JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay To Quantify Soluble β-Glucans in Oats and Barley

CHRISTOF RAMPITSCH,* NANCY AMES, JOANNE STORSLEY, AND LINDSAY MARIEN

Cereal Research Centre, Agriculture and Agri-Food Canada, 195 Dafoe Road, Winnipeg, Manitoba, Canada R3T 2M9

A set of 31 murine monoclonal antibodies was produced against (1→3,1→4)β-D-glucan from oats (*Avena sativa* L.) chemically cross-linked to keyhole limpet hemocyanin. Monoclonal antibodies were tested for their cross-reactivity to related and unrelated polysaccharides. The antibodies reacted strongly to unmodified β-glucan from oats and barley (*Hordeum vulgare* L.) and to lichenan from Icelandic moss, a polysaccharide with a structure similar to that of β-glucan but which is not encountered in cereals. Cross-reaction to other polysaccharides tested was minimal at physiological levels. An enzyme-linked immunosorbent assay (ELISA) that could routinely detect and quantify nanogram levels of soluble β-glucan extracted from the flour of oats or barley was designed with one of these monoclonal antibodies. The β-glucan extraction procedure from ground oat and barley samples and the ELISA were both optimized for reproducibility, accuracy, and throughput, and results were compared to values obtained from an established, commercially available enzyme-based assay. Correlations between the two assays were consistently high ($r^2 > 0.9$), indicating that the ELISA presented in this paper is a valuable alternative for assaying β-glucan levels in cereals and cereal products, both routinely and in preparations in which β-glucans are present in nanogram amounts. Development of the extraction procedure for ELISA is discussed.

KEYWORDS: ELISA; β-glucan; nonstarch polysaccharide; *Avena sativa*; *Hordeum vulgare*

INTRODUCTION

β-D-Glucans are major structural polysaccharide components of the seed of oats and barley and together with cellulose and arabinoxylans are the main contributors of dietary fiber in these grains. β-Glucans are unbranched linear polymers of glucose linked by β(1→3) and β(1→4) linkages. There are minor variations in the ratios of these linkages, but β-glucans from all sources are at least partially soluble in aqueous buffers and form viscous solutions, although β-glucans from oats are more readily solubilized than those of barley (*1*). Nonstarch polysaccharides have health benefits for humans. For β-glucans there is a large body of evidence showing an ability to lower serum cholesterol levels in humans and rats (*2*−*4*). It has been shown, for example, that rats fed a diet rich in β-glucan had serum cholesterol levels reduced 2.5-fold, low-density lipoprotein (LDL) cholesterol 1.6-fold, and triglycerides 1.3-fold, compared to rats fed a control diet (*3*). These reductions contribute to lowering the incidence of heart disease. More recently, Cavallero et al. (*5*) have measured decreased glucose levels in the postprandial blood of human subjects given a diet with a high glycemic index when eating barley products rich in β-glucans.

Oats are an important ingredient of many familiar foods and food products, such as breakfast cereals, baked goods, and snacks, in which high levels of β-glucans are clearly beneficial. Barley is also beginning to find a niche market as an ingredient of baked foods for human consumption, with similar health benefits. In contrast to this, both oats and barley are used for animal feed for which, particularly in the poultry and hog industries, β-glucans are undesirable (*6*). Barley is also grown for malt used in the brewing industry; however, β-glucans adversely affect processing because they can form gels or hazy precipitates during fermentation or beer storage (*7*, *8*). There is clearly a need for cereal breeders to tailor β-glucan levels in oat and barley cultivars to meet the requirements of their intended end-use.

The commercial importance of β-glucans has led to the development of at least two methods for their assay. The first is based on an intense fluorescence produced by a complex of Calcofluor with β-glucan (*9*), and the second is based on the enzymatic degradation of the polysaccharide by β-glucan hydrolases (*10*) followed by a spectrophotometric measurement of liberated reducing sugars. Both of these methods are used routinely in the brewing and food industries, but neither is entirely suitable as a high-throughput assay of β-glucans in barley or oat flour, of the kind that would be required by cereal

* Author to whom correspondence should be addressed [telephone (204) 983-2385; fax (204) 983-4604; e-mail crampitsch@agr.gc.ca].

10.1021/jf030173i CCC: $25.00 Published 2003 by the American Chemical Society
Published on Web 08/30/2003

breeders. The main throughput barriers for any large-scale assay are sample preparation and cost, the latter of which is greatly reduced for the enzyme-linked immunosorbent assay (ELISA). ELISA has the further advantage that it can be performed in 96-well (or larger) arrays and can be adapted to existing liquid-handling robots. Monoclonal antibodies have been made successfully to detect β-glucans in the cell wall of wheat by immunotransmission electron microscopy (*11*), but an ELISA suitable for high-throughput assay of β-glucans in ground cereal samples is still lacking.

One of the challenges of producing any assay to detect β-glucans is the development of an extraction protocol that solubilizes and thus permits accurate measurement of total β-glucans (*12*, *13*). This is of special concern for barley cultivars, which have widely varying ratios of soluble to insoluble β-glucans (*14*). Furthermore, the extraction method must be nondestructive to preserve β-glucan epitopes for the ELISA. This precludes use of enzymes such as lichenase and β-glucanase, which form the core of the standard enzymatic measurement method (*15*), relying instead on chemical extraction.

We describe an ELISA suitable for low- and high-throughput measurement of β-glucan levels in ground cereal. Efforts have been made to select an optimal monoclonal antibody, to optimize ELISA conditions, and to develop a method capable of processing hundreds of samples with little cost in time and labor. Methods for extracting β-glucans for ELISA have been investigated, and the extraction method used for the ELISA reported here is designed to maximize the extraction of soluble and insoluble β-glucans from samples. Data comparing this ELISA with β-glucan measurements made by the established assay of McCleary and Codd (*10*) are presented.

MATERIALS AND METHODS

Preparation of Antigen and Production of Antibodies. Oat (1→3,1→4)β-D-glucan (medium viscosity) was purchased from Megazyme (Bray, Ireland), chemically modified and cross-linked to keyhole limpet hemocyanin (Pierce, Rockford, IL) exactly as described by Meikle et al. (*11*). This antigen was used to prepare murine monoclonal antibodies following standard procedures (*16*). Four mice were immunized by three intraperitoneal injections of ~1 mg of conjugate and a final intravenous injection on the day before the fusion. Antigenic response was determined by ELISA performed on serum collected by a tail bleed. A single fusion was performed on the best responder. A total of 31 antibody-secreting hybridomas that bound to β-glucan were identified by ELISA, and seven of these were single cell cloned twice by limiting dilution.

Antibody Purification. Antibodies were purified from tissue culture supernatant (TCS) by chromatography. The TCS was first precipitated from 50% saturated ammonium sulfate and the pellet redissolved in 0.1 volume of phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.2). Antibodies were then purified by protein G affinity chromatography using a 1 mL HiTrap column under conditions recommended by the manufacturer (Amersham Biosciences, Little Chalfont, U.K.). Antibodies were eluted from the column with 0.1 M glycine, pH 2.8, which was immediately neutralized with 20 μL/mL 10% (v/v) diethanolamine. Antibody purity was assessed by denaturing polyacrylamide gel electrophoresis. Protein content was determined using the Bradford dye-binding assay with an IgG control (Bio-Rad, Hercules CA).

For large-scale production of monoclonal antibodies, hybridomas were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum until maximum growth was achieved. At this stage the medium was removed and centrifuged to remove cells, and antibody was purified by affinity chromatography as described above.

Sample Preparation and Assays. Standard enzymatic determinations of β-glucans are described by AACC Standard Method 32-23 (*15*). A kit designed for the application of this method was purchased from Megazyme and used for all enzyme assays. A standard curve of commercially prepared β-glucan from oats and A_{510} were used to calculate the amount of β-glucan present in oat and barley samples.

Three extraction agents, 20 mM sodium phosphate buffer, 1 N NaOH, and 0.2 N HNO_3, were evaluated for their ability to solubilize β-glucan from oats and barley. This was done by assaying solubilized β-glucan by ELISA and the enzymatic procedure, as described below, and assaying unextracted β-glucan remaining in the pellet using the enzymatic assay.

Samples were obtained from cereal breeding and quality analysis programs located at Winnipeg or Brandon (MB, Canada) and Saskatoon (SK, Canada) (**Table 1**). Hulled seeds were mechanically dehulled and milled to pass through a 0.5 mm screen. The resulting wholemeal and/or fractions, hereafter termed flour, were dried and stored desiccated until needed. In a glass test tube, 20 mg of flour was wetted with 200 μL of ethanol and then resuspended in 2 mL of 1 N NaOH. Samples were left at room temperature for 10 min with occasional vortexing. Debris was then centrifuged briefly at low speed (3200*g* for 2 min). The supernatant was diluted 10−50000-fold with PBS before transferring 100 μL into the wells of Maxisorp ELISA plates (Nunc, Roskilde, Denmark).

Table 1. Sources of Barley and Oat Samples Used in This Study

	sample/cultivar	year	location	fraction/description
barley	AC Metcalfe	2001	Brandon, MB[a]	malt
	CDC Alamo	2000	SK	flour
	CDC Dawn	2000	SK	shorts
	Condor	2000	SK	dusted flour from bran
	CDC 92-55-06-54	2000	SK	shorts
	CDC Alamo	2000	SK	shorts
oats	OT7051	2001	Indian Head, SK	wholemeal
	AC Ronald	2001	Glenlea, MB	wholemeal
	WO1323	2001	Brandon, MB	wholemeal
	commercial ground oats			wholemeal
	WO1321	2001	Brandon, MB	wholemeal
	commercial bran A			bran
	commercial bran B			bran
wheat	Glenlea	2001		flour

[a] Canadian provinces (MB = Manitoba, SK = Saskatchewan).

For processing large numbers of samples rapidly, the same method was used, but scaled down: 5 mg of flour was weighed onto a 15 mm GF/A glass fiber disk (Whatman, Maidstone, U.K.). The entire disk was then placed into a 2 mL well of a deep-well microtiter plate (Whatman) containing a single 2 mm glass bead and extracted with 1 mL of 1 N NaOH with vigorous shaking at room temperature in a commercial paint shaker for 20 min. After extraction and centrifugation, samples were diluted 50000-fold in PBS, and then 100 μL was transferred to wells of an ELISA plate. All plates were kept overnight at 4 °C.

The following day, wells were emptied and washed three times with PBS. A hand-held Immuno-Wash 8 plate washer (Nunc) was used initially and after each subsequent step. Plates were blocked with 2% BSA in PBS with 0.1% (v/v) Tween 20 for 1 h at room temperature (~22 °C). Monoclonal antibody 9E11 was then added to each well at 25 ng/μL in PBS with 0.2% BSA and incubated at room temperature for 1 h. Finally, plates were incubated with an alkaline phosphatase-linked goat anti-mouse conjugate (Sigma, St. Louis, MO) diluted as recommended by the manufacturer in PBS with 0.2% BSA. After 1 h, plates were incubated with 5 mg/mL *p*-nitrophenyl phosphate in 10% (v/v) diethanolamine. The absorbance of wells was read after 1 and 3 h at 405 and 620 nm in a multiwell μQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT). All ELISA absorbance values are reported as A_{405} minus A_{620} after 3 h. The effect of the GF/A disk on the outcome of the ELISA was assessed; template-negative controls (blanks) were run on each plate.

All samples were assayed in duplicate on each plate, and each assay was carried out on two ELISA plates.

Modifications to the Extraction Procedure. For ethanol precipitation of β-glucans, the supernatant from the NaOH extraction was neutralized with an equal volume of 1 N HCl and 100 μL was aspirated and precipitated from 80% ethanol for 30 min at −20 °C, followed by centrifugation at 3200*g* for 10 min. The pellet was redissolved in 400 μL of PBS, diluted, and loaded into the well of a microtiter plate.

To compare the ELISA and enzymatic procedure directly, dissolved β-glucans were obtained by chemical extraction without lichenase as follows: flour samples were extracted with NaOH as before, but flour was removed by centrifugation before the addition of lichenase. The supernatant only, containing dissolved β-glucan, was then used for β-glucan analysis using both ELISA and enzymatic assays. Residual β-glucan remaining in the pellet was measured using the enzymatic procedure, treating the pellet as a new sample of flour for analysis.

For extractions needing enzymes, care was taken to ensure that the enzyme was placed into and incubated in a suitable buffer at a suitable temperature.

Antibody Characterization. The isotype of all 31 monoclonal antibodies obtained was determined by an indirect ELISA using anti-isotype specific antibodies (Sigma). Ambiguous results were confirmed using a reagent-strip-based isotyping assay (HyCult). Antibody sensitivity was measured by ELISA by assaying the amount of antibody needed in nanograms to detect a nonlimiting amount of β-glucan (100 ng) after 1 h of substrate incubation. "Detection" was defined as an absorbance reading (A_{405} minus A_{620}) greater than the mean plus 3 standard deviations of 15 negative samples (wheat flours).

Direct ELISA to test for cross-reactivity to dextran, amylose, amylopectin, arabinoxylan, pectin, locust bean gum, guar gum, cellulose, and lichenan was performed as described above. Competitive ELISA was performed as follows. Plates were coated with 100 ng of β-glucan per well as described and allowed to bind overnight at 4 °C. After blocking and washing as before, 50 μL of competing polysaccharide was added followed immediately by 50 μL of monoclonal antibody in PBS with 0.5% BSA, giving final polysaccharide concentrations of 100, 10, 1, and 0 μg/100 μL. The competition reaction proceeded for 1 h at 22 °C. Sample negative controls, without initial antigen, were made on the same plate; all samples were run in duplicate and on duplicate plates. The ELISA was completed as before. Results were expressed as the percentage of signal reduction (averaged), relative to the maximum signal wells (i.e., those without competing polysaccharide).

RESULTS

Monoclonal Antibodies. The myeloma/splenocyte fusion yielded 31 stable hybridomas producing monoclonal antibodies to β-glucan. All antibodies gave strong ELISA values with oat and barley β-glucan after a 3 h substrate incubation period, and ELISA values to Icelandic moss lichenan ranged from an approximately equal signal to a 3-fold reduced signal (**Table 2**). Icelandic moss lichenan has a structure very similar to that of β-glucan but is not encountered in cereals. All other polysaccharides tested, including ones that are commonly found in cereals such as amylose, amylopectin, cellulose, and pentosans, gave ELISA readings which were at background. Zero-target blanks, zero-antibody blanks, and flour that was essentially β-glucan-free (wheat) all gave similar ELISA signals, which were at background. To ensure that polysaccharides were able to bind to the plate, competitive ELISAs were also run. **Table 2** shows competition ELISA results of the two most cross-reactive polysaccharides tested, expressed as a ratio of absorbances obtained for wells with 10-fold excess of competing polysaccharide compared to the zero-competitor well. When a 1000-fold excess was used, the monoclonal antibodies tested again cross-reacted mainly with lichenan and arabinoxylan (between 84 and 94% reduced signal, unpublished observation). However, because the assay format used for β-glucan analysis was a direct ELISA, the effect of this cross-reactivity on the ELISA results presented is minimal.

Table 2. Properties of 8 of 31 of the Anti-β-glucan Monoclonal Antibodies Evaluated

			cross-reactivity by ELISA[b]			
			competitive[c]		direct	
antibody	isotype	sensitivity[a] (ng)	lichenan	arabino-xylan	lichenan	arabino-xylan
4G9	IgG1	3.25	0.16	0.41	0.75	0[d]
5B6	IgG1	120	ND[e]	ND	0.86	0
5G4	IgG1	3.25	0.34	0.85	0.80	0
6C8	IgG2a	60	ND	ND	0.32	0
6E3	IgG1	120	ND	ND	0.88	0.01
9E11	IgG1	3.25	0.84	0.89	0.61	0
10F11	IgG1	3.25	0.68	0.89	0.65	0
14E8	IgG1	3.25	0.70	0.84	0.60	0.02

[a] Sensitivity is the minimal amount of antibody required to give a signal; "detection" is defined as an absorbance reading (A_{405} minus A_{620}) greater than the mean plus 3 standard deviations of 15 negative samples (wheat flours). [b] Values for the most cross-reactive polysaccharide shown as A_{405} (polysaccharide)/A_{405} β-glucan. [c] Values for 10-fold excess competing polysaccharide. [d] Values of <0.01 are shown as 0. [e] Not determined.

Table 2 summarizes the properties of eight of the monoclonal antibodies that were used for further study. They were chosen on the basis of sensitivity, serotype, and ease of culturing the parent hybridoma. The others have been partially characterized, including 10 IgMs, (unpublished data), and are being stored either freeze-dried or frozen. Monoclonal antibody 9E11 and its parent hybridoma line had the best overall properties, and it was used for all subsequent ELISAs.

ELISA: Linear Range. The response of the ELISA to commercially available, purified oat β-glucans using 9E11 (**Figure 1**) and 10F11 (not shown) was linear over a range of ∼1−20 ng of β-glucan/mL, with a linear regression value, $r^2 = 0.958$. This range was established empirically after it was observed that higher levels of β-glucan failed to increase the ELISA response, which reached a plateau when β-glucan was >25 ng/mL (unpublished observation). This was also reported by Meikle et al. (*11*). Correlation coefficients (r^2) for the 10 most recent standard curves run during this study ranged from 0.902 to 0.998 and the dynamic range (absorbance maxima) from 0.3 to 0.8. In subsequent ELISA experiments extracted flour samples were diluted so that their β-glucan content would fall approximately within the linear range. A linear relationship between β-glucan amounts and A_{510} with $r^2 = 0.997$ was also obtained with the enzymatic assay under the conditions shown in **Figure 1**.

The relationship between β-glucan amounts measured by ELISA and by the enzyme assay was also linear, after suitable dilution of the ELISA samples. Lower dilutions of β-glucan extracted from grain failed to reach a plateau, instead showing reduced absorbance values.

ELISA: Ground Barley and Oat Samples. The oat and barley flours and brans that were used for this study are listed in **Table 1**. They were chosen to cover a range of β-glucan values (**Table 3**). Results of an ELISA of these samples, together with levels of β-glucan measured by the enzymatic method, are plotted as a curve of β-glucan amount (micrograms per milliliter) and corrected absorbance (A_{405} minus A_{620}) (**Figure 2**). The r^2 values for these curves were 0.990 (barley) and 0.955 (oats). These r^2 values show a good correlation between results of the two assays.

A more detailed analysis of the extraction procedure showed that some 5% of β-glucans remained in the pellet of barley flour

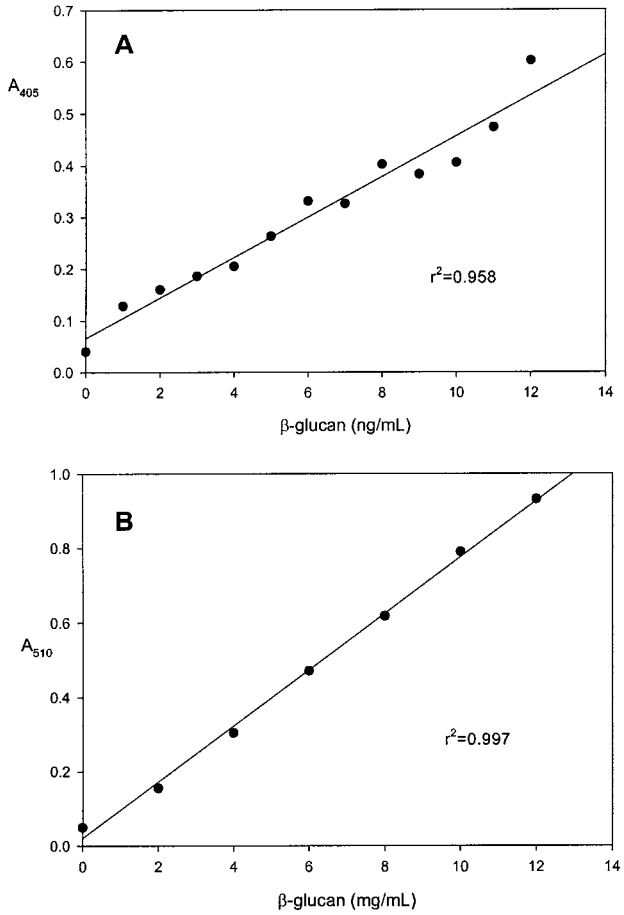

Figure 1. Standard curves of (A) ELISA data quantifying commercially purified oat β-glucan amounts with monoclonal antibody 9E11 and (B) enzyme-based measurement (*15*) of the same commercial oat β-glucan.

Table 3. Amount of Unextracted β-Glucan Remaining in the Pellets after Extraction by NaOH or HNO_3

	% unextracted β-glucan[a]		
variety	NaOH	HNO_3	%βG[a]
AC Metcalfe malt	4.9	1.3	0.6
CDC Alamo flour	3.46	1.74	5.1
CDC Dawn shorts	3.18	3.6	6.6
Condor bran flour	2.56	3.79	7.7
CDC 92-55-06-54 shorts	3.88	4.89	8.9
CDC Alamo shorts	4.96	3.89	11.4
OT7051 wholemeal	0[b]	0	3.7
AC Ronald wholemeal	0	0.45	4.8
WO1323 wholemeal	0	1.65	5.9
commercial ground oats	0	2.13	6.8
WO1321 wholemeal	0	1.22	7.3
commercial bran A	0	3.54	7.9
commercial bran B	0	2.63	8.5

[a] β-Glucan was measured by standard enzyme assay (*15*) and is shown as mg of β-glucan per 100 mg of flour. [b] A zero reading indicates that the value was below the threshold of the assay.

after NaOH extraction, as measured by the enzymatic assay, whereas all of the measurable β-glucan was extracted from oats (**Table 3**). Attempts were made to improve recovery of β-glucan from the pellet by using 50 mM nitric acid as suggested by Manzanares and Sendra (*17*); this resulted in reduced yield in oats and approximately the same yield in barley, with minor variations between samples (**Table 3**). Attempts were made to improve correlation between the two assays by measuring chemically extracted β-glucans (i.e., without lichenase or other

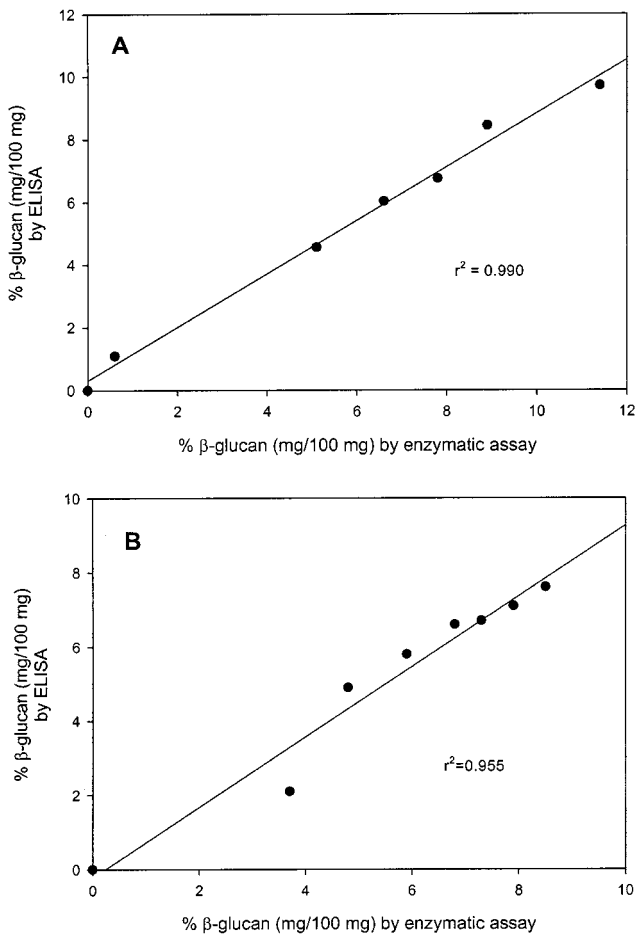

Figure 2. Comparison of assays used to quantify β-glucan prepared from ground (A) barley and (B) oat samples. Independent extraction methods were used to measure total β-glucan by ELISA and with the enzyme-based measurement (*15*).

Table 4. Effect of Various Components on the Extraction of β-Glucan from Oat and Barley Flour for ELISA

		A_{405}[a]	
treatment		oat WO 1323	barley CDC Dawn
1	1 N NaOH extraction[b]	0.767	0.65
2	0.2 M sodium phosphate extraction	0.445	0.563
3	1 + ethanol precipitation	0.661	0.584
4	1 + proteinase K	0.714	0.446
5	1 + α-amylase	0.718	0.547
7	1 + lichenase	0.035	0.029
8	1 + lysozyme	0.244	0.176
	% β-glucan	5.9	6.6

[a] Absorbance measured after a 3 h substrate incubation; 10 ng/mL β-glucan. [b] Control method described in text.

enzymes); this effectively allows both assays to measure exactly the same pool of β-glucan. However, correlation coefficients were consistently in the same range ($r^2 > 0.95$), suggesting that the unextractable β-glucan does not seriously affect the accuracy of the ELISA.

Other variations in the extraction procedure included extracting with 20 mM phosphate buffer, pH 6.8, with boiling or adding an enzyme step to the extraction. Lichenase, proteinase K, and α-amylase (and lysozyme as a control) all failed to improve the response of the ELISA (**Table 4**). Inclusion of an ethanol precipitation step gave inconsistent results, again with a reduced

ELISA signal. Deviation from the NaOH extraction did not improve the assay and merely added steps to the procedure.

DISCUSSION

The monoclonal antibodies raised to chemically modified β-glucan gave a specific, linear response to β-glucan by ELISA. Because a linear response is also obtained from the standard enzymatic assay, the ELISA can be used in its place for soluble β-glucan measurements. It is also possible to use the ELISA to get an accurate estimate of relative β-glucan levels in flour samples if the described extraction procedure is followed exactly. Even the type of plate used (Nunc with MaxiSorp surface) was critical to obtaining good standard curves and correlations. Two other types of plate were assessed (Costar and Nunc polystyrene), but these were less sensitive and gave inferior results with low correlation coefficients (not shown).

Care must be taken in interpretation of results, especially when the measurement of absolute amounts is attempted, because residual β-glucans remaining in the flour can amount to 5% (**Table 3**), although higher values, up to 20%, have been reported in some cases (*12*). This value varies between cultivars. We have shown that this does not seriously affect the results of the ELISA. The ELISA is thus a valid screening tool for early generation identification of high- or low-β-glucan lines and is an excellent low-cost, high-throughput assay for dissolved β-glucan measurement.

The ELISA has a limited linear range. Results are not accurate if β-glucan levels approximately >20 ng/mL are used, so dilution into this range is necessary. This is both an advantage and a disadvantage; the high sensitivity of the assay permits testing of very small samples and also suggests that the monoclonal antibodies could be used in other experiments in which sensitivity is paramount. Small sample size also makes the assay compatible to 96- or 384-well arrays. The drawback is that large dilutions have to be made accurately to bring β-glucan samples into the correct range for accurate ELISA. When wholemeal from breeder's lines is assayed, varieties exceeding 12% β-glucan are rarely encountered, most being in the 2−9% range. The 50000-fold dilution brings samples with β-glucan <20% into the linear range of the assay. Enriched samples, such as brans, with β-glucan levels in excess of this are encountered, and the user should try several dilutions. Malting barley varieties or other samples with very low β-glucan (<1%) are best assayed at lower dilutions. Some caution should be used when enriched fractions suspected of being very high in arabinoxylan (>10-fold excess relative to β-glucan) are assayed, because all of the monoclonal antibodies tested show some cross-reactivity to this pentosan in a competitive ELISA. Such high levels of arabinoxylan are not encountered in wholemeal, which typically contains <10%, nor in flour, which is typically <3% arabinoxylan (*18*). It is also important to point out that during the direct ELISA the monoclonal antibody is never in solution with the extracted sample, as it is in a competitive assay, and only minor levels of interfering polysaccharides liberated from the plate during incubation could reduce the ELISA signal. Cross-reaction to non-β-glucan polysaccharides was thus not a concern and was further reduced by keeping the primary antibody concentration well above its sensitivity limit.

During the design of the ELISA it became clear that results of the assay would depend on the efficiency of the extraction procedure. Many variations were attempted, the most important of which have been described in the text, with the ultimate goal of extracting as much β-glucan as possible without destroying its antigenic properties and if possible maintaining the 96-well array format for throughput. Endogenous components of the grain, such as enzymes, other proteins, and possibly secondary phenolics, can interfere with the ELISA by reducing the signal at low dilutions of sample, but their effects were eliminated by the high dilution required by the assay. Addition of enzymes to the extraction resulted in a reduced response even if the enzyme was unrelated (e.g., lysozyme) and even at low concentrations. This suggests that protein is binding to the plate in competition with the β-glucan or otherwise interfering with the assay, resulting in a reduced ELISA signal. In the case of lichenase there was a reduced signal even with short incubation times, presumably because of the destruction of epitopes, a process that may continue during antigen incubation on the ELISA plate. When samples from oats or barley were extracted, it was found that extraction conditions which are damaging to proteins (i.e., in NaOH or HNO_3) gave more consistent ELISA values than milder extractions (i.e., phosphate buffer). Using NaOH as an extracting agent has the further advantage that it is more efficient at extracting β-glucans of lower solubility from barley (*14*). No advantage was noted when sodium borohydride was used to prevent alkaline peeling of β-glucan. We also found that using 96-well deep-well plates as described gave more consistent results than using glass test tubes, probably because the more convenient format of the plate permits more synchronized handling of samples.

Precipitating β-glucans in 80% ethanol after the NaOH extraction and then redissolving in PBS removes protein and other contaminants such as secondary phenolics that are soluble in ethanol. This gave a good response, but did not consistently correlate well to enzymatic β-glucan measurements, presumably because of the difficulty of recovering and redissolving all of the precipitated β-glucan. Despite the loss of some unextractable β-glucan, we have found that the NaOH method described gave the most consistent results. It should be emphasized that even small deviations from the described procedure could give rise to poor ELISA results, but these were easily recognized from the standards run on each plate. Since working on the initial design of the ELISA procedure we have found that the use of a liquid-handling robot reduces errors and eases the burden of multiple pipetting steps, especially when dealing with large sample numbers.

Comparison of the ELISA procedure directly to the enzymatic procedure is difficult because both rely on different extraction procedures. The ELISA uses NaOH to extract β-glucan, whereas the enzymatic procedure uses a mild phosphate buffer, heat, and lichenase. However, because lichenase destroys the antigenicity of β-glucan for the monoclonal antibodies used, it is not possible to measure β-glucan accurately by ELISA after this extraction and an accurate comparison of the two methods is therefore complicated. It is likely that each procedure liberates different amounts of β-glucans, but the high correlation coefficients obtained indicate that this is not a serious problem. It is important for any β-glucan assay to be based on an extraction protocol that solubilizes as much of the β-glucan as possible, especially in barley, where less soluble forms of β-glucan are present and where variability between cultivars is higher. Investigation of β-glucan left in the flour pellets after extraction confirmed this (**Table 4**), showing no β-glucan in oat flour pellets and an average of 3.8% β-glucan in barley flour pellets when 1 N NaOH was used to extract as described under Materials and Methods. Nitric acid was not as effective, and the procedure, which involves boiling the acid, was more hazardous to execute.

We have designed an ELISA based on a specific monoclonal antibody to detect β-glucan in oat and barley flour and in solution. In principle, the assay should work for any food-related products, for example, bran, which is a milling fraction enriched with β-glucan, and thus should find use in food quality laboratories as well as cereal-breeding programs. The ELISA is being evaluated by Canadian oat and barley breeders who are mandated to introduce varieties with both high and low levels of β-glucan. The high sensitivity of the assay, low cost, and nonreliance on β-glucan hydrolases make the ELISA especially attractive for measuring levels of dissolved β-glucan in various liquid products, such as beer. The assay is quantitative, and because of the compatibility of ELISA with scale-up and liquid-handling robotic equipment, it has the potential to become a high-throughput assay, which is currently lacking for β-glucan determination.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TCS, tissue culture supernatant.

ACKNOWLEDGMENT

We thank Peter Wood for the kind gift of polysaccharides.

LITERATURE CITED

(1) Wood, P. J.; Weisz, J.; Blackwell, B. A. Molecular characterization of cereal β-D-glucans. Structural analysis of oat β-D-glucan and rapid structural evaluation of β-D-glucans from different sources by high-performance liquid chromatography of oligosaccharides released by lichenase. *Cereal Chem.* **1991**, *68*, 31−39.

(2) Hecker, K. D.; Meier, M. L.; Newman, R. K.; Newman, C. W. Barley β-glucan is effective as a hypercholesterolemic agent in foods. *J. Sci. Food Agric*. **1988**, *77*, 179−183.

(3) Kalra, S.; Jood, S. Effect of dietary barley β-glucan on cholesterol and lipoprotein fractions in rats. *J. Cereal Sci*. **2000**, *31*, 141−45.

(4) Bell, S.; Goldman, V. M.; Bistrian, B. R.; Arnold, A. H.; Ostroff, G.; Forse, R. A. Effect of β-glucan from oats and yeast on serum lipids. *Crit. Rev. Food Sci. Nutr*. **1999**, *39*, 189−202.

(5) Cavallero, A.; Empilli, S.; Brighenti, F.; Stanca, A. M. High (1→3,1→4)β-glucan barley fractions in bread making and their effects on human glycemic response. *J. Cereal Chem.* **2002**, *36*, 59−66.

(6) McNab, J. M.; Smithard, R. R. Barley β-glucan: An antinutritional factor in poultry feeding. *Nutr. Res. Rev*. **1992**, *5*, 45−60.

(7) Vis, R. B.; Lorenz, K. β-glucans: Importance in brewing and methods of analysis. *Lebensm. Wiss. Technol.* **1997**, *30*, 331−336.

(8) Grimm, A.; Krueger, E.; Burchard, W. Solution properties of β-D-(1→3,1→4)-glucan isolated from beer. *Carbohydr. Polym.* **1995**, *27*, 205−214.

(9) Wood, P. J.; Weisz, J. Use of calcoflour in analysis of oat β-D-glucan. *Cereal Chem*. **1984**, *61*, 73−75.

(10) McCleary, B. V.; Codd, R. Measurement of (1−3),(1−4)-β-D-glucan in barley and oats: A streamlined enzymic procedure. *J. Sci. Food Agric*. **1991**, *55*, 303−312.

(11) Meikle, P. J.; Hoogenraad, N. J.; Bonig, I.; Clarke, A. E.; Stone, B. A. A (1→3,1→4)-β-glucan-specific monoclonal antibody and its use in the quantitation and immunocytochemical location of (1→3,1→4)-β-glucans. *Plant J.* **1994**, *5*, 1−9.

(12) Bhatty, R. S. Extraction and enrichment of (1−3),(1−4)-β-D-glucan from barley and oat brans. *Cereal Chem*. **1993**, *70*, 73−77.

(13) Burkus, Z.; Timelli, F. Effect of extraction conditions on yield, composition and viscosity stability of barley β-glucan gum. *Cereal Chem*. **1998**, *75*, 805−809.

(14) Izydorczyk, M. S.; Storsley, J.; Labossiere, D.; MacGregor, A. W.; Rossnagel, B. Variation in total and soluble β-glucan content in hull-less barley: Effects of thermal, physical and enzymatic treatments. *J. Agric. Food Chem.* **2000**, *48*, 982−989.

(15) American Association of Cereal Chemists. Method 32-23. β-Glucan content of barley and oats—Rapid enzymatic procedure. *Official Methods of the American Association of Cereal Chemists*; AACC: Minneapolis, MN, 1995.

(16) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Publishing: Cold Spring Harbor, NY, 1988.

(17) Manzanares, P.; Sendra, J. M. Determination of total (1−3), (1−4)-β-D-glucan in barley and malt flour samples. *J. Cereal Sci.* **1996**, *23*, 293−296.

(18) Andersson, R.; Aman, P. Cereal arabinoxylans: occurrence, structure and properties. In *Advanced Dietary Fibre Technology*; McCleary, B., Prosky, L., Eds.; Blackwell Scientific: Oxford, U.K., 2000; pp 301−314.

Received for review March 10, 2003. Revised manuscript received July 17, 2003. Accepted July 18, 2003. This work was funded by a grant to C.R. and N.A. from the Western Grains Research Foundation and a Matching Investment Initiative grant from Agriculture and Agri-food Canada. Contribution 1855 of the Cereal Research Centre.

JF030173I