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STUDIES OF THE FINE STRUCTURE OF β-D-GLUCANS OF BARLEYS EXTRACTED AT DIFFERENT TEMPERATURES*

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ABSTRACT

Enzymic analyses of aqueous extracts of barley obtained at 25–100° demonstrated an exponential relationship between β-D-glucan content and temperature. Purified β-D-glucans, in particular those from extracts made at 40° and 100°, were compared by the Smith-degradation method followed by g.l.c. analysis of the methyl and the trimethylsilyl ethers of the products. All extracts yielded qualitatively the same products, including oligosaccharides which were characteristic of sequences of adjacent (1→3)-linkages. The material extracted at 100° contained relatively more of these sequences, and also showed a much higher specific viscosity, than did the material extracted at low temperatures. The structural implications of these findings are discussed.

INTRODUCTION

Barley β-D-glucan consists[1] of ~70% of (1→4)-linked and 30% of (1→3)-linked β-D-glucosyl residues. However, there are varying reports concerning the exact ratio of the linkages[2,3], the yields of glucan[4,5], and, in particular, the molecular weight[3,5,6]. The results seem to vary according to the conditions of extraction, *e.g.*, temperature and, for the molecular weights, the methods used, *e.g.*, viscosity, sedimentation, and gel filtration. Application of a differential enzymic method[7] for β-D-glucan in barley extracts, *e.g.*, beer, produced additional evidence that the amount and nature of the glucan varied considerably according to the conditions of extraction. The presence of sequences of (1→3)-β-D linkages in barley glucan has been established by chemical[6,8–10] and enzymic[11–13] methods, despite the conclusions of a recent reviewer[14]. We now report an attempt to provide a structural explanation for these previous observations by relating the yields and physical properties of barley β-D-glucan to the presence and proportion of sequences of (1→3)-linkages within molecules isolated by aqueous extraction at different temperatures.

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RESULTS AND DISCUSSION

The estimation[7] of β-D-glucan in barley involved a differential enzymic method in which the D-glucose derived from the total glucan in dialysed barley extracts was measured by a D-glucose oxidase method[15] after total hydrolysis with acid, and the α-D-glucan content was estimated by D-glucose oxidase analysis of amyloglucosidase digests. The difference between these values represents the content of β-D-glucan. Application of this method to aqueous extracts of barley obtained at different temperatures yielded the results in Table I, which demonstrate a marked increase in yield with increasing temperature of extraction. Indeed, for two typical samples, the

TABLE I

BARLEY-GLUCAN CONTENTS (% DRY WEIGHT) OBTAINED FOR DIFFERENT TEMPERATURE EXTRACTS OF DIFFERENT BARLEY VARIETIES[a]

Barley variety	*Extraction temperature*		
	40°	*65°*	*100°*
Julia	0.90	2.09	6.62
	1.00	2.15	7.24
Zephyr	0.87	2.13	6.40
	0.89	2.20	6.68
Golden Promise	0.78	1.77	6.52
	0.71	1.51	6.20

[a]The duplicates involved repetition of the whole extraction and analysis procedures from fresh samples of grain. A further value of 0.38% was obtained for Golden Promise extracted at 25°.

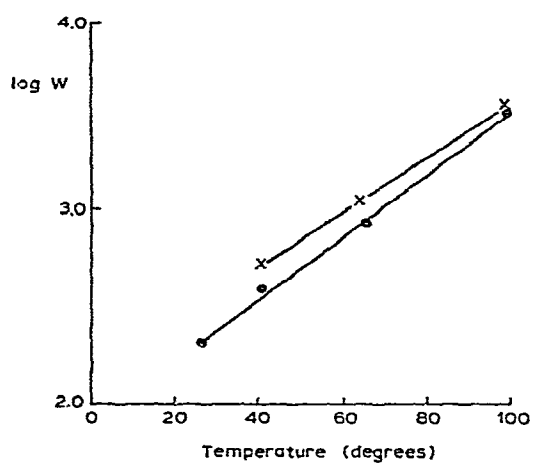

Fig. 1. Effect of extraction temperature on barley-glucan content: W = weight (mg) of barley glucan per 50 g of dry grain; Julia barley, —×—; Golden Promise, —●—.

relationship was exponential (Fig. 1), and the 100° extracts provided barley glucan contents ~10 times higher than did the corresponding 25° extracts. This increase appeared to be much greater than could be explained by solubility factors. The variation of polysaccharide content with temperature of the extract agrees with previous observations[4,5]. There were minor differences in the extraction procedures used at lower temperatures and at 100°, due to the high viscosity of the 100° extracts resulting from the gelatinisation of the starch. These modifications involved a preincubation with alpha-amylase and increases in the extract volumes. The alpha-amylase, which was free from β-D-glucanase, did not diminish the viscosity of solutions of pure β-D-glucan, nor affect the contents of β-D-glucan obtained for standard solutions of β-D-glucan. Adjustments of the extraction volumes from 3 to 8 times the dry weight of the barley used did not significantly affect the amount of extracted β-D-glucan. The exponential relationship of β-D-glucan content/temperature was maintained for different barley varieties, although the overall yields varied.

The β-D-glucans extracted from Julia barley at 40°, 65°, and 100°, respectively, were purified by the method of Preece and Mackenzie[16], and were coded G40, G65, and G100, respectively. The 65° extraction was performed on spent grain from a previous 40° extraction. It follows that G65 did not contain β-D-glucan extractable at 40°. Total glucan contents of G65 were obtained by adding the values for G40 and G65. Figures obtained in this way correspond to glucan contents estimated directly from 65° extracts of fresh grain. The other extracts were prepared from fresh grain. In general, subsequent experiments were on G40 and G100, but, wherever a G65 analysis was included, results which were intermediate between the other two were obtained, as expected. The purified glucans did not stain with iodine and were therefore assumed to be free of α-D-glucan. Total, acid hydrolysates were chromatographically free from arabinose and xylose, indicating purification from the other main arabinoxylan contaminant.

The main structural feature in cereal glucan consists of long segments in which sequences of two or three (1→4)-β-D linkages are separated by isolated (1→3)-β-D linkages[17,18] (the straight-line sections of Fig. 2). However, it has been established in these[6,8] and other[6,9,13] laboratories that sequences of up to at least five contiguous (1→3)-β-D linkages also exist as minor structural features (the zigzag lines in Fig. 2). The number of these linking sequences would be related to the physical properties of the barley glucan. It appeared possible that, at 40°, only the smaller molecules were extracted, representing glucan that had previously been degraded at a number of these linking sequences, whereas, at 100°, the extract contained material of higher molecular weight in which more of these sequences remained intact.

The first investigations of this theory were by the Smith-degradation procedure[19], involving the sequential application of periodate oxidation, borohydride reduction, and mild hydrolysis with acid. This method cleaves acetal linkages derived from the original 4-substituted glucose residues, but leaves intact the glycosidic linkages from the original, periodate-resistant, 3-substituted residues. Thus, erythritol is obtained from adjacent (1→4)-linked residues, glucosyl-erythritol from alternating

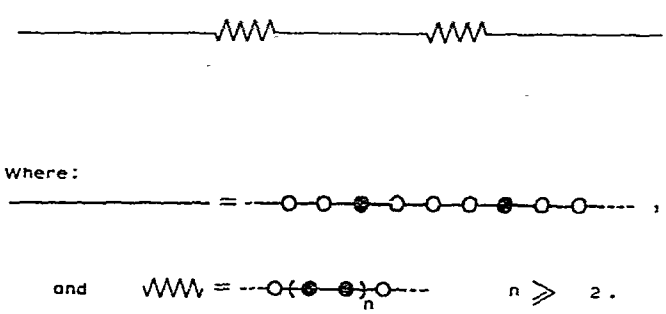

Fig. 2. Schematic diagram[6] of proposed structure of barley β-glucan: 4-substituted hexosyl residue, —O—; 3-substituted hexosyl residue, —●—.

(1→4)-(1→3)-linked residues, and the laminarisaccharide series of erythritol oligosaccharides from sequences of (1→3)-β-D linkages. Chromatography of Smith-degraded barley glucans extracted at different temperatures revealed all these products up to at least the pentasaccharide-erythritol. The chromatographic mobilities were comparable with those previously obtained[8] and in the literature[6]. The spots corresponding to members of the series up to laminaritriosyl-erythritol were characterised by acid and enzymic hydrolyses and by methylation analysis (ratios of 2,4,6-tri-*O*-methyl-D-glucose, 2,3,4,6-tetra-*O*-methyl-D-glucose, and 1,3,4-tri-*O*-methyl-D-erythritol). Moreover, a linear relationship was observed when $-\log R$ (erythritol) was plotted against degree of polymerisation for the series laminaribiosyl-erythritol to laminaripentaosyl-erythritol. The identification of the oligosaccharide fragments characteristic of sequences of adjacent (1→3)-β-D linkages in all of the barley extracts is therefore established.

Although, qualitatively, the Smith-degraded glucans extracted at different temperatures produced the same series of oligosaccharides, quantitative differences in the intensities of the component spots were apparent, the high-temperature extracts showing more of the components of higher molecular weight. In order to determine these differences, purified glucans G40 and G100 were subjected to Smith-degradation, and the *O*-trimethylsilyl derivatives of the products (together with D-ribose as internal standard) were analysed by g.l.c. The total D-glucose contents of the Smith-degraded mixtures were determined by the D-glucose oxidase method after total, acid hydrolysis. The results (Table II) demonstrated a significant increase in the proportion of D-glucose derived from the 100° extract relative to that from the 40° extract, indicating the formation of relatively more D-glucose-containing oligosaccharides from the degraded glucan, and hence the presence of more sequences of (1→3)-β-D linkages in the glucan extracted at the higher temperature, or else an increased proportion of alternating residues. However, previous work[2] indicated that few if any alternating sequences were present in barley glucan, and this is substantiated by the similar proportions of glucosyl-erythritol and erythritol obtained from the Smith-degraded glucans in the present work.

TABLE II

ANALYSIS OF FREE ERYTHRITOL AND BOUND D-GLUCOSE IN SMITH-DEGRADED BARLEY GLUCAN

Erythritol (*μmol*)	D-*Glucose* (*μmol*)	D-*Glucose*/*Erythritol*	*Extraction temperature* (*degrees*)
19.3	34.9	1.81	40
6.7	13.7	2.04	65
5.0	16.8	3.36	100

Since the major products of the Smith-degradation were erythritol and glucosyl-erythritol, a linkage analysis of the degraded glucan based on these two components produces a result which approximates to the overall composition of the original glucan extract. These components were separated by preparative paper chromatography (the large spot due to glucosyl-erythritol tended to run into and mask the minor spot due to laminaribiosyl-erythritol). The free erythritol was determined directly by g.l.c., and the bound erythritol was likewise determined by g.l.c. after hydrolysis of the D-glucosyl-erythritol. The D-glucose moiety in the latter was determined by hydrolysis followed by D-glucose oxidase analysis and also, for comparison, by g.l.c. analysis of the *O*-trimethylsilyl derivative. The results are presented in Table III; the ratio of total erythritol ($A+B$) and the total D-glucose + erythritol ($A+B+C$) is approximately the proportion of (1→4)-β-D linkages within the glucan. The 40° and 100° extracts gave values of 73% and 65%, respectively, which correspond approximately to the upper and lower limits recorded in the literature[2,3]. This range was small, and therefore the overall ratio of (1→4)- to (1→3)-linkages is not sufficiently sensitive to be a criterion for variations in the fine structure of barley glucan, although an increase in the proportion of (1→3)-β-D linkages is indicated. The ratio of free/bound erythritol, which was a measure of the proportion of adjacent (1→4)-β-D linkages, showed, if anything, a small decrease when G40 and G100 are compared.

TABLE III

DETERMINATION OF ERYTHRITOL AND D-GLUCOSYL-ERYTHRITOL IN SMITH-DEGRADED BARLEY GLUCANS

Extraction temperature[a] (*degrees*)	*Erythritol*[b] (A) (*μmol*)	*Glucosyl-erythritol*		(A+B)/(A+B+C)	A/B
		D-*Erythritol moiety*[b] (B) (*μmol*)	D-*Glucose moiety*[c] (C) (*μmol*)		
40	26.0	17.2	15.6 (15.1)	0.73	1.67
65	7.5	5.3	6.3 (6.3)	0.67	1.19
100	7.2	9.0	8.7 (10.0)	0.65	0.83

[a]The 65° extraction was on spent grain from a previous 40° extraction. [b]Determined by g.l.c. of the *O*-trimethylsilyl derivative. [c]Determined by the D-glucose oxidase method; the figures in parentheses were obtained by g.l.c. analysis.

The relative proportions of the oligosaccharide products obtained by Smith-degradation of glucan G40 were determined by comparing the free erythritol content with the bound D-glucose contents of the eluted, fractionated components. The results (Table IV), which did not include laminaribiosyl-erythritol because of difficulties in purification, confirmed that the proportions of erythritol and 2-*O*-*β*-D-glucosylerythritol were approximately equal. Those for 2-*O*-*β*-laminaritriosyl-erythritol and 2-*O*-*β*-laminaritetraosylerythritol were also similar, but the ratio of each of the former pair to each of the latter was >50:1. This is numerically different from, but similar in degree to, literature data[9], namely, 28:3:1 for D-glucosyl-, laminaribiosyl-, and laminaritriosyl-erythritols.

TABLE IV

DETERMINATION[a] OF THE SMITH-DEGRADATION PRODUCTS OF BARLEY GLUCAN EXTRACTED AT 40°

Experiment[b]	*Erythritol*	D-*Glucosyl-erythritol*	*Laminaritriosyl-erythritol*	*Laminaritetraosyl-erythritol*
1	5.90 (74)	4.58 (57)	0.08 (1)	0.07 (0.9)
2	8.69 (72)	8.06 (67)	0.12 (1)	0.12 (1.0)

[a]The figures in parentheses refer to the molar proportions relative to laminaritriosyl-erythritol. [b]Experiments 1 and 2 refer to duplicate degradations and analyses of the same sample of glucan G40.

The results of the Smith-degradation analyses therefore confirmed the infrequent presence of sequences of (1→3)-*β*-D linkages in barley glucans, and that the relative proportions of oligosaccharides containing (1→3)-linked *β*-D-glucose residues were higher for glucans extracted at higher temperatures. These results were rather similar to previous observations[6] with regard to the composition of Smith-degraded extracts of barley and of green malt obtained at different temperatures. The differences noted therein could therefore have been due to temperature factors and/or the state of germination of the grain. In the present work, the exponential increase in glucan content with extraction temperature could, in part, reflect the increased solubility at the higher temperatures of larger molecules containing more sequences of (1→3)-*β*-D-linked units.

EXPERIMENTAL

Materials. — D-Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, peroxidase (EC 1.11.1.7) from horseradish, and alpha-amylase (EC 3.2.1.1) from hog pancreas were obtained from Sigma Chemical Company. Amyloglucosidase (EC 3.2.1.3) from *Aspergillus* was obtained from B.D.H. Chemicals Ltd. The amyloglucosidase preparation was specified to have an activity of 3,000 units/g, where 1 unit yields 25 mg of D-glucose/h from a starch slurry at pH 4.5. The preparation was assayed against potato starch after 2 years and retained 82% of its specified activity. Barley samples were kindly donated by Professor A. M. MacLeod.

Paper chromatography (p.c.). — Qualitative descending p.c. was performed on

Whatman No. 1 paper with ethyl acetate–pyridine–water[20] (10:4:3). Chromatograms were developed with the silver nitrate reagent[21]. Quantitative chromatography was similarly performed with Whatman 3MM paper. Components were recovered by cutting out bands corresponding to developed guide-strips, and eluting with water.

Extraction of barley at 40° and 65°. — Samples of barley were ground by using a Casella Mill with a 1-mm sieve. The ground flour (50 g, dry weight) was treated with boiling 80% ethanol (150 ml) for 1 h to inactivate the barley enzymes. The hot mixture was filtered under vacuum and the product was allowed to air-dry overnight. The barley grist was extracted three times with water (250, 200, and 150 ml) at 40° for 30 min, with continuous stirring before filtering through muslin. The combined extracts were centrifuged at 19,000 *g* at 20° for 15 min. The supernatant solution was dialysed against running water to remove material of low molecular weight. The extract was stored, if necessary, under toluene at 2°. Extraction at 65° was either performed by the same procedure on fresh grain, or by using the spent grain from a 40° extraction. Both alternatives provided the same β-D-glucan contents when assayed by the enzymic method; in the latter case, the 65° figure was added to the 40° result to provide the appropriate total.

Extraction of barley at 100°. — At elevated temperatures, filtration problems were encounted, due to the elevated levels of polysaccharide in the extracts. Ground and inactivated barley (50 g, dry weight) was heated with water (500 ml) in a boiling water-bath for 10 min. Alpha-amylase (500 mg) was added to the cooled mixture, which was then incubated overnight at room temperature, heated in a boiling water-bath for 30 min with frequent stirring, and filtered through muslin. Two successive extractions with distilled water (2 × 400 ml) at 100° for 30 min were then carried out. The subsequent filtration, dialysis, and centrifugation procedures were as described for the 40° extraction.

Purification of β-D-glucan. — This was effected on the crude, aqueous extracts by the method of Preece and Mackenzie[16]. The extract was centrifuged for preliminary clarification, filtered through acid-washed Kieselguhr, and concentrated to ~450 ml under reduced pressure at a temperature not exceeding 50°. The β-D-glucan was precipitated with 20% ammonium sulphate, collected by centrifugation, redissolved in water (equal volume) in a boiling water-bath, and then reprecipitated by adding an equal volume of acetone. This procedure was repeated a further four times. The final solution, which did not stain with iodine, was dialysed overnight and then freeze-dried. Yields of ~80% of the enzymically estimated β-D-glucan in the crude extracts were obtained.

Enzymic estimation of barley glucan. — This was accomplished by the procedures described elsewhere[7]. The difference between the D-glucose oxidase[15] analysis of total, acid hydrolysates of the extracts (total D-glucose) and the D-glucose oxidase analysis of amyloglucosidase digests (40 μg of amyloglucosidase/ml of digest at pH 4.5) of the extracts (total D-glucose derived from α-D-glucan) provided the total β-D-glucan content of the extracts after adjustments for the dilution factors and for the conversion of D-glucosyl residues into D-glucose.

Smith degradation of purified β-D-glucan[19]. — Glucan (80 mg) was dissolved in water (5 ml) and allowed to oxidise with sodium metaperiodate (0.3M) in the dark at room temperature for 10 days with occasional stirring. The oxidised sample was dialysed against running water for 24 h. The non-diffusible material and washings were reduced with potassium borohydride (120 mg) for 20 h at room temperature. More (30 mg) potassium borohydride was then added; after 4 h, the solution was neutralised to pH 7.0 with glacial acetic acid and evaporated to ~3 ml under reduced pressure at 40°. The polyalcohol was precipitated with ethanol (30 ml), recovered by centrifugation, redissolved in water, reprecipitated with ethanol, washed with ethanol and ether, and dried. The polyalcohol was hydrolysed with 125mM sulphuric acid for 24 h at room temperature (mild, acid hydrolysis). The hydrolysate was neutralised with barium carbonate and centrifuged. The supernatant solution and washings were deionised with Zerolit DM-F, and concentrated to a few drops by rotary evaporation at 40°, and the component sugar alcohols were separated by p.c.

Acid hydrolysis. — Total and partial hydrolyses with acid were performed, as for the mild, acid hydrolysis, with M sulphuric acid at 100° for 3 h, and 165mM sulphuric acid at 100° for 1 h.

Methylation[22]. — Smith-degraded oligosaccharides (2 mg) were methylated in a sealed tube by using redistilled *N,N*-dimethylformamide (0.2 ml), redistilled methyl iodide (0.2 ml), and silver oxide (150 mg) in the dark at room temperature for 48 h with continuous shaking. The methylated mixture was filtered, and the residue washed with chloroform. The filtrate and washings were repeatedly evaporated to dryness *in vacuo* at 40° with toluene. The product was treated with 3% methanolic hydrogen chloride at 100° for 16 h, and the solution was repeatedly evaporated to dryness as before but with dry methanol. The residue was dissolved in chloroform, and analysed by g.l.c.[23]. G.l.c. was performed with a Perkin–Elmer Gas Chromatograph F-11 and a glass column (200 × 0.3 cm) packed with 15% of 1,4-butanediol succinate polyester on Chromosorb W (80–100 mesh) at 180° (isothermal), with injection and detector temperatures of 265° and 200°, respectively, and a nitrogen flow-rate of 40 ml/min (30 p.s.i.).

Trimethylsilylation[24]. — Standard solutions (5 ml) containing D-glucose and erythritol (125–500 μg), or a deionised solution of Smith-degraded oligosaccharide (5 ml) before or after preparative chromatography, together with D-ribose (250 μg) as internal standard, were freeze-dried. Each residue was dissolved in dry pyridine (0.2 ml), with heating at ~80° where appropriate. Hexamethyldisilazane (0.04 ml) and chlorotrimethylsilane (0.2 ml) were added, and the solution was shaken vigorously at room temperature for 1 min. After being kept for at least 15 min, the samples were analysed by g.l.c. A glass column (200 × 0.3 cm) packed with 5% of silicone GE SE30 on Chromosorb W (80–100 mesh) was used. Samples were chromatographed at 170° for the erythritol derivative, or at 230° for the glucosyl-erythritol derivative. Otherwise, the conditions were similar to those used for the methylated derivatives.

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