Enzymic Analysis of Beta-D-Glucans in Cereal Grains

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Cereal Chem. 57(3):198-202

ABSTRACT

Crude β-D-glucans were extracted from cereal grains and assayed by their hydrolysis to glucose with a partially purified β-glucanase complex from *Trichoderma viride*. The required β-glucanase was separated from contaminating amyloglucosidase by treatment of the fungal enzymes in 0.02*M* acetate, pH 4.6, with diethylaminoethyl cellulose equilibrated with this buffer. The positively charged β-glucanases were removed by filtration, whereas the anionic amyloglucosidases reacted with the cationic form of the diethylaminoethyl cellulose. The level of β-D-glucan in Larker barley was 7.2% when extracted at 80°C but only 2.8% when extracted at 45°C. β-D-Glucan quantities extracted at 80° C ranged from 1.2% for triticale to 8.2% for Birgitta barley. β-D-Glucan in malting barley varieties varied from 4.6% for Beacon to 8.2% for Birgitta. Oats contained 6.6% and wheat 1.4% β-D-glucan.

Barley endosperm cell walls are composed of a complex mixture of β-D-glucans, pentosans, and protein. Although the pentosans do not appear to be covalently linked with the β-D-glucans, some of the protein component is chemically bound to the β-D-glucans (Fleming and Kawakami 1977, Forrest and Wainwright 1977). β-D-Glucans apparently have molecular weights up to 4×10^7 and require a spectrum of enzymes for complete hydrolysis to glucose (Bathgate and Dalgliesh 1975). They are composed of β-1,4 and β-1,3-D-glucopyranosyl units in the proportion of about 7:3 (Bathgate et al 1974, Bourne and Pierce 1972).

When barley malt is used in brewing, the β-D-glucans must be degraded during the malting and mashing stages; otherwise, high wort viscosity due to undegraded β-D-glucans makes filtration of the mash difficult. Therefore, the level of β-glucans in a malting barley, about 6.5% (Fleming and Kawakami 1977), and the activity of β-glucanases in the malt are important characteristics of the malting quality of a barley.

Levels of β-D-glucans in oats have a range of about 1–3% (Wood et al 1977). Wheat and rye, on the other hand, have high levels of pentosans but little β-D-glucans (Perlin and Suzuki 1965, Preece and Hobkirk 1953).

β-D-Glucans are usually extracted from ground barley in a suitable buffer. A portion of the extract is treated with amyloglucosidase, which hydrolyzes the α-D-glucans to glucose, and another aliquot of the extract is hydrolyzed with acid. The difference between the quantity of glucose in the acid and enzyme hydrolysates is a measure of the β-D-glucan (Fleming et al 1974).

This report describes the assay of β-D-glucan in cereals by a method involving extraction and direct hydrolysis of β-D-glucan in the extract to glucose with a β-glucanase complex from *Trichoderma viride*.

MATERIALS AND METHODS

Cereals

The cereals analyzed and their location of growth and crop year are listed in Table I.

β-D-Glucan Extraction

The extraction procedure was similar to that of Wood et al (1977). Cereals were ground in a Udy cyclone mill (Tecator Udy Co., Boulder, CO) and 10-g portions were refluxed with two successive 50-ml portions of 70% ethanol, each for 2 hr. The solids were pelleted by centrifugation at 8,000 × *g* for 30 min, suspended in 300 ml of sodium carbonate-sodium bicarbonate buffer (0.2*M*, pH 10) in a 500-ml flask, and shaken (150 oscillations per minute) at an appropriate temperature (45 or 80°C) for 20 hr. The pH was adjusted to about 7 with HCl and the suspension was centrifuged at 35,000 × *g* for 20 min. The supernatant was dialyzed against glass-distilled water (4 × 1,300 ml) for 48 hr. The nondialyzable material was centrifuged as before, and the supernatant was lyophilized. The lyophilyized residue was weighed and stored over P_2O_5.

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[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Preparation of β-Glucanase

The enzyme source was "Cellulase Onazuka SS," a crude culture filtrate of *T. viride* (Yakult Biochemicals Co., Ltd., Tokyo, Japan) that has been described elsewhere (Moore et al 1972, Toda et al 1971, Tomita et al 1974). A 0.75-g portion of this crude enzyme complex, which contained β-glucanases, amyloglucosidase, and xylanase, was dissolved in 30 ml of 0.02*M* acetate buffer, pH 4.6. It was dialyzed to equilibrium at 4° C against this buffer and diluted to 80 ml with the buffer. Diethylaminoethyl (DEAE) cellulose (Whatman DE-52, Whatman Co., Clifton, NJ 07014) was regenerated according to the manufacturer's instructions, equilibrated with the same buffer, and filtered through a coarse sintered glass filter under reduced pressure. The dialyzed enzyme solution was applied to 300 g of wet DEAE cellulose and sufficient buffer was added to make a slurry. The suspension was filtered with the glass filter and washed with five 75-ml portions of buffer. Particulate matter, including fines of DEAE, was removed from the combined filtrate and washings by centrifugation at 150,000 × *g* at 4°C for 30 min. The supernatant was lyophilized, and the solids were dissolved in distilled water to a concentration of 50–100 μg of N per milliliter.

Electrofocusing

A quantity of Cellulase Onazuka SS, which contained 2.4 mg of N, was dissolved in 1% glycine, dialyzed against this solution, and electrofocused at 4°C with a 8100-1 (110-ml) LKB column (LKB Instruments Co., Rockville, MD 20852) according to the manufacturer's instructions. Ampholytes of pH 3–6 and 5–8 were used with a glycerol density gradient. The anode was at the bottom of the column. Initial conditions were 1.6 kv (Emax) and 12 W, and the final conditions after 20 hr were 1.6 kv and 4 W. Two-milliliter portions of the electrofocused fractions were collected from the column, and pHs were measured at 4°C. Enzyme activities (β-glucanase, amyloglucosidase, laminaranase, cellobiase, and xylanase) of the fractions were determined.

Enzyme Assays

β-Glucanase. The substrate, barley β-D-glucan (Biocon, Inc., Rathduff, Grenagh, Co. Cork, Ireland), prepared according to Bathgate and Dalgliesh (1975), was dissolved in 0.01*M* acetate, pH 4.8, such that the concentration was 2.5 mg/ml. One milliliter of this solution and from 1 to 10 μg of β-glucanase N were incubated from 1 to 20 hr at 50°C. Reaction rate was determined from measurement of reducing power (Luchsinger and Coresky 1962)

under linear conditons. In experiments on thermal stability of the enzyme, enzyme solutions were maintained at 35 or 50°C for up to 1 hr before activity was determined. Activity was expressed as the change in absorbance per microgram of enzyme N per hour.

Amyloglucosidase. Soluble starch (1.25%) was prepared in a solution of 0.01 *M* acetate and 0.005 *M* $CaCl_2$, pH 4.3. One milliliter of this solution and an appropriate amount of enzyme were incubated at 50°C for varying times, then heated to 100°C for 5 min to inactivate the enzyme. Where activity was insignificant, as much as 5 μg of enzyme N was used with a 20-hr incubation. The activity was measured by determining either increase in reducing power (Luchsinger and Coresky 1962) or increase in glucose level by the glucose oxidase-peroxidase method (Haugawa and Smolensky 1970). Activities were expressed either as change in absorbance per microgram of N per hour, or as micrograms of glucose produced per microgram of N per hour.

Cellobiase. Different amounts of the β-glucanase complex in 1 ml of 0.25 *M* cellobiose, 0.02 *M* acetate, pH 4.7, were incubated at 50°C for up to 3 hr and then heated to 100°C for 5 min. Glucose in the reaction mixtures was assayed with glucose oxidase-peroxidase (Haugawa and Smolensky 1970). Activities were expressed as micrograms of glucose produced per microgram of enzyme N per hour.

Laminaranase and Xylanase. The substrate was a suspension of 675 mg of laminaran, a β-1,3-glucopyranosyl polysaccharide (Nutritional Biochemical Co., Cleveland, OH 44128), in 100 ml of 0.01 *M* acetate, pH 4.7. One-milliliter suspensions and from 4 to 10 μg of enzyme N were agitated at 150 oscillations per minute at 50°C for up to 2 hr. The suspensions were kept at 100°C for 5 min after incubation, cooled, and centrifuged at 50,000 × *g* to remove unreacted substrate. Reducing power (Luchsinger and Coresky 1962) of the supernatant was measured, and activity was expressed as change in absorbance per microgram of enzyme N per hour.

Xylanase was assayed in the same way except that the buffer was 0.005 *M* citrate, pH 5.0. Xylan was obtained from the Sigma Chemical Co., St. Louis, MO.

TABLE I
Cereals Examined

Cereal	Crop Year	Location[a]
Malting barleys		
Larker	1976	ND
Vanguard	1976	WA
Klages	1977	ID
Karl	1977	ID
Kristina	1970	ID
Birgitta	1970	ID
Ingrid	1970	ID
Piroline	1971	ID
Beacon		
1	1973	ND
2	1978	ND (Minot)
3	1978	ND (Carrington)
4	1978	ND (Fargo)
5	1978	ND (Langdon)
Morex	1978	ND
Bonanza	1977	ND
Barbless	1977	ND
Manchuria	1978	ND
Manker	1978	ND
Conquest	1973	ND
Dickson	1976	ND
Traill	1976	ND
Feed barleys		
Steptoe	1976	ID
Trebi	1974	ID
Ubamer	1977	ID
Himalaya	1963	ID
Hiproly	1976	ID
Godiva	1976	ID
Everest	1976	ID
Good Delta	1976	ID
Luther	1976	ID
Other cereals		
Kenosha winter wheat	1976	WI
Polk spring wheat	1976	WI
Lodi oats (with hulls)	1976	WI
Goodland oats (with hulls)	1976	WI
Coloma rye	1976	WI
Adams rye	1976	WI
Van Luchow rye	1976	WI
Triticale	1977	ND
Sorghum TAM 680	1976	TX

[a]ND = North Dakota, WA = Washington, ID = Idaho, WI = Wisconsin, TX = Texas.

TABLE II
Percent of Commercial Barley β-D-Glucan Converted to Glucose[a] by β-Glucanase from Onazuka SS Cellulase[b]

Incubation Time at 50°C (hr)	Enzyme N, μg/ml of Substrate Solution			
	2.35	4.70	7.05	9.40
3	38	52	62	71
5	57	67	81	86
7	71	86	90	95
10.5	86	90	100	104
21	90	104	···	···

[a]By acid hydrolysis and amyloglucosidase hydrolysis, the substrate was 90–95% glucan; less than 2% starch was present.
[b]A crude culture filtrate of *Trichoderma viride.*

β-D-Glucan Analysis

Acid Hydrolysis—Amyloglucosidase Procedure. Barley extract (30 mg) was dissolved in 3 ml of 3 *N* H_2SO_4 in a test tube. The tube was sealed and maintained at 100°C for 4 hr. The acidic solution was neutralized with $BaCO_3$ and centrifuged, and total glucose in the supernatant was determined by the chromatographic procedure of Brobst et al (1973). The glucose from starch was subtracted from this total. The difference, adjusted to an anhydro basis, was the weight of β-D-glucan.

β-Glucanase Procedure. To determine the optimum amount of β-glucanase, 2.5 mg of β-D-glucan (Biocon, Inc.) per milliliter was dissolved in 0.01 *M* acetate, pH 4.8, and 0.0018 *M* sodium azide. One milliliter aliquots were treated with four levels (2.4–9.4 μg) of enzyme N (1.1 ml total volume) for 20 hr at 50°C and then heated to 100°C for 5 min. Glucose was measured by the glucose oxidase-peroxidase method (Haugawa and Smolensky 1970). Table II shows that at least 5 μg of enzyme N was needed.

Cereal extract (10 mg/ml) was dissolved in 0.01 *M* acetate, pH 4.8, and 0.0018 *M* sodium azide. β-Glucanase preparation was added to 0.5-ml solutions of cereal extract in an amount (50–200 μl, containing 5–10 μg of enzyme N) sufficient to hydrolyze the β-D-glucan completely to glucose. The reaction mixture was incubated for 8–10 hr at 50°C and then heated at 100°C for 5 min. Glucose was determined by the chromatographic method (Brobst et al 1973). The anhydro value was calculated; this was the amount of β-D-glucan in the quantity of substrate used. Finally, the β-D-glucan content of the toal extract was calculated; this was the amount from the original 10 g (dry basis) of cereal. Substrate and enzyme blanks were treated similarly.

Determination of Starch in β-D-Glucan Preparations

The method was described by Banks et al (1970). α-Amylase, (Type IIA, Sigma Chemical Co., St. Louis, MO) was dissolved in water to give 2,500 units per milliliter and, similarly, amyloglucosidase (Grade IV, same source) was prepared so that the solution contained 1,400 units per milliliter. The starch substrate prepared as above in 0.01 *N* acetate and 0.005 *M* $CaCl_2$, pH 4.8, contained 2.2 mg of starch per milliliter. To each of 0.1–1.0 ml aliquots of substrate solution, 10 μl of α-amylase solution and 10 μl of amyloglucosidase solution were added; all volumes were adjusted to 1.02 ml with buffer, and the reaction mixtures were incubated for 3–20 hr at 50°C. Appropriate enzyme and substrate blanks were included. All solutions were heated at 100°C for 5 min

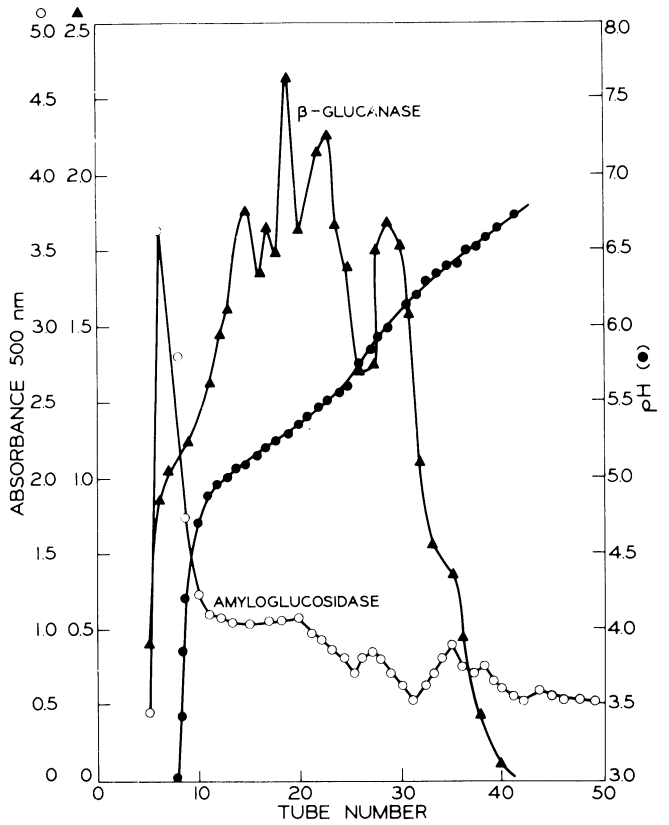

Fig. 1. Isoelectrofocusing (pH 3–6) of *Trichoderma viride* β-glucanase and amyloglucosidase.

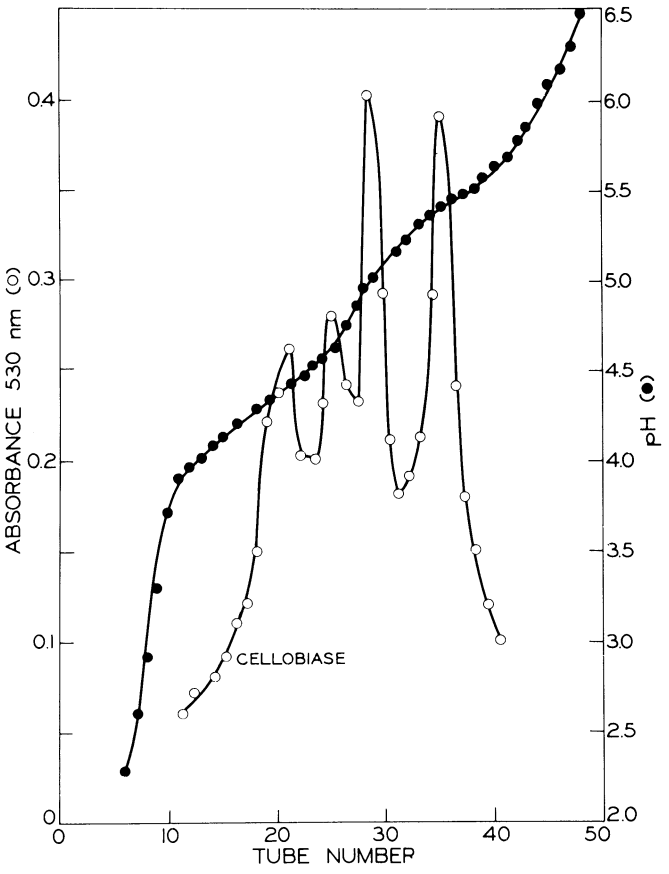

Fig. 2. Isoelectrofocusing (pH 3–) of *Trichoderma viride* cellobiase.

after incubation. Glucose in the final solutions was measured chromatographically (Brobst et al 1973). The conversion of starch to glucose was 90–97% with both reaction times. Unknown solutions containing this range of starch concentrations were treated similarly. Essentially the same starch values were obtained when α-Amylase was omitted.

Nitrogen Analysis

This was done by the micromethod of Johnson (1941).

Test for Traces of Amyloglucosidase in β-Glucanase Preparations

The barley extracts prepared at 45 and 80°C contained about 19 and 78% starch, respectively, which could be hydrolyzed by any amyloglucosidase present in the β-glucanase preparation. A starch

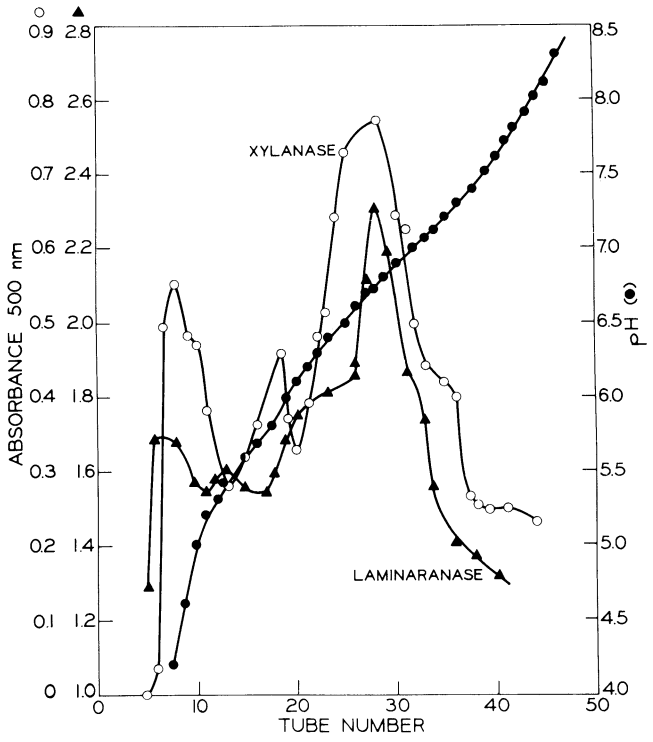

Fig. 3. Isoelectrofocusing (pH 5–8) of *Trichoderma viride* laminaranase and xylanase.

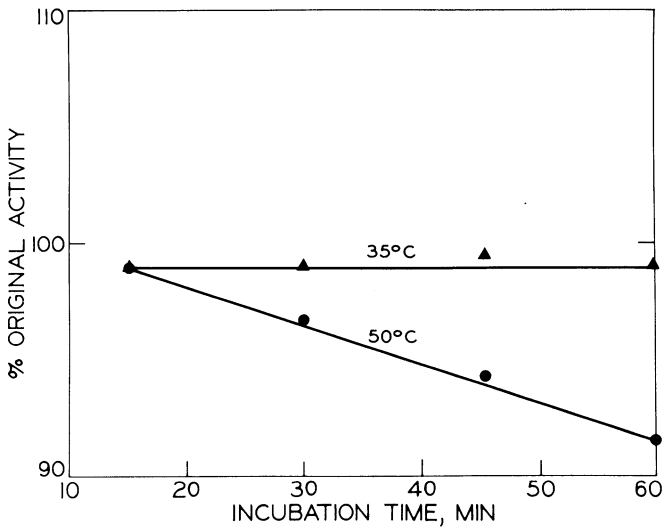

Fig. 4. Thermal stability of β-glucanase. Enzyme solution was maintained at 35°C or 50°C for the indicated times before activity was determined.

solution was therefore made to contain 2.5 mg per milliliter in buffer (0.01*M* acetate, 0.005*M* $CaCl_2$, 0.00185*M* NaN_3, pH 4.8); and 1-ml aliquots were treated with the β-glucanase preparation (10 μg of enzyme N) for 20 hr at 50°C. Glucose was determined chromatographically (Brobst et al 1973) in the reaction mixture after enzyme inactivation (100°C for 5 min).

RESULTS AND DISCUSSION

Purification of the β-Glucanase Complex from Cellulase Onazuka SS

If β-D-glucan in starch-containing extracts of cereals is to be estimated from its enzymic conversion to glucose, the enzyme preparation must be essentially free of amyloglucosidase. Figure 1 shows that the β-glucanase complex of *T. viride* was isoelectric between pH 5 and 6, whereas most of the amyloglucosidase, amylase, and α-glucosidase was isoelectric at about pH 3. Shown in Fig. 2 are the cellobiase isozymes, isoelectric between pH 4.4 and 5.4. Figure 3 shows most of the laminaranase and xylanase to be isoelectric at about pH 6.5–6.7. The separation of much of the undesirable amyloglucosidase seemed possible by the use of an anion exchanger that would strongly retain this negatively charged amyloglucosidase but allow the positively charged β-glucanase complex, the cellobiase isozymes, and most of the laminaranse to be removed by filtration.

These enzymes were separated from Onazuka SS with DEAE cellulose at pH 4.6. Treatment of the commerically purified barley

TABLE III
Activities of Parent *Trichoderma viride* Cellulase and the β-Glucanase

	Parent Cellulase (U/μg N[a])	β-Glucanase Preparation (U/μg N[a])	% Recovery[b]
Amyloglucosidase	0.71	0.006	0.5
β-Glucanase	4.1	6.9	27
Laminaranase	0.30	0.66	4
Cellobiase	32	19	15
Xylanase	1.0	2.3	35

[a] A unit of amyloglucosidase, β-glucanase, laminaranase, and xylanase is an increase of 1.0 absorbance at 500 nm/hr. A unit of cellobiase is an increase of 1 μg of glucose per hour.
[b] Percent of total parent activity recovered in the purified β-glucanase preparation.

TABLE IV
Analyses of Replicate Extracts of Larker Barley for β-D-Glucan with Replicate Enzyme Preparations

Enzyme Replicate No.	Barley Extract Replicate No.	β-D-Glucan (% of barley)
Extracted at 45°C		
2	1	2.8
	2	2.8
Extracted at 80°C		
1	1	7.7
	2	7.7
	3	7.2
2	1	7.4
	2	7.1
	3	7.1
3	1	7.3
	2	7.2
	3	7.4
4	1	7.0
	2	6.6
	3	7.0
Mean		7.2
Standard deviation		0.3

TABLE V
Replicate Analysis of Larker Barley for β-D Glucan by the Acid Hydrolysis-Amyloglucosidase Method

Extraction Temperature	β-D-Glucan (% of Barley) in Replications 1	2	3	4	5	6	7	Mean	SD
45°C	1.9	2.1	2.0	2.2	2.0	2.1	1.9	2.0	0.1
80°C	7.7	8.0	7.9	7.4	7.0	7.0	...	7.5	0.4

TABLE VI
Composition of Larker Barley Extracts

Extraction Temp	Trial No.	Weight of Sample (g)	Weight of Extracted Solids (g)	% of Sample	Protein (%)	Starch (%)	β-Glucan (%)
45° C	1	10.0050	0.4241	4.4	11.6	19.0	64.9
	2	10.0047	0.4391	4.5	13.0	19.0	64.6
80° C	1	10.0042	3.5142	35.1	4.0	77.0	20.0
	2	10.0015	3.2273	32.3	3.6	75.0	20.4
	3	10.0032	3.4482	34.5	3.4	78.0	20.3

TABLE VII
β-D-Glucan in Cereals

Cereal	Replications	Kernel Weight (mg)	β-D-Glucan % of Cereal Mean	SD
Malting barleys				
Larker	12	42.3	7.2	0.3
Vanguard	5	39.6	7.4	0.6
Klages	5	44.2	7.8	0.5
Karl	5	38.0	7.7	0.2
Kristina	6	46.5	7.6	0.4
Birgitta	9	50.4	8.2	0.3
Ingrid	7	46.2	7.9	0.3
Piroline	4	45.8	6.0	0.4
Beacon				
1	4	36.6	4.7	0.3
2	4	36.7	4.6	0.5
3	4	34.4	4.5	0.6
4	4	30.4	5.3	0.2
5	4	35.5	5.2	0.4
Morex	4	33.1	5.4	0.4
Bonanza	2	30.3	6.9	0.3
Barbless	4	34.8	7.5	0.3
Manchuria	3	32.6	5.5	0.2
Manker	3	35.8	6.1	0.3
Conquest	3	36.7	5.8	0.3
Feed barleys				
Steptoe	4	43.2	5.7	0.5
Trebi	4	47.9	6.6	0.4
Ubamer	4	47.9	5.6	0.3
Himalaya	4	50.8	6.3	0.5
Hiproly	4	38.7	6.6	0.4
Pearled barley	4	26.3	5.1	0.3
Godiva	3	40.7	7.2	0.5
Everest	4	42.3	5.7	0.5
Good Delta	4	37.5	6.2	0.3
Luther	4	38.1	5.7	0.1
Other cereals				
Kenosha winter wheat	4	29.0	1.4	0.4
Polk spring wheat	2	35.8	1.4	0.5
Lodi oats	4	30.2	6.6	0.4
Goodland oats	4	31.4	4.8	0.2
Coloma rye	4	29.8	1.9	0.2
Adams rye	3	26.5	2.3	0.1
Van Luchow rye	4	28.1	2.9	0.2
Triticale	4	39.5	1.2	0.3
Sorghum TAM 680	4	27.9	1.0	0.4

β-D-glucan with β-glucanase prepared from the *T. viride* source yielded complete recovery as glucose after 10 hr (Table II). Because β-glucanase appeared to be almost as stable at 50°C as at 35°C during 1 hr of inbcubation (Fig. 4) a reaction temperature at 50°C was chosen to preclude microibal growth. Activities of the β-glucanase complex and of the parent *T. viride* cellulase and percent recovery of enzyme activities in the purified β-glucanase preparation are given in Table III. Although the β-glucanase activities, as expressed by development of reducing power (Table III), are a measure of substrate hydrolysis, an enzyme complex that gives high activity measured in this way does not necessarily have good glucose-producing activity so that it can be used for β-glucan assay. This property must be determined by prolonged action on the substrate, as was done for the data in Table II.

Treatment of starch solutions for 8–10 hr at 50°C with the purified β-glucanase showed negligible (0.5%) starch hydrolysis.

Analysis of Cereals

In some studies (Wood et al 1977) on β-D-glucan levels of oats and barley, the glucan was extracted at 45°C. Table IV shows that extraction of Larker barley at this temperature gave a β-D-glucan level of 2.8% when the extract was hydrolyzed with β-glucanase. Similarly, the average β-D-glucan in the 45°C extract was 2.0% when measured by the acid hydrolysis and amyloglucosidase treatment (Table V). This is in agreement with the values of Wood et al (1977), who used this extraction temperature.

Fleming and Kawakami (1977) and Wood et al (1978) showed that the level of β-D-glucan obtainable depended on the temperature of extraction. Table IV shows that when the extraction temperature was 80°C, replicate assays of Larker barley with β-glucanase gave a β-D-glucan level of 7.2%. Similarly, hydrolysis of the extracted solids with acid and amyloglucosidase gave a β-D-glucan level of 7.5% (Table V). Much more material was extracted at 80°C than at 45°C, and, relative to the material extracted at 45°C, it contained a lower percentage of protein and β-D-glucan and a higher percentage of starch (Table VI). Replication of the analysis (extraction temperature 80°C) with three extracts of Larker barley and four different enzyme preparations (Table IV) showed the coefficient of variability to be about 4%.

Other cereals besides Larker barley were analyzed by the 80°C extraction and β-glucanase method. Table VII shows marked differences in their β-glucan contents. Representative values for oats, wheat, rye, and triticale were 6.6, 1.4, 2.9, and 1.2%, respectively. β-D-Glucan in malting barleys ranged from 4.6 (Beacon) to 8.2% (Birgitta). The low β-D-glucan level of Beacon barley occurred in the five samples that represented two crop years and several locations. For the malting barleys listed in Tables I and VII, the correlation coefficient for kernel size and β-D-glucan level is 0.84. This may reflect the fact that the starchy endosperm (where the β-D-glucan is located) is smaller in proportion to the bran and hull tissue in the small kernels than it is in the large ones. Exceptions are Piroline barley, which has a kernel weight close to that of Ingrid but about 25% less β-D-glucan, and Barbless barley, which has a kernel weight similar to the Beacon samples but about 50% more β-D-glucan.

The assay for β-D-glucan with β-glucanase is less time-consuming than that involving acid and amyloglucosidase-catalyzed hydrolyses. The β-D-glucan assay requires only a small amount of the lyophilized β-glucanase, which can be prepared in quantity and stored in the freezer indefinitely.

The glucose oxidase-peroxidase assay is adequate for estimating glucose from the action of β-glucanase on pure β-D-glucan and starch substrates. Glucose formed by the enzyme acting on barley extracts must be measured by a chromatographic method, however, because glucose oxidase and/or peroxidase activity is inhibited by one or more components of the solids extracted from barley, and only about 55% of the glucose present is indicated by the assay.

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[Received June 11, 1979. Accepted August 16, 1979]