 65°C water-soluble β-glucan was characterized by a lower percentage of (1→4)-linked β-glucosyl residues (69%, cf. 72% for the 40°C soluble fraction). Although this difference is small and values obtained here by methylation analysis were subject to approximately 1% error, the lower (1→4)-linkage content of the 65°C water-soluble β-glucan was reflected in the lower overall abundance of the longer blocks of adjacent (1→4)-linkages in the polysaccharide, as determined by enzymic hydrolysis (Table 2). The ratios of 3-*O*-β-D-cellobiosyl-D-glucose (DP3, generated from blocks of two adjacent (1→4)-linkages) to 3-*O*-β-D-cellotriosyl-D-glucose (DP4, generated from blocks of three adjacent (1→4)-linkages) in the 65°C and 40°C soluble fractions, were 2·0 and 1·7, respectively. In addition, levels of water-insoluble products formed during enzymic hydrolysis were slightly lower in the 65°C water-soluble fraction (Table 2). Indeed, we could find no evidence to support the notion that the 65°C water-soluble β-glucan contains longer blocks of adjacent (1→4)-linkages in greater abundance than the 40°C water-soluble fraction; this suggestion was advanced to explain differences in β-glucan solubilities (Woodward & Fincher, 1983). No contiguous (1→3)-linkages were detected in the 40°C water-soluble fraction (cf. Woodward *et al.*, 1983*b*) although these have been reported in some barley β-glucan preparations (Bathgate *et al.*, 1974; Fleming & Kawakami, 1977). We have not yet investigated the presence of adjacent (1→3)-linkages in the 65°C water-soluble fraction.

The 65°C water-soluble barley β-glucan studied here had a higher nitrogen content (0·4% w/w, Table 1) than the 40°C fraction (0·18% w/w; Woodward *et al.*, 1983*b*), but it is not known whether the nitrogen is proteinaceous in nature. Forrest (1977) showed that the 65°C soluble polysaccharide fraction from isolated barley endosperm cell walls is covalently associated with protein, and gel-filtration chromatography of the same material indicated an apparent molecular weight of 3×10^7 to 4×10^7 (Forrest & Wainwright, 1977). Sedimentation equilibrium ultracentrifugation of the 65°C water-soluble fraction examined here revealed a weight average molecular weight of 150000, which is considerably lower than the value of 290000 obtained by the same procedure for a 40°C water-soluble β-glucan prepared from the same sample of Clipper barley, but is similar to the value of 160000 calculated for a commercially available β-glucan extracted from barley flour at 40°C (Woodward *et al.*, 1983*a*). In addition, the 65°C water-soluble fraction from Clipper barley is characterized by a high degree of polydispersity with respect to

molecular weight (Fig. 4; cf. Woodward *et al.*, 1983*a*), with an upper, limiting value of approximately 220 000 (Fig. 4).

The lower overall molecular weight of the 65°C water-soluble fraction is reflected in its intrinsic viscosity value of 4·04 dl g^{-1} (Fig. 2), which is lower than 6·90 dl g^{-1} reported for the 40°C water-soluble fraction from Clipper. It is, however, similar to the value (4·26 dl g^{-1}) obtained for a commercial 40°C water-soluble β-glucan (Woodward *et al.*, 1983*a*). Axial ratios of the fractions bear a similar relationship. Care was exercised to prevent microbial contamination during the isolation, and the methods used, with the exception of the water extraction temperature, were exactly as described previously for the isolation of the 40°C water-soluble β-glucan (Woodward *et al.*, 1983*a*, *b*). Thus, the lower molecular weight and intrinsic viscosity of the 65°C water-soluble β-glucan, and its polydispersity, are unlikely to result from partial degradation during the isolation procedure or during the preparation of solutions for the physicochemical experiments.

The freeze-dried 65°C water-soluble β-glucan proved difficult to dissolve in aqueous buffer, requiring several hours at 90°C with constant stirring before dissolution was complete. Solutions were buffered to prevent hydrolysis. In contrast, the 40°C water-soluble β-glucan dissolved after 1–2 h at 70°C (Woodward *et al.*, 1983*a*). The 65°C water-soluble fraction also showed a greater tendency to precipitate from solution when the temperature was lowered. It is clear that the lower solubility of the 65°C water-soluble β-glucan is not due to a higher molecular weight or increased asymmetry. It has been suggested that the irregular spacing of (1→3)-linked β-glucosyl residues in the barley β-glucan chain is responsible for the irregular overall conformation of the polysaccharide (Woodward & Fincher, 1983). Thus, the polysaccharide remains in solution because the chains are unable to align closely over extended regions, and hence do not aggregate (Woodward & Fincher, 1983). One possible explanation for the apparently lower solubility of the 65°C water-soluble β-glucan relates to the lower abundance of long blocks of adjacent (1→4)-linkages in the polysaccharide chain; this might render the 65°C water-soluble β-glucan more regular in its overall conformation and hence more likely to aggregate. Indeed, lichenin, a (1→3,1→4)-β-glucan from Iceland moss (*Cetraria islandica*), has few long blocks of adjacent (1→4)-linkages relative to the 40°C water-soluble barley β-glucan (Parrish *et al.*, 1960; Reese & Perlin, 1963), it exhibits significant conformational regularity (Tvaroska *et al.*, 1983), and is sparingly soluble in cold water. Similarly, the (1→3,1→4)-β-glucan RSIII, which is prepared from a pneumococcal polysaccharide, is a structurally regular molecule consisting of strictly alternating (1→3)-

and (1→4)-linkages; it is extremely insoluble in aqueous media (Anderson & Stone, 1975).

Thus, the reduced solubility of the 65°C water-soluble barley β-glucan preparation compared with the 40°C water-soluble fraction might be attributable to small differences in fine structure which permit more extensive molecular aggregation. Whether the higher protein and uronic acid levels in the 65°C water-soluble β-glucan (Table 1) contribute to its solution behaviour is not clear. It is apparent, however, that the β-glucans of barley endosperm cell walls represent a family of polysaccharides of varying molecular size and fine structure, and that a precise description of their interactions with other β-glucan molecules, proteins, arabinoxylans and with fibrillar polysaccharides will be crucial in understanding the behaviour of the polysaccharides in the endosperm cell wall and in commercial processes.

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