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Note

Chromatofocusing as a method for comparing β-D-glucan hydrolases from germinating cereals

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Interest in barley β-D-glucan has arisen from the problems caused by the viscous nature of the polysaccharide, which can give filtration difficulties in the brewing industry[1]. Analysis of both β-D-glucan and β-D-glucan hydrolases in malted barley has therefore been the subject of several studies[2–4]. There is, however, also an interest in using sorghum, either as an adjunct[5,6] or directly as a sorghum malt, for brewing beer[7]. In such cases, viscosity problems do not exist because the content of β-D-glucan in sorghum is so low[8]. Recent work by Aisien *et al.*[9] has suggested that sorghum β-D-glucanases may be very different from barley β-D-glucanases[9]. This view accords with recent work which shows that the sorghum cell-wall is not modified in the same way as that of barley during malting[10].

Studies of germinated and malted barley have indicated that there are at least five enzymes which are capable of hydrolysing β-D-glucosidic linkages[4], and detailed studies of two barley-endo-β-D-glucanases have been the subject of recent papers[11,12]. It was therefore of interest to compare the enzymes, which hydrolyse β-D-glucosidic linkages, from samples of germinated barley and germinated sorghum, using chromatofocusing.

In initial experiments, the activity of enzyme extracts of the germinated cereals against various β-D-glucans (Table I) indicated that the samples were capable of hydrolysing all the substrates. However, the enzyme extract from barley appeared to be about twice as active as the enzyme extracts from sorghum. In addition, the barley extract rapidly reduced the viscosity of solutions of barley β-D-glucan (Table II). Sorghum extracts were less active, but hydrolysed the 90°-β-D-glucan more readily than the 40°-β-D-glucan. Since the 90°-β-D-glucan contains occasional, consecutive (1→3)-β-D-linkages[2], it is more susceptible to the action of endo-(1→3)-β-D-glucanase than is the 40°-β-D-glucan.

When the enzyme extracts were subjected to chromatofocusing[13] (Figs. 1a

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TABLE I

ACTIVITY OF EXTRACTS BY MEASUREMENT OF REDUCING POWER

Enzyme extract	*Glucose equivalent (μg) released/mg of protein/min from*		
	Laminarin	*Lichenin*	*Barley β-D-glucan*
Barley	53.3	5.0	9.3
Red sorghum	37.5	2.7	5.1
Chalky sorghum	39.2	2.8	5.9

TABLE II

RELATIVE ACTIVITY OF EXTRACTS BY VISCOSITY MEASUREMENTS

Enzyme extract	*Relative decrease (%) in viscosity of solutions of barley β-D-glucan*	
	40°-β-D-Glucan	*90°-β-D-Glucan*
Barley	100	100
Red sorghum	13	25
Chalky sorghum	8	15

and 2a), a much greater difference was apparent. A basic pattern was found in both barley and sorghum extracts with separation of activities against *p*-nitrophenyl β-D-glucopyranoside and laminarin. However, the activities against barley β-D-glucan (Figs. 1b and 2b) were very different. A profile of the crude enzyme extract from barley (Fig. 1a) showed several peaks of activity against *p*-nitrophenyl β-D-glucopyranoside, which were eluted over a wide pH-range (9.55–7.8). The fractions corresponding to these peaks all hydrolysed laminaribiose and cellobiose rapidly and hydrolysed laminarin and barley β-D-glucan slowly to D-glucose, properties indicative of β-D-glucosidases. Two distinct peaks of activity occurred against laminarin (Fig. 1a, A1 and A2), which were eluted at pH 9.7 and 9.6. Trace activity against laminarin was also found in later fractions, with hydrolysis to glucose, probably due to the action of β-D-glucosidases. The enzymes present in peaks A1 and A2 hydrolysed laminarin to laminaribiose, laminaritriose, and the higher series of (1→3)-β-oligosaccharides, plus a trace of glucose. However, they did not hydrolyse laminaribiose or cellobiose. This is indicative of endo-(1→3)-β-D-glucanase activity. One strong peak of activity against barley β-D-glucan was eluted at pH 9.65 (Fig. 1b, B1). This fraction produced two oligosaccharides from the hydrolysis of barley β-D-glucan, which corresponded chromatographically to 3-*O*-cellobiosyl-D-glucose and 3-*O*-cellotriosyl-D-glucose and are indicative of barley endo-β-D-glucanase activity. No glucose was formed, and peak B1 did not hydrolyse laminaribiose or cellobiose. Trace activity against barley β-D-glucan was found in later fractions, with hydrolysis to glucose probably attributable to β-D-glucosidases. In addition, a small area of activity (B2) was the result of hydrolysis of arabinoxylan, which was

a minor contaminant of the barley β-D-glucan substrate (arabinose, xylose, and a trace of glucose were revealed by paper chromatography). A second chromatofocusing experiment was carried out with the barley-enzyme extract between pH 11 and 8, but the resolution of the laminarin-hydrolysing and the barley β-D-glucan-hydrolysing enzymes was not improved.

Chromatofocusing of the chalky sorghum extract (Fig. 2a) showed several peaks of activity in the elution range pH 9.3–7.8 with *p*-nitrophenyl β-D-glucopyranoside as substrate. These fractions showed the same properties as described for the barley extracts, *i.e.*, they were probably β-D-glucosidases. One

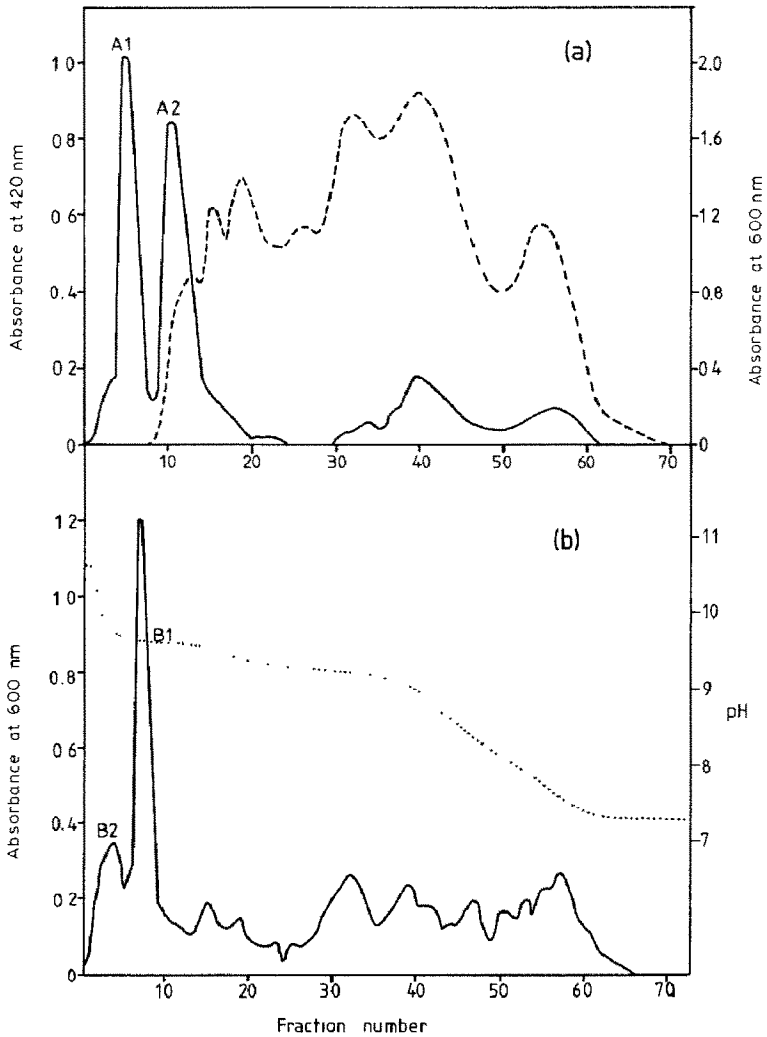

Fig. 1. Fractionation of germinated barley extract by chromatofocusing: (a) laminarin as substrate, A_{600} ——; *p*-nitrophenyl β-D-glucopyranoside as substrate, A_{420} -----; (b) barley β-D-glucan as substrate, A_{600}. The pH of the fractions is also shown ·····.

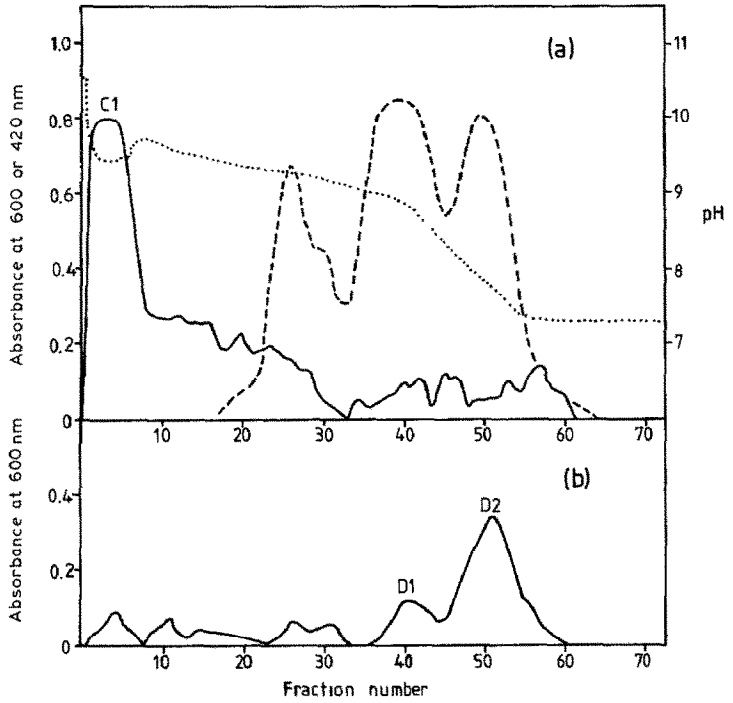

Fig. 2. Fractionation of germinated sorghum extract by chromatofocusing: (a) laminarin as substrate, A_{600} ——; *p*-nitrophenyl β-D-glucopyranoside as substrate, A_{420} ----; (b) barley β-D-glucan as substrate, A_{600}. The pH of the fractions is also shown ·····.

large peak of activity against laminarin occurred (Fig. 2, C1) at the exclusion volume with some tailing into later fractions. This peak, and the tubes up to fraction 20, produced the same series of β-(1→3)-linked oligosaccharides from laminarin as did the barley-enzyme extract. Using barley β-D-glucan as substrate, the greatest activity occurred at peaks D1 and D2 (Fig. 2b) corresponding with peaks of β-D-glucosidase activity. Glucose was the only product detected. (However, if any oligosaccharides had been formed by an endo-(1→3; 1→4)-β-D-glucanase, they would probably have been hydrolysed by the β-D-glucosidase in this fraction.) The chromatofocusing profile of the extract from red sorghum showed features very similar to those of chalky sorghum extract.

The sorghum extracts attack barley β-D-glucan to a very limited extent, but it is likely that this action is due to exo-acting enzymes and not to true endo-(1→3; 1→4)-β-D-glucanase activity which is present in barley extracts. These experiments therefore illustrate the usefulness of chromatofocusing as a means of fingerprinting β-D-glucanases and β-D-glucosidases in cereal extracts.

EXPERIMENTAL

Materials. — Two varieties of sorghum (a red French RS3 and a chalky white

variety of Nigerian sorghum CN4) were obtained from Lawson Donaldson Seeds Ltd., Edinburgh. A sample of Golden Promise barley was kindly provided by Dr. G. H. Palmer. Laminarin and laminaribiose, from *Laminaria hyperborea*, were laboratory preparations. Barley β-D-glucan was extracted[14] with distilled water from Golden Promise barley at a striking temperature of either 40° or 90°. These samples were kindly prepared and provided by Dr. Pamela Brunswick. Lichenin from *Cetraria islandica*, *p*-nitrophenyl β-D-glucopyranoside, and cellobiose were commercial products (Sigma).

General methods. — Reducing sugars were determined by a modification of the Somogyi–Nelson method[15], and protein was determined by a modification[16] of the method of Lowry. Descending paper chromatography was performed on Whatman No. 1 paper with ethyl acetate–pyridine–water (10:4:3); reducing sugars were detected with alkaline silver nitrate[17].

Germination of sorghum and barley. — Grains of red sorghum, chalky sorghum, and barley were surface sterilised by soaking in aqueous sodium hypochlorite (1% available chlorine) for 20 min followed by several rinses with distilled water. The grains were blotted dry and allowed to germinate on filter paper, which was kept moist throughout the germination period, at 25° for 4 days (red and chalky sorghum) or 3 days (barley). The grains were then removed and frozen.

Extraction of sorghum and barley enzymes. — Germinated grain (100 g) was macerated with 0.1M sodium acetate buffer (200 mL, pH 5.0) for 60 s at 2°. The blended samples were extracted by stirring for 3 h at 2° and the crude extract was clarified by centrifugation at 16,000*g* for 15 min. The supernatant solution was dialysed against cold distilled water for 48 h and then stored frozen. The extracts were centrifuged at 16,000*g* for 15 min before use, to remove any precipitated material. The protein contents were: red sorghum, 0.78; chalky sorghum, 0.98; and barley, 1.04 mg/mL.

β-D-Glucanase activity. — All enzyme assays were carried out in the presence of excess of substrate, and values of activity relate to the linear portion of the progress curve for enzyme action. The release of reducing sugars from specific polysaccharides by endo- and exo-activities in the enzyme extracts was studied using substrate solutions which were 0.2% (w/v) in sodium acetate buffer (40mM, pH 5.0). Laminarin solution (6.7 mL) was added to enzyme solution (0.3 mL), and the mixture incubated at 37° for 1 h. Lichenin solution (5.0 mL) or barley-β-D-glucan solution (5.0 mL) was added to enzyme solution (2.0 mL), and the mixture incubated at 37° for 1 h. Samples (1 mL) were taken at 10-min intervals and analysed for reducing sugars against a boiled enzyme blank. The results were expressed as μg of glucose equivalent released/mg of protein/min.

Endo-β-D-glucanase activity. — The change in viscosity was measured using digests containing 0.5% barley β-D-glucan (10 mL; 40°- or 90°-β-D-glucan preparation in 40mM sodium acetate buffer, pH 5.0) and enzyme solution containing 0.5 mg of protein. The flow time at 25° was measured at intervals using a modified Ubbelohde viscometer (SLSU, No. 3), and the reciprocal specific viscosity was cal-

culated. The results were expressed as a percentage of the rate of increase of the reciprocal viscosity. The buffer had a flow time of 98.20 s in the viscometer used.

Enzyme fractionation by chromatofocusing. — All procedures were carried out at 4°. An aliquot of enzyme extract containing 10 mg of protein was applied to a column (1.5 × 30 cm) containing a slurry (30 mL) of Polybuffer Exchanger 118 (Pharmacia) which had been equilibrated with 25mM triethylamine–HCl buffer (pH 11). The column was then eluted with pharmalyte (diluted 1:45 with distilled water)–HCl buffer (pH 7.0) at 30 mL/h. Fractions (3 mL) were collected and the pH was measured before analysis for enzyme activity against *p*-nitrophenyl β-D-glucopyranoside, laminarin, and barley β-D-glucan (90°-β-D-glucan preparation).

Enzyme assays. — *p*-Nitrophenyl β-D-glucopyranoside (0.40 mL of a 0.3% solution in 0.1M sodium acetate buffer, pH 5.0) was added to an aliquot (0.30 mL) of the column fraction, and the mixture was incubated at 37° for 1 h. Incubation was followed by the addition of M potassium hydrogencarbonate (0.35 mL) and, after mixing and centrifuging at 5,000*g* for 2 min, the absorbance was read at 420 nm against a reagent blank. Laminarin (0.40 mL of a 0.5% solution in 40mM sodium acetate buffer, pH 5.0) was added to an aliquot (0.30 mL) of the column fraction, and the mixture was incubated at 37° for 2 h. Barley β-D-glucan (0.35 mL of a 0.5% solution in 40mM sodium acetate buffer, pH 5.0) was added to an aliquot (0.35 mL) of the column fraction, and the mixture was incubated at 37° for 5 h. After incubation, samples (0.5 mL) of the laminarin and barley-β-D-glucan digests were tested for reducing sugar against a reagent blank. The digests were then incubated at 37° for 24 h (laminarin) or 48 h (barley β-D-glucan) for analysis by paper chromatography. An aliquot (0.1 mL) of the column fraction was added to a 1% solution of laminaribiose or cellobiose in 0.1M sodium acetate buffer (pH 5.0, 0.1 mL) and, after incubation at 37° for 24 h, analysis was carried out by paper chromatography.

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