ELSEVIER

PII: S0144-8617(96)00126-9

Carbohydrate Polymers 32 (1997) 7–15

0144-8617/97/$17.00 + .00

Physical and structural properties of barley (1→3),(1→4)-β-D-glucan. Part I. Determination of molecular weight and macromolecular radius by light scattering

C. Gómez[a], A. Navarro[a]*, P. Manzanares[a], A. Horta[b] & J. V. Carbonell[c]

[a]*Asociación de Investigación de Cerveza y Malta (INVESCEMA). P.O. Box 73, 46100-Burjassot, Valencia, Spain*
[b]*Departamento de Ciencias y Técnicas Fisicoquímicas, Facultad de Ciencias, Universidad a Distancia (UNED), C/Senda del Rey s/n, 28040 Madrid, Spain*
[c]*Instituto de Agroquímica y Tecnología de Alimentos (IATA, CSIC), P.O. Box 73, 46100-Burjassot, Valencia, Spain*

(Received 24 January 1996; revised version received 9 August 1996; accepted 16 August 1996)

(1→3),(1→4)-β-D-Glucan purified from the 65°C water extract of barley flour, commercial β-glucans and samples obtained by controlled depolymerisation of the former, covering a range from 9×10^3 to 6×10^5 dalton, were characterised by size exclusion chromatography and light scattering. The on-line measurement of molecular weight with a multiangle laser-light scattering photometer gave reproducible and consistent values according to: (a) the process followed in sample preparation; (b) their chromatographic elution in terms of hydrodynamic volume; and (c) the kinetics of depolymerization. However, batch light scattering measurements of β-glucan molecular weights did not give concordant and reproducible results. These last data, together with the study of the very early stages of enzymatic degradation of high molecular weight β-glucans by barley endo-β-glucanases as monitored by the Calcofluor–F.I.A. method, seem to suggest the formation of very labile molecular aggregates. © 1997 Elsevier Science Ltd

INTRODUCTION

The major structural component in barley endosperm cell wall is (1→3),(1→4)-β-D-glucan, (β-glucan). It can be considered as a linear chain of β(1→3)-linked cellotriosyl and cellotetraosyl units, arranged randomly. It represents only a minor fraction of the total carbohydrate content of barley kernels, but accounts for about 75% of total carbohydrates (Bamforth, 1982) in the cell walls of the endosperm, the remainder being 20–25% arabinoxylan and minor amounts of cellulose, glucomannan and protein (Fincher, 1992).

During barley germination, these polymers are degraded into shorter chains by the endo-action of β-glucanases, synthesised *de novo* in the aleurone layer and in the scutellum. Two isoenzymes of (1→3),(1→4)-β-D-glucan 4-glucanohydrolase [barley endo-(1→3),(1→4)-β-D-glucanase or licheninase (EC 3.2.1.73)] are mainly responsible for the degradation of barley β-glucan to lower molecular weight β-glucan (Woodward & Fincher, 1982). These isoenzymes hydrolyse a (1→4)-linkage in mixed linkage (1→3),(1→4)-β-D-glucans, where the D-glycosyl residue is substituted at the three position, that is where the (1→4) linkage is adjacent to a (1→3) linkage.

The degradation is often incomplete and long chains of β-glucans are still present in resulting malts. In the brewing process, these chains are transferred into the wort during the mashing process, most of them remaining in final beers, unless exogenous β-glucanases are added. An excess of β-glucans can cause several problems, such as slow filtration of worts and beers, decreased brewhouse yield, and formation of haze or precipitates in stored beers (Bamforth, 1982). The magnitude of these problems depends on the total β-glucan content and on its molecular weight (Wagner *et al.*, 1988). Otherwise, β-glucans, as constituents of dietary fibre, can be responsible for positive physiological effects in humans (McIntosh *et al.*, 1991). Therefore, it seems obvious that knowledge of the physico-chemical

*Author to whom correspondence should be addressed.

properties of β-glucans in solution is of basic significance.

There is disagreement among the data on β-glucan molecular weights reported in the literature, and also important differences in values of the viscosities of their aqueous solutions. These differences had, up to now, been attributed to differences in barley varieties, extraction conditions, and analytical techniques employed. Only a few systematic studies, focused on elucidating the structure and conformation of β-glucan molecules, have been published (Forrest & Wainwright, 1977; Woodward *et al.*, 1983a; Buliga *et al.*, 1986; Vårum *et al.*, 1991), but the results also showed some discrepancies. Even the most recent studies (Vårum *et al.*, 1992; Grimm *et al.*, 1995) were in clear disagreement about the aggregation behaviour and the stability of aggregates of β-glucan molecules.

In the present work, we have prepared a wide range of β-glucan samples differing in their weight average molecular weight (from 9×10^3 to 6×10^5 dalton), verified their purity, analysed them by high performance size exclusion chromatography (HPSEC) and by light scattering, both on line with chromatography and in separate batch experiments. From the results obtained, the β-glucan molecules are characterised with respect to molecular weights and macromolecular radius, and the results compared with other data in the literature.

MATERIALS AND METHODS

Reagents and standards

Commercial barley β-glucan samples were obtained from Sigma (Sigma Chemical Co., St. Louis, MI; lots no. 81H0472—sample D in Table 1—and 92H0787—sample G) and Megazyme (Megazyme Australia Pty Ltd, Warriewood, Sydney; lot no. 30108, sample F). Commercial oat β-glucan (lot no. 0BG20606, sample C) was also obtained from Megazyme. Pullulan standards of average molecular weight 853 000, 380 000, 186 000 and 23 000 were purchased from Polymer (Polymer Lab. Ltd, Shropshire, UK). Stock solutions (3–5 g/l) were made by dissolving the reference compounds in hot water, followed by filtration of the solution through a 0·45 μm nylon membrane filter. Standard solutions were prepared from stock solutions by dilution in water.

Heat-stable α-amylase (Brewers Amyliq. TS, 6500 TAU/g) was kindly provided by Gist Brocades (Gist Brocades S. A., Sedin, France). Commercial licheninase {(1→3),(1→4)-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73] (1 U/μl)} was obtained from Megazyme (lot no. 011011). Proteinase K (lot no. 14080328) was purchased from Boehringer (Boehringer Mannheim Gmbh Biochemica, Mannheim, Germany). Amyloglucosidase (lot no. 318E218532) was supplied by Merck (E. Merck, Darmstadt, Germany). α-glucosidase (lot no. 51446/1693) was purchased from Fluka (Fluka Chemika-Biochemika, Buchs, Switzerland). β-Amylase was from Sturge Enzymes (USA).

Table 1. Origin, chemical characterisation and intrinsic viscosity of ß-glucan samples

Sample	Origin	Protein (%, d.b.)	Starch (%, d.b.)	Intrinsic viscosity (dl/g)
A	Barley extraction	1.50	1.30	5.2
B	Barley extraction	n.d.	0.80	4.6
C	Commercial (Megazyme, from oat)	n.a.	n.a.	2.8
D	Commercial (Sigma, from barley, lot no. 1)	n.d.	n.d.	2.8
E	Barley extraction	0.28	n.d.	3.0
F	Commercial (Megazyme, from barley)	n.d.	n.d.	2.7
G	Commercial (Sigma, from barley, lot no. 2)	n.d.	n.d.	2.7
H	Barley extraction	0.16	n.d.	n.a.
I	Enzymic degradation of barley extracted samples	0.16	n.d.	1.77
J	Enzymic degradation of barley extracted samples	0.40	n.d.	1.38
K	Enzymic degradation of barley extracted samples	0.16	n.d.	n.a.
L	Enzymic degradation of barley extracted samples	0.02	0.20	0.73
M	Enzymic degradation of barley extracted samples	0.06	n.d.	0.76
N	Enzymic degradation of barley extracted samples	n.d.	n.d.	0.28

n.d. = not detected.
n.a. = not analysed.

To inactivate residual β-glucanase activity, α-amylase was incubated at 100°C for 2 h, then the suspension centrifuged (1000 g for 10 min), and the supernatant used as such.

Silica hydrogel, commercial name Glucilite, was purchased from Crossfield Group (Warrington, UK).

Water used was Milli-Q grade.

Extraction and purification of β-glucan samples from barley

β-Glucan samples were prepared from an industrial batch of barley harvested in Spain in 1992. Barley grains were ground in a DLFU Disc Mill (Buhler-Miag) with a 0·2 μm gap (fine grind); 200 g of barley flour were suspended in 1 l of 80% (v/v) ethanol. Enzyme inactivation was achieved after incubation for 30 min in a boiling water bath. The slurry was filtered through filter paper and washed with 1 l of 80% (v/v) aqueous ethanol and then with 1 l of 96% (v/v) ethanol.

β-Glucan extraction and purification was carried out according to McCleary's procedure (McCleary, 1988) with some modifications. Inactivated barley flour (approximately 1 kg) was suspended in 10 l of water containing 5 ml of heat-treated α-amylase and incubated at 65°C for 2 h with continuous stirring. Aqueous extract was separated by centrifugation at 3000 **g** for 15 min and the supernatant incubated overnight at 90°C with 15 ml of heat-treated α-amylase and afterwards with 6 ml of proteinase K (20 mg/ml, 20 U/mg) for 12 h at 37°C. After enzymic treatment the solution was cooled to room temperature, treated with ammonium sulphate (20 g/100 ml) and stored at 4°C for 20 h. The precipitate was collected by centrifugation at 3000 **g** for 15 min. The ammonium sulphate treatment was repeated twice.

The precipitate collected was suspended in 20% (v/v) aqueous ethanol, recovered by centrifugation (3000 **g** for 15 min), washed again with 20% (v/v) aqueous ethanol and then with 96% (v/v) ethanol. The precipitate was recovered by centrifugation (3000 **g** for 15 min) dissolved in boiling water (approx. 2–3 g/l) and treated again with α-amylase (0·5 ml/l) at 90°C for 3 h to remove starch traces. The cold solution was treated with 100 ml/l of an aqueous silica hydrogel suspension (10% w/v) and stored at 25°C for 20 h to remove protein traces. After centrifugation (3000 **g** for 15 min) the supernatant was diluted with an equal volume of ethanol (96% v/v) and stored at 4°C for 20 h. The precipitate was centrifuged (3000 **g** during 15 min), successively washed with 96% (v/v) ethanol, acetone and ethyl ether, and dried under vacuum.

Enzymic degradation of extracted β-glucan samples from barley

Some β-glucan samples (I, J, K, L, M and N) were obtained by enzymic degradation of high molecular weight barley β-glucan with licheninase (1·3 U/l of solution containing about 6·5 g β-glucan and incubated at 30°C), stopping the degradation by heating at 100°C for 2 h. The degradation of β-glucan was monitored in an HPSEC–F.I.A. system (Manzanares *et al.*, 1993).

Preparation of β-glucan samples prior to analysis

The extracted, degraded and commercial β-glucan samples were analysed for their protein (Bradford, 1976) and starch contents. Residual starch was determined by treating aqueous β-glucan solutions (6 g/l), diluted in acetate buffer (0·1 M, pH5) and treated successively with the following enzymes: 100 μl of α-amylase for 1 h at 90°C, then with 100 μl of amyloglucosidase (10 mg/ml, 75 U/mg) during 1 h at 50°C, and finally with 100 μl of β-amylase (10 mg/ml) and 100 μl of α-glucosidase (10 mg/ml, 57 U/mg) for 1 h at 37°C. Glucose liberated was measured by means of the hexokinase/glucose-6-phosphate dehydrogenase system (Kunst *et al.*, 1984).

Finally, the β-glucan powder was dissolved in boiling water previously filtered (0·22 μm, nylon filter), and then the solution is filtered again through a 0·45 μm nylon filter and left at room temperature until the analysis.

HPSEC-MALLS-RI

The liquid chromatograph consisted of an isocratic pump (Hewlett Packard, series 1050), a sample injection valve (Rheodyne, model 7125) with a 50 μl loop, column oven (Waters, model TCM), two PL-GFC columns in series (1000 Å, 8 μm, 7·5×300 mm and 4000 Å, 8 μm, 7·5×300 mm) with a guard column (7·5×50 mm) and a differential refractive index detector (Waters, model 410). Columns were operated at 80°C, whereas the temperature of the refractometer cell was 40°C. The mobile phase was degassed Milli-Q water at a flow rate of 1 ml/min.

A multi-angle laser light scattering photometer (Wyatt, model Dawn F) equipped with an argon-ion laser-light source ($\lambda = 488$ nm), connected to the liquid chromatograph through a flow cell (Wyatt, model F-2), was used for the on-line measurement of β-glucan molecular weights. The temperature of the photometer cell was 25°C. A compatible personal computer using Astra software (Wyatt) was applied to collect and analyse data.

At each elution volume, V_i, the signal from the differential refractive index detector is proportional to β-glucan concentration, c_i. Also, at each elution volume, V_i, we have a set of signals from the light scattering detector, one for each scattering angle, θ. These signals give $R_i(\theta)$, the excess Rayleigh ratio at angle θ for elution volume V_i (where $R_i(\theta) = R_i(\theta)_{solution} - R_i(\theta)_{solvent}$).

The extrapolation to 0 angle is performed in the

Debye type plot, namely, $R_i(\theta)/K{\cdot}c_i$ vs $\sin^2\theta/2$, from which $M_{w,i}$ is obtained as intercept, K being the optical constant, $K=(4\pi^2 n_o^2/\lambda_o^4\ N_A)\ (dn/dc)^2$. The initial slope of such extrapolation also yields the mean square radius of gyration, $<s^2>_i$, of the macromolecule having $M_{w,i}$. With our samples, the extrapolation was linear except for the samples of higher molecular weight, which required a quadratic regression in $\sin^2\theta/2$.

The molecular weight average and macromolecular radius corresponding to the samples can be calculated according to the following equations:

$$M_n = \left(\frac{\sum c_i/M_{w,i}}{\sum C_i}\right)^{-1} \qquad M_w = \frac{\sum c_i \cdot M_{w,i}}{\sum c_i}$$

$$M_z = \frac{\sum c_i \cdot M_{w,i}^2}{\sum c_i \cdot M_{w,i}} \qquad <S>_w^2 = \frac{\sum c_i \cdot <s>_i^2}{\sum c_i}$$

The differential refractive index increment (dn/dc) of barley β-glucan was determined with an interferometric refractometer (Wyatt, model Optilab 903), calibrated with aqueous NaCl and operating at the laser photometer wavelength. Aqueous β-glucan solutions used were in the range $0{\cdot}2\times10^{-3}$–4×10^{-3} g/ml, and the dn/dc value, calculated by linear regression analysis data, was 0·151 ml/g.

RESULTS AND DISCUSSION

Molecular weights and radii

Tables 1 and 2 show the physical and chemical characteristics of commercial, laboratory extracted and degraded β-glucan samples used in the experiments.

Several chromatographic injections of a given sample were performed, changing either the solute concentration or the total volume injected, Table 2 shows the average values. The reproducibility of the results was good. For most of the cases, the deviations were below ±5% in weight average molecular weight, M_w, or polydispersity index, M_w/M_n, and below ±10% in the root-mean-square radius, $<s^2>_w^{1/2}$.

The molecular weight range covered by these samples is quite wide ($M_w=9\times10^3$–6×10^5), and the polydispersity indexes obtained were not high ($M_w/M_n=1{\cdot}1$–$1{\cdot}7$). These values agree with those reported by Vårum *et al.* (1992) in oat β-glucans (polydispersity indices between 1·2 and 1·7, weight average molecular weights from 2×10^4 to $1{\cdot}4\times10^5$) and those of Woodward *et al.* (1983b) in barley β-glucans (polydispersity of 1·1–1·4, weight average molecular weights of $2{\cdot}1\times10^5$ and $2{\cdot}9\times10^5$) in spite of the different extraction conditions. Nevertheless, Saulnier *et al.* (1994) gave a higher polydispersity index (2·2 for a sample of $M_W=1{\cdot}5\times10^5$).

Table 2 also contains the molecular weight corresponding to the maximum of the chromatographic peak as detected by the differential refractometer, M_p(RI), and the molecular weight corresponding to the maximum of the peak described by the light scattering intensity at $\theta=90°$, M_p(LS).

The values corresponding to the commercial pullulan standards are shown in Figs 1 and 2.

Chromatographic calibration

The plot of log molecular weight vs elution volume (V_e) for β-glucans and pullulans is shown in Fig. 1. The molecular weight corresponding to the maximum of the chromatographic peak recorded by the refractive index detector, M_p(RI), was chosen in this graph. With polydisperse samples, it is more rigorous to use average values, $\bar{M}$ and $\bar{V}_e$, calculated from the whole peak profile by some adequate method (Barrales-Rienda *et al.*, 1985), but in our case the polydispersity indexes were not too high, giving an excuse for the simplification.

Table 2. Molecular characterisation of ß-glucan samples

Sample	M_n ($\times10^{-3}$)	M_w ($\times10^{-3}$)	M_z ($\times10^{-3}$)	M_p(LS) ($\times10^{-3}$)	M_p(RI) ($\times10^{-3}$)	$<s^2>_w^{1/2}$ (nm)	M_w/M_n
A	482	573	668	641	616	43.3	1.18
B	308	456	591	671	585	35.7	1.48
C	197	278	400	309	215	35.0	1.41
D	151	253	337	338	251	27.0	1.67
E	186	235	291	312	188	28.4	1.26
F	179	231	295	267	205	28.2	1.30
G	164	231	339	245	173	27.9	1.41
H	143	205	284	267	193	26.8	1.43
I	78	105	139	128	96	22.5	1.35
J	69	106	437	101	70	22.5	1.54
K	68	102	144	147	94	18.6	1.50
L	31	36	41	40	29	17.0	1.16
M	24	27	32	31	21	15.3	1.12
N	8.4	9.2	12.9	7.8	6.9	12.2	1.10

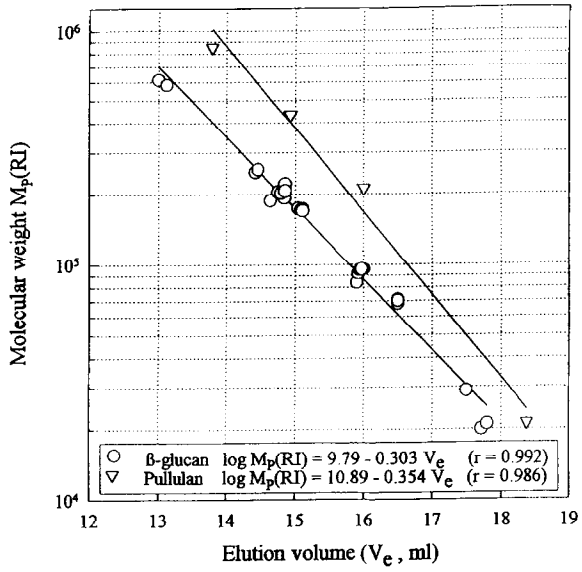

Fig. 1. Semi-logarithmic plot of molecular weight vs elution volume for β-glucans and pullulans.

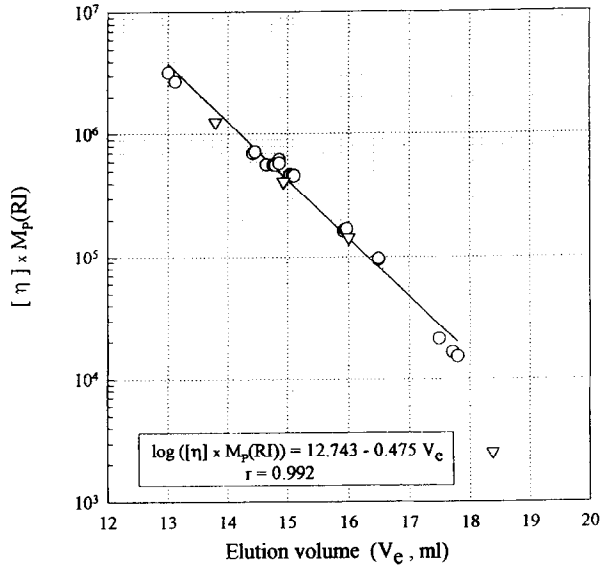

Fig. 2. Semi-logarithmic plot of the hydrodynamic volume, [η] x M_p(RI), vs elution volume for β-glucans and pullulans.

As it can be seen in Fig. 1, the calibration curve for β-glucans, in the broad range of molecular weights assayed, was almost linear with only a slight curvature. The coefficients of the linear fit are given in the figure insert. β-Glucan macromolecules showed advanced elution with respect to the flexible pullulan coils of equal molecular weight. So, if the calibration with pullulan standards were used to determine molecular weight of β-glucans, these would be highly overestimated, with an error of more than 100% of the real molecular weight in almost the whole range covered. The macromolecules of β-glucans are more extended than random coils of pullulans and thus elute at an earlier time, behaving as if their molecular weights were much higher than they actually are.

When no other secondary process such as adsorptions or strong interactions occur in the column, the calibration can be made universal by using the hydrodynamic volume, $[\eta]M$, instead of M, where $[\eta]$ is intrinsic viscosity. Figure 2 shows the universal calibration for β-glucans samples together with the pullulan standards. Intrinsic viscosities have been taken from the accompanying paper (Gómez *et al.*, 1996). As can be seen, the two polymers, β-glucans and pullulans, fall within the same curve, so the universal calibration concept holds well for the β-glucan macromolecules, confirming over a wider range of molecular weights the results reported by Vårum *et al.* (1991). β-Glucans are more extended than molecules of pullulans, but when this effect is taken into account through the use of the hydrodynamic volume occupied by them, then a single calibration is obtained. Thus, no secondary processes of fractionation are apparent in this case. Water is not an especially good solvent for β-glucans, so the more extended macromolecule occupying a higher hydrodynamic volume should be the consequence of chain stiffness rather than of coil swelling or excluded volume.

With the on-line light scattering detector mode we got reproducible results of β-glucan molecular weights which, together with the ones on pullulan molecules, give us a good universal calibration. Certainly the molecular weight values in Table 2 keep consistency among them and in relation to other molecular properties.

Depolymerization kinetics

Moreover, we could get further support for the consistency of our molecular weight values by following the kinetics of change in β-glucan molecular weight by the action of endo-β-glucanases (Fig. 3). The highest molecular weight sample (sample A) was depolymerised in aqueous solution, under controlled conditions, by a bacterial licheninase, an endo-β-glucanase having the same mechanism of action as malt licheninase, that is, it also randomly cleaves β-(1→4) bonds that are adjacent to a β-(1→3) bond. Aliquots of the incubation mixture of the β-glucan/licheninase solution were periodically taken, stopped by heating and immediately injected in the HPSEC system and their elution volume determined. Using the β-glucan calibration curve shown in Fig. 1, the molecular weight of corresponding injected aliquots was calculated. The experimental values fitted closely to the equation expected from the mechanism of depolymerization of the enzyme (see below).

This equation can be deduced derivating $W = S \cdot M_n$ with respect to time, t, where W is the concentration (w/v) of β-glucans and S their molar concentration.

$$dW/dt = S dM_n/dt + M_n dS/dt$$

It could be assumed that, during the majority of the depolymerisation process, most of the molecular fragments produced by the action of this endo-enzyme can

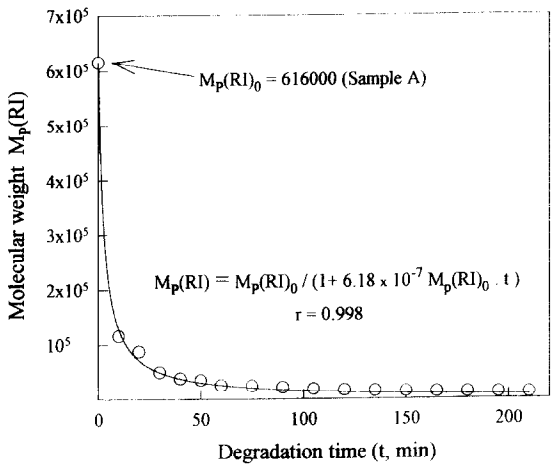

Fig. 3. Evolution of the molecular weight of β-glucan during their degradation by endo-β-glucanases.

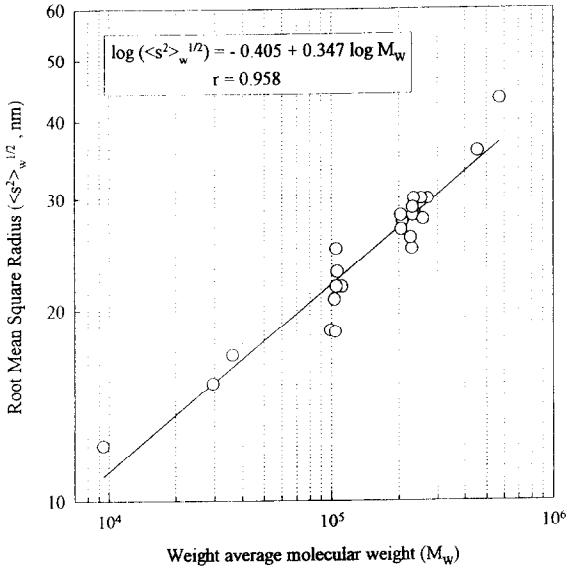

Fig. 4. Double logarithmic plot of root-mean-square radius of β-glucan molecules vs their weight average molecular weight in different β-glucan samples.

be the target of new enzymic attacks, the production of true product (mainly tri- and tetrassacharides) being negligible. In this condition:

$$dW/dt = 0 \quad W = W_0 \quad dS/dt = k'[ES] = k'k''W_0$$

[ES] being the molar concentration of the enzyme–substrate complex, and k' and k'' proportionality constants. Then

$$(W_0/M_n)dM_n/dt + M_n.k'.k''W_0 = 0$$

$$(1/M_n^2)dM_n = -k'.k''.dt = -k.dt$$

and integrating between times 0 and t

$$M_n = M_{n0}/(1 + k.M_{n0}.t)$$

This equation-type, initially deduced for the number average molecular weight, can be applied using any other average molecular weight, just by modifying the constant factor k.

Scaling of root-mean-square radius

The variation of $<s^2>_w^{1/2}$ with M_w is shown in Fig. 4 as double logarithmic plot. The results follow a scaling law with exponent ≈0·35, below that corresponding to a random coil and more akin to that of a sphere. This is surprising in view of the molecular characteristics of the β-glucan chain, which should adopt a more extended conformation. However, the results for this type of exponent reported in the literature vary widely. Thus, Vårum et al. (1992) found a higher value ($<s^2>_w^{1/2} = 0{\cdot}03\ M_w^{0{\cdot}59}$), but using degraded oat β-glucan of a narrower range of molecular weights; and very recently, Grimm *et al.* (1995), described a very low exponent of 0·22 in the molecular weight dependence of the root-mean-square radius, but from gel-forming β-glucan isolated from beer and showing concentration-dependent aggregation. Therefore, similar aggregation could be suspected as origin for our low exponent, but our $<s^2>_w^{1/2}$ values were from on-line light scattering data where no aggregation of β-glucan could be detected.

As a further check for this anomalous low exponent we analysed the individual chain lengths composing a given chromatographic peak. The on-line configuration allows for the simultaneous determination of $<s^2>_{wi}^{1/2}$ and M_{wi} for each V_i in the peak. In this way, a $<s^2>_{wi}^{1/2}$ vs M_{wi} scaling can be determined for each sample. As an example, that of sample A is shown in Fig. 5. The scaling exponents thus determined that each sample may differ from the global value (0·35) found with the set of averages $<s^2>_w^{1/2}$ vs M_w, but they also

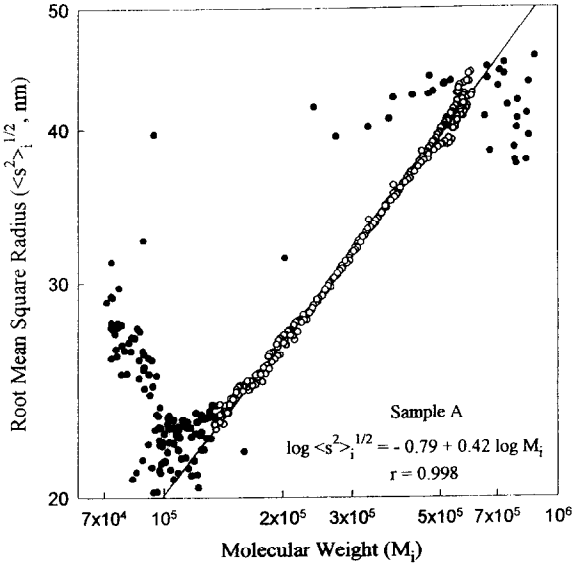

Fig. 5. Double logarithmic plot of root-mean-square radius of β-glucan molecules vs their weight average molecular weight in individual chain lengths composing the sample A (black symbols were not used in the regression fitting).

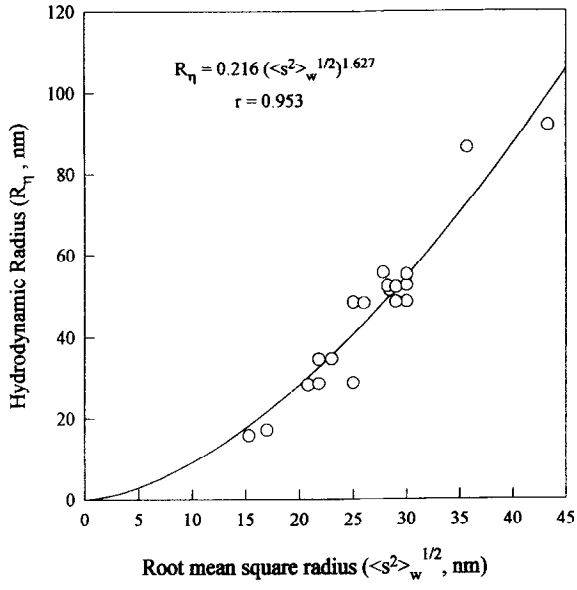

Fig. 6. Relationship between the hydrodynamic radius and the root-mean-square radius of β-glucan molecules.

fall below the 0·5 random coil value (for example, in Fig. 5 it is 0·42 for the range $M_{wi} = 1{\cdot}3 \times 10^5$ to 6×10^5). Thus, a consistent determination of the scaling exponent is obtained from averages for the different samples and from the chromatogram of an individual sample.

The scaling exponent adopts a value higher than 0·5 when the radius of the macromolecule is obtained from a different property. Thus, if we consider the hydrodynamic radius, R_η, as $(3/4\pi)^{1/3}([\eta]M)^{1/3}$, then the scaling exponent is $R_\eta \approx M_w^{0.57}$. It remains to be explained why $<s^2>_w^{1/2}$ gives a lower value. The relationship between both types of radii is shown in Fig. 6.

Light scattering analysis in batch mode

The measurement of molecular weight and root-mean-square radius of β-glucans by the batch mode did not give concordant results with the former findings using the on-line flow system. Although the monitoring technique is essentially the same (light scattering), the batch mode and the on-line flow mode used here differ substantially in procedure. In the batch mode, solutions of several concentrations are prepared, filtered, poured into the batch cell and measured statically. In the on-line flow mode, one solution is injected, fractionated through a chromatographic column and monitored while flowing. Therefore, the β-glucan concentrations passing through the flow cell are much lower than those commonly used in the vials introduced inside the batch cell. We have performed light scattering experiments in the batch mode with most of our samples and have found molecular weights higher than those of Table 2 and extending up to the million range. We have detected in these experiments many deficiencies in reproducibility, such as can be observed comparing the Zimm plots in Fig. 7, corresponding to two separate determinations of the same sample (G). This type of problem has also been reported by Vårum *et al.* (1992) with oat β-glucan, by Berth *et al.* (1994) with citrus pectin, and by Lang and Burchard (1993) with tamarind seed polysaccharide (a cellulose derivative carrying xylose and galactoxylose side chains).

According to Vårum *et al.* (1992) a possible explanation of the apparently anomalous behaviour of β-glucan molecules in the Zimm plots would be their tendency to the formation of labile molecular aggregates. These may disappear by the effect of very weak shear forces, such as those occuring in a pipeline, a flow-cell or a size exclusion column. Thus, the light scattering analysis in batch mode would be influenced by uncontrolled factors affecting the stability of those aggregates.

To try to remove these aggregates, the filtration of samples through different pore size membranes was investigated. In spite of the reproducibility problems, the big differences in molecular weight values seemed to establish a clear correspondence between those values and the pore sizes of membrane filters (Table 3). However, the β-glucan concentration in the stock solutions showed a very small decrease, not compatible with the mass balance in the filtration. This result would

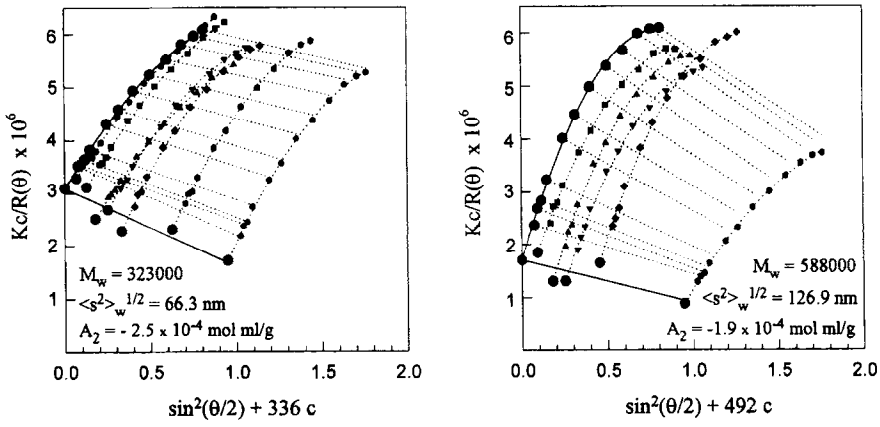

Fig. 7. Zimm plots corresponding to two separate assays of the same β-glucan sample (G).

Table 3. Variation of the apparent values of weight average molecular weight and root-mean-square radius of the β-glucan sample G according to the pore size of the membrane filter employed prior to analysis by static light scattering at 70°C

Sample treatment	Mw ($\times 10^{-6}$)	$<s^2>_w^{1/2}$ (nm)	Concentration of the β-glucan stock solution (mg/l)
Unfiltered	221.0	951	4430
Filtered up to 0.22 μm	43.4	362	4130
Filtered up to 0.022 μm	3.2	226	3910

suggest the breakdown of most of the molecular aggregates because of the shear forces produced through the membranes (Vårum *et al.*, 1992).

The analysis of the samples by light scattering in batch mode was performed at 70°C and 25°C. The absolute values of scattered light intensity were higher (about 70%) at 70°C than at 25°C in all samples, giving higher apparent molecular weights at 70°C (Table 3). This finding suggests that if the high molecular weight species are aggregates of β-glucan molecules, then in our case this aggregation is enhanced by the temperature. This hypothesis is not unrealistic, since in certain water-soluble polymers a decrease in solubility occurs with increasing temperature (Molyneux, 1983). Furthermore, another recent study (Chambers *et al.*, 1994) has reported, by means of the same HPSEC-MALLS technique, the formation of aggregates on the heating of a water-soluble natural polysaccharide: κ-carrageenan.

Moreover, in some experiments of degradation of β-glucans (231 kD) by endo-β-glucanases a very slight increase in the concentration of β-glucan, as measured by the fluorimetric calcofluor–F.I.A. method was occasionally detected during the very early stages of degradation (Navarro et al., 1995). A series of assays to amplify this initial phase of depolymerisation, using very weak enzyme solution, was performed. The results are shown in Fig. 8. As can be observed, a clear increase in the amount of β-glucan detected appears in the sample of higher molecular weight (573 kD); this increase is quite a bit lower in the sample of 456 kD and practically negligible in the degradation of the sample G (231 kD). This increase could be the effect of the rapid disaggregation of β-glucans, which make their linkage to the calcofluor easier and would enhance the fluorescent signal, confirming the results reported by Vårum *et al.* (1992) and Grimm *et al.* (1995) about the aggregation of β-glucan molecules.

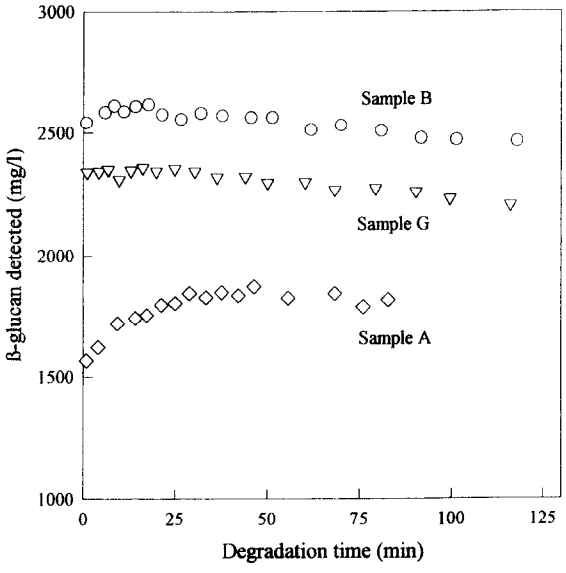

Fig. 8. Increase in concentration of β-glucan detected by the fluorimetric calcofluor–F.I.A. method during the very early stages of enzymic depolymerisation of high molecular weight samples.

ACKNOWLEDGEMENTS

This work was supported by Cerveceros de España and the Comisión Interministerial de Ciencia y Tecnología (CICyT), Project ALI 93-0067.

REFERENCES

Bamforth, C.W. (1982). Barley β-glucans. Their role in malting and brewing. *Brew. Dig.*, **35**, 22–27.

Barrales-Rienda, J.M., Galera, P.A., Horta, A. & Sáiz, E. (1985). Gel permeation chromatography of poly (N-vinyl-3,6-dibromocarbozole). Universal calibration, non-exclusion effects and incompatibility. *Macromolecules*, **18**, 2572–2579.

Berth, G., Dautzenberg, H. & Rother, G. (1994). Static light scattering technique applied to pectin in dilute solution. Part I: reliability of experiments and model calculations. *Carbohydr. Polym.*, **25**, 177–185.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.*, **72**, 248–254.

Buliga, G.S., Brant, D.A. & Fincher, G.B. (1986). The sequence statistics and solution configuration of a barley (1→3),(1→4)-β-D-glucan. *Carbohydr. Res.*, **157**, 139–156.

Chambers, S.J., Langdon, M.J., Williams, P.A. & White, R.J. (1994). Combined differential light scattering with size exclusion chromatography techniques for monitoring conformational changes in κ-carrageenan. In *Gums and Stabilisers for the Food Industry*, Vol. 7, eds G.O. Phillips, P.A. Williams & D.J. Wedlock. IRL Press, Oxford, pp. 37–53.

Fincher, G.B. (1992). Cell wall metabolism in barley. In *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*, ed. P.R. Shewry. Biotechnology in Agriculture Series no. 5, C.A.B. International, Wallingford, pp. 413–437.

Forrest, I.S. & Wainwright, T. (1977). Differentiation between desirable and troublesome β-glucans. *Eur. Brew. Conv., Proc. Congr. 16th*, Amsterdam, pp. 401–413.

Gómez, C., Navarro, A., Manzanares, P., Horta, A. & Carbonell, J.V. (1997). Physical and structural properties of barley (1→3),(1→4)-β-D-glucan. Part II. Viscosity, chain stiffness and macromolecular dimensions. *Carbohydr. Polym.*, **32**, 17–22.

Grimm, A., Krüger, E. & Burchard, W. (1995). Solution properties of β-D-(1,3)(1,4)-glucan isolated from beer. *Carbohydr. Polym.*, **27, 3,** 205–214.

Kunst, A., Draeger, B. & Ziegenhorn, J. (1984). UV methods with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods in Enzymatic Analysis* , 3rd edn, Vol. 6, ed. H.U. Bergmeyer. Verlag Chemie, FL, pp. 163–171.

Lang, P. & Burchard, W. (1993). Structure and aggregation behavior of tamarind seed polysaccharide in aqueous solution. *Makromol. Chem.*, **194,** 3157–3166.

Manzanares, P., Navarro, A., Sendra, J.M. & Carbonell, J.V. (1993). Determination of the average molecular weight of barley β-glucan within the range 30–100 kD by the Calcofluor–F.I.A. method. *J. Cereal Sci.*, **17,** 211–223.

McCleary, B.V. (1988). Purification of (1→3),(1→4)-β-D-glucan from barley flour. In *Methods in Enzymology*, Vol. 160, eds W.A. Wood & S.T. Kellogg. Academic Press, San Diego, pp. 74–86.

McIntosh, G.H., Whyte, J., McArthur, R. & Nestel, P.J. (1991). Barley and wheat foods influence on plasma cholesterol concentrations in hypercholesterolemic men. *Amer. J. Clin. Nutrit.*, **53,** 1205–1209.

Molyneux, P. (1983). *Water-Soluble Synthetic Polymers: Properties and Behaviour*, Vols I and II. Chemical Rubber Company, Boca Ratón, FL.

Navarro, A., Manzanares, P., Carbonell, J.V. & Sendra, J.M. (1995). Determination of (1→3),(1→4)-β-D-glucanase activity by a calcofluor-flow injection analysis method. *J. Cereal Sci.*, **22,** 275–284.

Saulnier, L., Gevaudan, S. & Thibault, J.F. (1994). Extraction and partial characterisation of β-glucan from the endosperm of two barley cultivars. *J. Cereal Sci.*, **19,** 171–178.

Vårum, K.M., Martinsen, A. & Smidsrød, O. (1991). Fractionation and viscometric characterization of a (1→3),(1→4)-β-D-glucan from oat and universal calibration of a high-performance size exclusion chromatographic system by the use of fractionated β-glucans, alginates and pullulans. *Food Hydrocoll.*, **5,** 363–374.

Vårum, K.M., Smidsrød, O. & Brant, D.A. (1992). Light scattering reveals micelle-like aggregation in the (1→3),(1→4)-β-D-glucans from oat aleurone. *Food Hydrocoll.*, **5,** 497–511.

Wagner, N., Esser, K.D. & Krüger, E. (1988). Analytik und bedeutung hochmolekularer β-glucane in bier . *Monatsschr. Brauwiss.*, **42,** 384–395.

Woodward, J.R. & Fincher, G.B. (1982). Purification and chemical properties of two (1→3),(1→4)-β-D-glucan endohydrolases from germinating barley. *Eur. J. Biochem.*, **121,** 663–669.

Woodward, J.R., Fincher, G.B. & Stone, B.A. (1983). Water-soluble (1→3),(1→4)-β-D-glucans from barley (*Hordeum vulgare*) endosperm. II. Fine structure. *Carbohydr. Polym.*, **3,** 207–225.

Woodward, J.R., Phillips, D.R. & Fincher, G.B. (1983). Water-soluble (1→3),(1→4)-β-D-glucans from barley (*Hordeum vulgare*) endosperm. I. Physico-chemical properties. *Carbohydr. Polym.*, **3,** 143–156.