

## Early Generation $\beta$ -Glucan Selection in Oat Using a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay

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Breeding oat cultivars high in (1 $\rightarrow$ 3)(1 $\rightarrow$ 4) $\beta$ -D-glucans (referred to as  $\beta$ -glucans here) is becoming increasingly important because of their proven ability to lower serum cholesterol (Braaten et al 1994; Kalra and Jood 2000). In spite of the importance of this trait, its inheritance is poorly understood (Buckeridge et al 2004) and cannot be predicted using molecular markers. Furthermore, most *Avena sativa* breeding germplasm has a narrow range of  $\beta$ -glucan—typically between 4.5 and 7.0% (w/w, dry), although a larger range of values is found in wild relatives such as *A. atlantica* (11.3%, w/w); *A. longiglumis* (2.3%, w/w) (Welch et al 2000). The environment also influences the final  $\beta$ -glucan levels in oat (Givens et al 2000; Cervantes-Martines et al 2002). This leaves breeders with the difficult task of breeding for a poorly understood trait lacking variation in elite germplasm, and with an important environmental component (Petersen et al 1995). Despite this, breeding for elevated  $\beta$ -glucan is an important priority and requires a high-throughput assay for rapid and economical measurement.

Traditionally breeders select for agronomic suitability early on, waiting several generations before making quality-based selections, even when quality is a major objective. This reduces the number of lines in advanced generations and ensures a reasonably large seed sample for quality testing but it means that poor-quality lines are advanced unnecessarily. It would be preferable to assay quality characteristics earlier to improve overall efficiency. The intermediate heritability of  $\beta$ -glucan in oats and the predominantly additive inheritance (Holthaus et al 1996; Kibite and Edney 1998) suggest the possibility of substantial improvement through early generation selections (Cervantes-Martines et al 2001).

The currently recommended Approved Method 32-23 (AACC International 2000) for testing  $\beta$ -glucans in flour employs the enzymatic degradation of  $\beta$ -glucans, followed by the spectrophotometric measurement of liberated glucose, and subtraction of non- $\beta$ -glucan-derived glucose with a mathematical formula. Although accurate and quantitative, this test is expensive and relatively slow and is thus unsuited for early generation screening for thousands of plants with low quantities of seed available. Other assays include viscosity measurement, near-infrared spectroscopy and calcofluor white fluorescence (Wood and Weisz 1984; Lim et al 1992; Doehlert et al 1997), each with their own advantages and disadvantages. With an enzyme-linked immunosorbent assay (ELISA), monoclonal antibodies are used to detect  $\beta$ -glucans directly and with great specificity. The ELISA brings significant advantages in cost, ease of use, and throughput, although the bottleneck during sample preparation remains.

We have recently developed a monoclonal antibody-based ELISA to measure  $\beta$ -glucans in ground oat and barley samples (Rampitsch et al 2003). A modified version of this assay is now being used to screen early generation ( $F_4$ ) oats for  $\beta$ -glucan levels. The ELISA is inexpensive, suitable for medium to high throughput, and reliably identifies plants with extreme  $\beta$ -glucan values.

Results from the assay of 105 oat lines derived from “low  $\times$  high”  $\beta$ -glucan crosses, 33 other oat samples with extreme  $\beta$ -glucan contents, and 6 control samples (144 samples in total) by ELISA are presented and compared with results obtained using Approved Method 32-23. Because ranking oats for  $\beta$ -glucan levels is more important at this early stage of breeding than knowing their absolute  $\beta$ -glucan content, the ELISA was primarily used to identify the 20th and 80th percentiles of the experimental population. Final  $\beta$ -glucan values can be measured accurately later, once interest in a particular breeding line warrants it. The ELISA can also be adapted to other sample types and is particularly well suited to measuring dissolved  $\beta$ -glucans.

### MATERIALS AND METHODS

#### Plant Material

Oat (*Avena sativa* L.) lines were derived from AC Assiniboia  $\times$  Ariane and AC Medallion  $\times$  Marion QC. AC Assiniboia and AC Medallion are high-yielding tan-hulled and white-hulled, respectively, cultivars adapted to western Canada (Brown et al 2001; Duguid et al 2001); Ariane (PI361884) and Marion QC (PI536549) have high  $\beta$ -glucan content and are from the USDA-ARS National Small Grains Collection. The crosses were made in a growth chamber and the resulting  $F_1$  plants were grown in pots in a greenhouse.  $F_2$  plants were grown in a field rust nursery at Glenlea, MB, Canada. Seed from selected  $F_2$  plants was planted in  $F_3$  progeny hills in a New Zealand winter nursery. Seeds from selected  $F_3$  plants were planted in  $F_4$  progeny hills at Glenlea, and seed from these hills were harvested and used in this study. The sample set was supplemented with 33 additional oat samples with high (>7.5%, w/w) and low (<4.0%, w/w)  $\beta$ -glucan. Wheat flour was used as a negative control. Flour  $\beta$ -glucan content was 0.34% (w/w), measured by a commercial assay (Mixed Linkage Beta-Glucan Assay Procedure, Megazyme Ireland). The purpose of this control was not to produce a zero value but rather to confirm a low background for each assay.

#### $\beta$ -Glucan Extraction

A modified extraction procedure (Rampitsch et al 2003) was used. Hulled whole oats were mechanically dehulled to produce groats and milled using a Retch laboratory mill to pass through a 0.5-mm screen. The resulting flour was autoclaved at 15 psi for 15 mins, dried in an oven at 65°C for 4 hr, and stored desiccated.

For ELISA, 25 mg of flour was weighed and transferred to a well in a 10-mL 24-well polypropylene plate (Whatman, Maidstone UK). To each well, 5 mL of 4M guanidinium isothiocyanate (GITC) and two 2-mm borosilicate glass balls were added. Plates were sealed with parafilm and a cap-mat and shaken vigorously

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for 5 min using a commercial paint-shaker. Each sample was then placed at 60°C for 20 min with gentle vortexing. Plates were then chilled to 4°C and centrifuged at 2000 × g. Supernatants were diluted 50,000-fold in chilled phosphate buffered saline (PBS) containing 0.3% (w/v) BSA (Fraction V, Sigma) 0.02% (v/v) Tween 20, sequentially, using two 24-well plates. Finally, 100 µL of each diluted sample was pipetted in triplicate into an ELISA plate (Maxisorp, Nunc, Denmark) that had been previously prepared and left at room temperature for 30 min.

### ELISA

The ELISA plates were prepared by pipetting 100 µL (300 ng) of monoclonal antibody 9E11 (Rampitsch et al 2003) in PBS into each well. Plates were kept at room temperature for 30 min, washed three times with PBS using an electronic plate washer (Biotrak II, Amersham Biosciences, UK) and then blocked with 3% (w/v) BSA, 0.2% (v/v) Tween 20 in PBS, 200 µL per well and incubated for 30 min. Plates were washed as before and then 100 µL of diluted sample was added per well and incubated for 30 min. After washing again, 100 µL of horseradish peroxidase-linked 9E11 prepared as outlined below was added, diluted with PBS containing 0.3% (w/v) BSA, 0.02% (v/v) Tween 20, and held for 30 min. Finally, TMB substrate (3,5,3',5'-tetramethylbenzidine, Pierce, Rockwell, MD) was added. The reaction was terminated after 5 min using 100 µL of 2N HCl, and A<sub>450</sub> was measured. Purified β-glucan for the standard curve was purchased commercially (Megazyme, Ireland).

### 9E11-HRP Conjugate

This was prepared in batches from affinity-purified 9E11 by covalently cross-linking the IgG<sub>1</sub> molecule to horseradish peroxidase. A commercial kit (EZ-link Plus, Pierce) was used according to manufacturer's instructions without modification. Because yields of conjugate differed, optimal dilutions were determined empirically for each batch.

### Other β-Glucan Measurements and Analyses

The enzymatic measurement of β-glucans in samples was done using a commercially available kit (Megazyme, Ireland). This kit conforms to AACC Approved Method 32-23 for measuring β-glucan in cereals. The kit was used as recommended by the manufacturer. Results were compiled and analyzed using Microsoft Excel version (v. 10) and Sigmaplot (v. 8).

## RESULTS AND DISCUSSION

The β-glucan content of all 144 samples was first determined using the enzymatic procedure. The range was narrow (3.0–13.9%, w/w) with 115 out of 144 (≈80%) falling within 4.0–7.0% (w/w). The range for the F<sub>4</sub> oats alone was even narrower (4.52–7.68%, w/w) with only one sample >7.0% (w/w).

The response of this ELISA to purified β-glucan was similar to that reported earlier, (Rampitsch et al 2003). It was linear over a short range (0–30 ng of β-glucan/mL) with a high linear correlation coefficient, typically R<sup>2</sup> > 0.95. Similar results have been reported by Meikle et al (1994) and Milton et al (2001). It was shown previously that 9E11, does not cross-react with cereal polysaccharides (Rampitsch et al 2003). Figure 1 shows a typical standard curve included on each plate to account for plate-to-plate variations. If this had R<sup>2</sup> < 0.9, the plate was rejected and repeated. The standard curve was used to calculate the levels of β-glucan, averaged from three wells.

Results of the enzymatic assay were plotted against those of the ELISA procedure (Fig. 2). This produced a linear relationship, R<sup>2</sup> = 0.601, with clustering near the center of the curve. During the execution of both methods, it became apparent that ranking of individual samples within this cluster was less consistent for the ELISA than for the enzymatic procedure, suggesting that the latter

gives more consistent results, and that deviant values in Fig. 2 were due mainly to the ELISA procedure.

When measuring oat β-glucan levels by flow-injection analysis, Lim et al (1992) attributed variations of up to 1.6% (w/w) to environmental factors alone with an average variation of ≈0.5% (w/w) in oats grown at the same location over two consecutive years. Thus, some assay variability is not a serious limitation in a plant breeding application. More importantly, extreme high (>7.5%, w/w) and low (<3.5%, w/w) samples were all identified correctly in both biological replicates and in all replicates by ELISA. Thus, for initial screening and for identifying high and low β-glucan lines, the ELISA is suitable for an oat breeding program.

The clustering of values in Fig. 2 is typical of polygenic traits that show a normal distribution in cross progenies, in contrast with mono- or oligogenic traits that usually show a binomial distribution. This contributes to inconsistent ranking. In practice however, the absolute β-glucan levels of F<sub>4</sub> breeding lines are not important if they consistently fall into percentage bins and if outliers with β-glucan levels significantly above the mean (e.g., 80th, 90th percentile, etc.) or below the mean (e.g., 20th, 10th percentile, etc.) are identified. The savings in time and money of the ELISA more than compensates for the lack of absolute values, especially as the environmental can account for a ≥1% (w/w) variation in β-glucan levels within the same cultivar (Lim et al 1992). The ELISA produced consistent rankings into bins covering a range of ≈1.0 % (w/w).

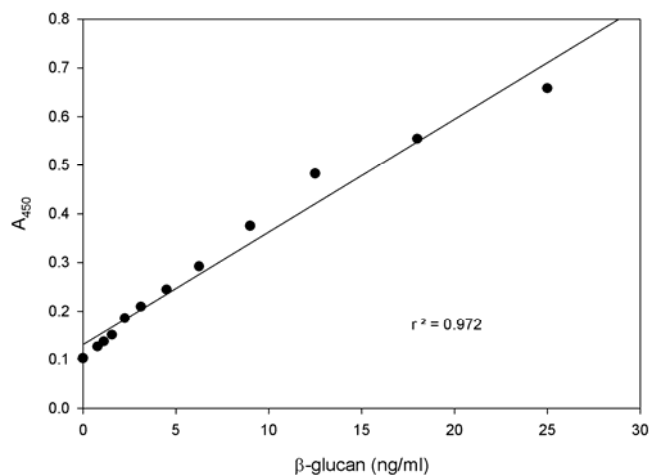


Fig. 1. Standard curve of ELISA data quantifying commercially purified oat β-glucan amounts.

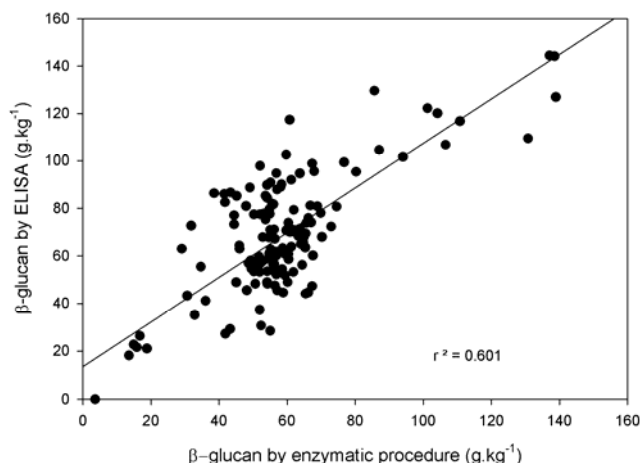


Fig. 2. Correlation between assays used to quantify β-glucan values prepared from 144 ground oat samples.

Two major modifications made to ELISA since it was reported by Rampitsch et al (2003) have enhanced reproducibility. First, the double-antibody sandwich format has replaced the plate-trapped version. This generally increases specificity and sensitivity because of the inclusion of an affinity step. Two striking features of the ELISA are its sensitivity and its short dynamic range; it is possible to detect dissolved  $\beta$ -glucans in the low ng/mL range, but standard curves start to plateau at >25–30 ng/mL of  $\beta$ -glucan. These limits hinder reproducibility that must be assured by careful dilution of samples into this range. On the other hand, sensitive detection of  $\beta$ -glucans in liquid samples is possible.

The second modification concerns the extraction procedure itself. The standard curve (Fig. 1) shows that a linear response to  $\beta$ -glucan that is 99% pure. This level of purity was not achieved for routine screening because the purification scheme used was designed to maximize yield and reproducibility, while keeping throughput and cost in mind. An autoclaving step has eliminated endogenous enzyme activity to prevent  $\beta$ -glucan degradation that renders the ELISA unreliable. Furthermore, it is likely that autoclaving also improves the extractability of  $\beta$ -glucans (Doehlert et al 1997). Four solvents were compared to determine their effectiveness in extracting  $\beta$ -glucan: 0.1N NaOH, 4M guanidinium hydrochloride, 4M GITC, and water. All of these gave similar results when <10 samples were compared; however, NaOH gave an elevated background. The 4M GITC consistently gave the best results and it was used for all extractions reported here.

The use of 10 mL/24-well plates permits the simultaneous handling of samples and the use of electronic dispensing equipment. We have also found that milling samples to pass a 0.5-mm screen (or finer) reduces variation between the same sample, presumably because of increased homogeneity of more finely ground material, for bran or wholemeal samples where large flakes of material rich in  $\beta$ -glucan are not evenly distributed (*unpublished observations*).

The ELISA reported here is very well suited to high throughput early generation selection in oat breeding, where throughput, approximate ranking, and identification of extremes are more important than absolute values. The assay is also applicable to barley (*Hordeum vulgare* L.), the only other cereal with significant levels of  $\beta$ -glucan. After early generation selection, more accurate testing can be applied to a smaller sample of elite germplasm.

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