

Release of β -Glucan from Cell Walls of Starchy Endosperm of Barley

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ABSTRACT

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β -Glucan can be solubilized from barley by warm water, with increasing solubilization as the temperature is increased. Substantially less glucan is extracted if the barley is dehusked using sulfuric acid, particularly if the dehusked barley is denatured. This indicates that enzymes capable of solubilizing glucan are present in barley. Various purified enzymes promote the solubilization of glucan from denatured and dehusked barley. Apart from endo- β -(1 \rightarrow 3)(1 \rightarrow 4)-glucanase, these enzymes in-

clude endo-xylanases, arabinofuranosidase, xyloacetyl esterase, and feruloyl esterase. Ferulic acid and, probably, acetyl groups are ester-linked to arabinoxylan, not β -glucan, in the cell walls of barley starchy endosperm, so the ability of the esterases, xylanases, and arabinofuranosidase to solubilize glucan indicates the pentosan component of the cell wall can restrict the extraction of glucan.

The cell walls of the starchy endosperm food reserve of barley are composed of \approx 75% β -(1 \rightarrow 3)(1 \rightarrow 4)-glucan, 20% arabinoxylan, and 5% protein, as well as traces of cellulose and ferulic acid (Fincher 1975). Their efficient degradation is a prerequisite for production of good malt and avoidance of a range of problems in brewing (Bamforth 1982, 1994). On the other hand, there is much interest in the positive impact that β -glucans can have on health, through their contribution of soluble fiber to the diet (Anderson et al 1990; Hecker et al 1998). There are also some indications that these molecules might have useful functional properties in food structures, for example in promoting whipping and emulsification (Morgan and Ofman 1998; Burkus and Temelli 2000). Irrespective of whether the need is to eliminate β -glucan or, alternatively, to encourage its extraction and retention with the appropriate structural features (e.g., visco-elastic properties), it is important to have an understanding of the processes involved in solubilization and digestion of the molecule.

The major enzyme involved in hydrolyzing the β -glucan component of barley is an endo- β -(1 \rightarrow 3)(1 \rightarrow 4)-glucanase, which develops during the germination of barley. Its properties have been extensively reported, among others its relative thermostability (Hrmova et al 1997).

One view is that this enzyme is solely responsible for the degradation of the glucan component of the walls, acting both on relatively insoluble glucan in the wall (hemicellulose) and glucan that is freely soluble (gum). Conversely, it has been claimed that the initial digestion of the hemicellulosic fraction involves other enzymes ("solubilases") and that it is the product of the action of these enzymes that represents the substrate for endo- β -glucanase. Among the candidates advanced for this role have been carboxypeptidase, acting in an esterolytic capacity (Bamforth et al 1979; Bamforth 1981); phospholipase (Palmer 1987); endo- β 1 \rightarrow 3-glucanase (Bathgate et al 1974); endo- β 1 \rightarrow 4-glucanase contributed from fungi populating the surface of the grain (Yin and MacGregor 1988); and ferulic acid esterase (Moore et al 1996). Bamforth et al (1997) found evidence that there are at least four solubilases in barley and that they include at least two types of esterase. Even so, the fraction most capable of releasing β -glucan, which could also release arabinoxylan from the walls, displayed no apparent esterase activity. Izydorczyk et al (2000) demonstrated that esterase and proteinase activities could promote the extractability of β -glucan from hull-less barleys.

This article describes a study on the solubilization of glucan from native, dehusked, and denatured-dehusked barley and reports

on the ability of a range of purified enzymes to solubilize β -glucan from the cell walls of barley.

MATERIALS AND METHODS

Preparation of Cell-Wall Substrate

Barley (cv. Morex) was dehusked, denatured, washed, depleted of starch, and milled in a Waring Blender according to the procedure of Moore et al (1996). Typically such preparations contain \approx 70% β -glucan, 8% pentosan, 1% lipid, and 1% protein, with the remainder made up of ash and residual starch. Although the product is composed primarily of cell wall material from the starchy endosperm, aleurone cell wall polymers are also present. Using values of 10 and 75% for the contribution of aleurone and starchy endosperm, respectively, to the dry weight of barley (Briggs 1998) and taking into consideration that the walls of the aleurone contain $<$ 10% β -glucan, the proportion of solubilized glucan originating from aleurone will be extremely small.

Assessment of β -Glucan Solubilization

Solubilase assays with added enzymes were conducted to assess solubilized β -glucan. Assay mixtures (1-mL total volume in 100mM sodium phosphate buffer, pH 6.4) at 65°C contained cell wall substrate (0.5 g) and enzyme (0.1 mL). After the required incubation period, the reaction was stopped by rapidly chilling the sample in ice and centrifuging prior to assay of β -glucan in the supernatant.

Barley, dehusked barley, and cell wall substrate (1 g) samples were mixed with 5 mL of 0.02% (w/v) sodium azide and incubated at 20, 40, 65 or 80°C for aqueous extraction of β -glucan. Samples were removed at intervals and centrifuged, and the supernatants were assayed for β -glucan. Solubilization was calculated as a percentage of the glucan present in the solid added.

Source of Enzymes

Enzymes tested for solubilase activity (Table I) were a gift from Novo Nordisk (Gentofte, Denmark). The enzymes were from *Aspergillus aculeatus* or *A. niger* recombinantly expressed in *A. oryzae*.

Chemical Analyses

Protein was measured using the method of Bradford (1976) and bovine serum albumin as the reference. β -Glucan was assayed according to Bamforth (1983). The latter assay was used because it involves the complete extraction and digestion of glucan, whereas other procedures do not provide a complete assessment of glucan (Ullrich et al 1991).

Enzyme Assays

Assays were conducted using published procedures for endo- β 1 \rightarrow 3;1 \rightarrow 4-glucanase (McCleary and Shameer 1987), arabinofuranosidase (Cleemput et al 1997), endo- β 1 \rightarrow 4-xylanase (Prentice et al 1980), and esterase (Burger et al 1968). The search for endo-

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β -glucanase contamination in purified enzymes was conducted using the radial diffusion assay of Bamforth and Martin (1983).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in 7.5% (pH 8.9) and 4.75% (pH 6.8) gels according to Ornstein (1964) and Reisfeld et al (1962), respectively. Protein was stained using Coomassie Brilliant Blue in 5% acetic acid and 50% methanol.

RESULTS AND DISCUSSION

There is no consensus in the literature on the nature of the differences, if any, between the hemicellulose and gum fractions of barley β -glucan. It has been suggested that glucan is associated with protein (Forrest 1977) and that this may be involved in restricting solubility. It has also been implied that ester linkages are involved in the architecture of the walls and that esterases are able to release glucan through the hydrolysis of these bonds (Bamforth 1981). The converse opinion is that there are no covalent associations in the walls, rather there is a steric hindrance limiting the extraction of glucan material that may be overcome by extraction at increased temperatures (Fleming and Kawakami 1977). It has been proposed that the cellulosic regions comprising contiguous β 1 \rightarrow 4-linked glucosyl residues may contribute to the insolubility of glucans (Woodward et al 1988). Alternatively, it also has been speculated that the accessibility of glucan to solvating water is restricted by an outer coating of arabinoxylan and that this needs to be removed before all of the glucan can be extracted (Palmer 1989).

Extraction of Glucan by Water Alone

The ability of water alone to extract β -glucan from barley was investigated. Three separate samples were assessed: native barley, dehusked barley, and dehusked-denatured barley with starch removed and endogenous enzyme activity destroyed (cell wall substrate). The total β -glucan contents of the three samples used in this experiment were 6.2, 8.7, and 63.7%, respectively. Table II shows the release of β -glucan from milled native barley, while Tables III and IV illustrate the release of glucan from milled dehusked and milled dehusked-denatured barley, respectively.

TABLE I
Enzymes Used in This Study

Enzyme	Activity (U/mL) ^a	PC ^b (μ g/mL)	SA ^c (U/mg protein)	GA ^d (U/mL)
β (1 \rightarrow 3)(1 \rightarrow 4)-glucanase	350	110	3,182	390
Xylanase I	763	6,640	115	ND
Xylanase II	653	2,740	238	200
Xylanase III	520	1,200	433	180
Arabinofuranosidase	82	1,240	66	ND
Xylacetylsterase	36	1,420	25	ND
Feruloylsterase	6	140	43	ND

^a U = units.

^b Protein concentration.

^c Specific activity.

^d β -Glucanase activity (GA) measured by radial diffusion assay (Bamforth and Martin 1983); ND = not detectable

TABLE II
Percent Solubilization of β -Glucan from Whole Barley by Water

Time (min)	Water Temperature			
	20°C	40°C	65°C	80°C
10	4.3	4.4	4.1	4.8
30	6.7	13.5	12.6	14.6
60	12.7	13.8	15.2	15.8
120	15.2	17.0	17.5	16.4
240	12.7	16.4	17.3	21.6
480	14.8	16.8	19.2	21.4

In all cases, the amount of glucan solubilized was higher at higher temperatures. The proportion extracted, however, was significantly higher for native barley than for dehusked barley, which in turn afforded much more glucan than dehusked-denatured barley. Although it is possible that the dehusking procedure and, more particularly, the denaturing process modified the solubility properties of the glucan, it seems most likely that the decrease in glucan solubility observed in the latter two samples reflects denaturation of endogenous solubilases by sulfuric acid and boiling ethanol, respectively. Sulfuric acid, which does not penetrate the pericarp-testa layer of the grain (Pollock et al 1955; Essery et al 1956), is assumed to inactivate enzymes contributed by the microflora populating the surface of the grain (Yin and MacGregor 1988). The boiling of milled dehusked barley in ethanol destroys all remaining enzymes native to the barley.

We might have anticipated that extraction of glucan would have been easier from dehusked barley than native barley, because a potential barrier to solubility had been removed. That this is not the case further supports the thesis that catalytic digestion is involved.

With native and dehusked barley there is a clear time-dependency for extraction over the first 180–240 min, which would be consistent with a progressive enzyme(s)-catalyzed event(s). That the solubilization continues even at 80°C testifies to the thermotolerance of at least one of the enzymes involved (Bamforth et al 1979; Bamforth et al 1997). The levels of endo- β 1 \rightarrow 3;1 \rightarrow 4-glucanase present in raw barley are very low (Stuart et al 1986), and in any event, this enzyme is palpably heat-labile (Bourne et al 1976). Solubilase activity is clearly not a function of this activity.

Extraction of Glucan Catalyzed by Enzymes

A series of enzymes were investigated for their ability to catalyze the release of glucan from denatured barley flour substrate. In all cases the data presented have been corrected for freely soluble glucan (Table IV).

All of the enzymes studied released glucan, to a greater or lesser extent (Fig. 1). The fastest release of glucan was caused by two of the three endo-xylanase preparations, although the most extensive release of glucan was caused by an endo- β -glucanase. The latter released \approx 90% of the glucan above that solubilized by water alone. It appears that \approx 7–8% of the total glucan was not accessible even to β -glucanase. None of the other enzymes was able to release the high proportion of glucan released by glucanase, although the xylanase I and II preparations caused solubilization of \approx 80% of the glucan.

TABLE III
Percent Solubilization of β -Glucan from Dehusked Barley by Water

Time (min)	Water Temperature			
	20°C	40°C	65°C	80°C
10	2.2	3.0	4.6	8.7
30	4.0	3.3	5.9	12.2
60	6.3	6.9	10.1	13.9
120	6.3	6.3	10.7	14.2
240	8.6	10.2	11.9	14.2
480	9.3	9.6	13.3	15.2

TABLE IV
Percent Solubilization of β -Glucan from Dehusked-Denatured Barley by Water

Time (min)	Water Temperature			
	20°C	40°C	65°C	80°C
10	0.89	1.39	1.40	1.86
30	1.22	1.37	1.57	1.95
60	1.37	1.37	1.83	1.91
120	1.37	1.50	1.56	2.11
240	1.30	1.64	1.72	1.95
480	1.42	1.65	2.02	2.34

That the other enzymes did not cause the same extent of solubilization could be due to their lack of survival for a sufficiently long period, due to some form of developing inhibition, or simply because the bonds they hydrolyze do not allow complete glucan extraction. Figure 2 illustrates the thermo-tolerance of the enzymes. Xylanase I was completely inactivated within 20 min, at which time glucan solubilization had reached its maximum. That the more heat-resistant xylanase II also failed to solubilize >80% of the glucan suggests that this is the maximum amount of glucan that can be released by xylanase activity. Xylanase III was largely destroyed within 20 min of heating to 65°C. The low levels of enzyme that survived, however, were able to continue to solubilize glucan, although solubilization ceased within 40 min when the enzyme had been completely destroyed.

It appears that arabinofuranosidase, which removes arabinose side-chains from arabinoxylan, has substantial solubilase activity. Furthermore, two esterases were capable of extracting glucan to a more limited extent, one of them hydrolyzing acetyl groups associated with xylan, the other breaking ferulic acid ester bonds. The latter are associated with arabinoxylan rather than β -glucan (Ahluwalia and Fry 1986), and this, together with the efficacy of the xylanases and the arabinofuranosidase in releasing glucan, supports the hypothesis that arabinoxylan can limit glucan extraction. The greater efficacy of the feruloyl, as opposed to acetyl esterase, might suggest that ester linkages involving ferulic acid are more important than those involving acetic acid for cell-wall structure. Ester-linked acetyl groups have been reported as a feature of the structure of arabinoxylans in the walls of the aleurone (Bacic and Stone 1981) but have not yet been reported in the walls from the starchy endosperm (Briggs 1998). Acetic acid esterase has been reported in malting barley (Humberstone and Briggs 2000b). Although the extent of release of glucan due to arabinofuranosidase, xyloacetyl esterase, and feruloyl esterase reached a maximum after 40, 40 and 60 min, respectively, these enzymes still had some residual activity, suggesting that the removal of arabinose, acetic acid, and ferulic acid side-chains from arabinoxylan only facilitated the release of a portion of the glucan.

All of the enzymes were added at a constant rate, but they differed considerably in their protein concentration and in the activity of their principal named activity (Table I). For example, the quantities of glucanase and xylanases added substantially exceeded those for other enzymes. When the specific solubilase activity in each enzyme is examined, it is evident that although β -glucanase was the most effective solubilizing agent feruloyl esterase was also very active. (A general assay was used to estimate esterase activity in

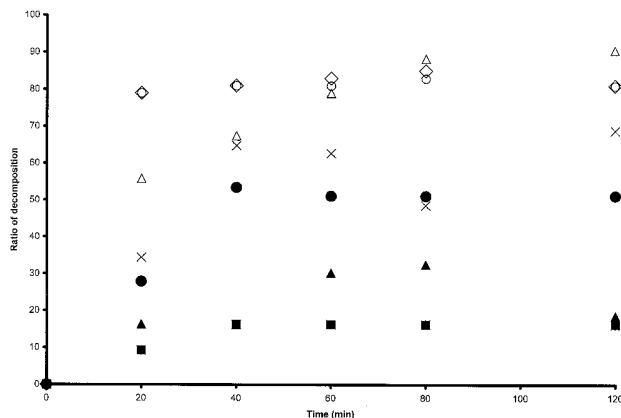


Fig. 1. Enzymic solubilization of β -glucan from barley using solubilase assays with added enzymes. Ratio of decomposition is defined as the percentage of total β -glucan released from the substrate by an enzyme and is corrected for freely soluble glucan. Δ , β -glucanase; \circ , xylanase I; \diamond , xylanase II; \times , xylanase III; \bullet , arabinofuranosidase; \blacktriangle , feruloyl esterase; \blacksquare , xyloacetyl esterase.

both the xyloacetyl esterase and feruloyl esterase preparations, and the specific activities would be expected to be substantially higher if acetyl xylan or a ferulic acid ester had been used.)

Although the enzymes studied were reasonably pure, electrophoresis showed a few contaminants in all of them. The feruloyl esterase and arabinofuranosidase were probably the cleanest. Of particular concern was the possible presence of contaminating β -glucanase. As shown in Table I, only the xylanase II and III preparations displayed any contaminating β -glucanase, and in each case, the specific activity of the contaminating β -glucanase was much lower than that in the purified β -glucanase. The converse investigation has not been made (i.e., we have not yet assessed whether there are xylanase, esterase, or arabinofuranosidase contaminants in the β -glucanase preparation that may contribute to glucan solubilization). However, this would not confound the argument that hydrolysis of arabinoxylans enables the solubilization of β -glucan.

Various combinations of these enzymes have been assessed for their ability to solubilize glucan. The data (not shown) reveal no evidence of additive or cumulative effects in the action of the various enzymes. For example, whereas xyloacetyl esterase and feruloyl esterases caused 23 and 29% solubilization, respectively, after 10 min of incubation, the combined addition caused only 31% solubilization. It appears that there is a limit to how much glucan can be solubilized solely by breaking ester bonds and that the rate of release of glucan is not restricted by the availability of any individual enzyme, but rather by the availability of substrate for digestion.

It is clear from this work that a range of enzymes is able to promote extraction of β -glucan from barley. Although it is not reasonable to conclude based on this study that such enzymes incontrovertibly have an *in vivo* responsibility for solubilization of β -glucan prior to the action of endo- β -glucanases, the evidence nonetheless indicates that a series of activities present in barley are capable of causing dissolution of cell wall material, including feruloyl esterase, which is present in barley (Sanch et al 1999; Humberstone and Briggs 2000a). There is an increasing realization that the efficient degradation of plant cell walls generally involves a series of enzymes, with esterases in particular having a key involvement (Christov and Prior 1993; Williamson et al 1998). The data reported in this study are consistent with the theory that arabinoxylan (ester linked to ferulic acid and possibly acetyl groups) limits the solubility of β -glucan from the walls of the starchy endosperm of barley, perhaps because the former is located in the outer regions of the wall. That β -glucanase is able to access glucan, however, suggests that the covering of glucan by pentosan is incomplete. Through studies using fluorescent staining of barley

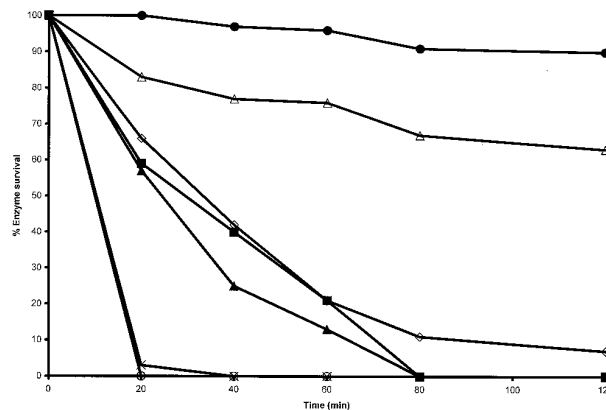


Fig. 2. Resistance of enzymic activity to heating at 65°C. Enzymes were heated in sealed tubes for various periods of time before rapidly chilling on ice, centrifuging, and assaying for either glucanase, xylanases, arabinofuranosidase, or esterase activity. Δ , β -glucanase; \circ , xylanase I; \diamond , xylanase II; \times , xylanase III; \bullet , arabinofuranosidase; \blacktriangle , feruloyl esterase; \blacksquare , xyloacetyl esterase.

sections, Autio et al (1996) demonstrated a role for xylanase in stripping away an outer layer of arabinoxylan in the walls of the aleurone, thereby exposing an inner glucan component. Although they did not report similar observations for the cells of the starchy endosperm, they did observe differences in the digestibility of walls in different regions of this tissue. The walls in the inner endosperm are much more resistant to hydrolysis.

CONCLUSIONS

β -Glucan from the walls surrounding starchy endosperm cells of barley can be solubilized by a range of enzymes, among them xylanases and enzymes capable of removing arabinosyl and ester side-chains from pentosan. This suggests that pentosan limits the solubility of glucan.

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