

Development of a Field Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of α -Amylase in Preharvest-Sprouted Wheat

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ABSTRACT

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A sandwich enzyme-linked immunosorbent assay (ELISA) has been developed for detection of α -amylase in preharvest sprouted wheat and adapted to rapid field-use formats requiring 15–20 min to perform. Polyclonal and monoclonal antibodies were prepared to detect a mixture of high and low pI isozymes of α -amylase and high pI isozymes only. All antibodies detected α -amylase on immunoblots of either a crude wheat extract or of purified enzyme, but only the polyclonal antibodies functioned in a sandwich ELISA. Depending on the antibody combination, the tube ELISA detected either the high and low pI isozymes of α -amylase or the high pI isozymes only with a detection limit of ≈ 0.5 – 1.0 ng/mL of amylase. Wheats with falling numbers (FN) of <350 sec could be discriminated

from sound wheats, with decreasing FN producing increasing assay color. Using 130 wheat grain samples, ELISA absorbances for detection of both high and low pI isozymes and of high pI isozymes only were highly positively correlated with amylase enzyme activity and negatively correlated with FN. The correlations were similar for detection of both isozyme families and for detection of high pI isozymes only. Analyses of three sets of wheat samples from different environments demonstrated that the relationship between ELISA absorbance and FN had little dependence on wheat cultivar. The precision of sample analysis using the field ELISA was similar to the precision of FN test apparatus.

Preharvest sprouting or weather damage in wheat can cause processing problems for the affected grain due to the action of hydrolytic enzymes (amylases, proteases, and lipases) in the grain endosperm (Orth and Moss 1987, Derera 1989). These enzymes (triggered by rain at or just before harvest) accelerate the breakdown of starch granules and protein in the endosperm of germinating grain (Meredith and Pomeranz 1985). Wheat that is weather damaged has a significantly lower market value as it is rendered less suitable for human consumption. Products that are made from sprouted wheat (for example breads) have gray color and poorer crumb texture, loaf structure, and volume; noodles have poor color and cooking qualities. These deficiencies are due to the action of hydrolytic enzymes (Meredith and Pomeranz 1985, Derera 1989).

At grain receipt, or during harvesting, mixing a small quantity of highly sprouted grain with larger amounts of sound grain can downgrade an entire batch of grain. The necessity for accurately discriminating sprouted from sound wheat highlights the need for a quick, easy, and reliable test for preharvest sprouting. The most common method for detecting preharvest sprouting at elevators measures α -amylase activity using the Falling Number (FN) method, in which the consequences of enzymic hydrolysis of starch caused by amylase production is assessed as the time required for a plunger to fall through a heated slurry of whole meal and water (Hagberg 1960, Perten 1964). However, the capital cost of the FN instruments means that it is only feasible to install them at a limited number of major grain elevators. The method is also relatively low in throughput, and results can be affected by variation in starch pasting characteristics (D'Appolonia et al 1982, Ringlund 1983). The cheaper option of visual assessment is both unreliable and unobjective (Jensen et al 1984, Moot and Every 1990). Other methods such as the Rapid ViscoAnalyser (Ross et al 1987a,b) and near-infrared (NIR) analysis (Czuchajowska and Pomeranz 1992), although faster, involve high capital cost. NIR predictions of FN are also of relatively low precision and can only discriminate relatively large differences in FN (Osborne 1984, Williams 1989, Czuchajowska and Pomeranz 1992, Shashikumar et al 1993). Direct enzyme activity assays for α -amylase (Barnes and Blakeney 1974, McCleary and Sheehan 1987) are not suitable for elevator

or on-farm use because they require technical expertise and equipment such as water baths and filtration devices.

Enzyme-linked immunosorbent assays (ELISA) provide an alternative method for detection of preharvest sprouting through the use of antibodies that are specific for α -amylase isozymes. Earlier research showed that specific antisera can be developed for separate recognition of the two major groups of α -amylase isozymes (Daussant and Renaud 1976, Lazarus et al 1985). The ELISA technique has an added potential advantage over enzyme activity assays in that, by using appropriate amylase antibodies, it should be possible to specifically measure different amylase isozyme families and to assess whether quantitation of particular isozymes is better correlated with FN than total amylase activity. ELISA kits are generally quite robust and suitable for shipping and for use in harsh environments and can be used by individuals with little training. Such a test could not only be used by grain handlers or traders at elevators, but also by individual wheat growers. This would allow them to detect sprouting on-farm before harvesting in order to prevent contamination of sound wheat by sprouted grain. The present study describes the development and evaluation of a simple tube sandwich ELISA using polyclonal antibodies to (PAb) wheat α -amylase and its use in detecting preharvest sprouting in wheat.

MATERIALS AND METHODS

Purification of α -Amylases

Grain from two wheat cultivars with quite different pedigrees (Janz and Osprey) was germinated for four days at 20°C. Germinated grain was frozen by immersion in liquid nitrogen and freeze-dried. Roots and shoots were removed and the grain was ground into whole meal flour using an electric cereal grinder (Jupiter, Schorndorf, Germany). α -Amylase was extracted by stirring whole meal flour in 20 mM sodium acetate buffer (extraction buffer), pH 5.5, containing 1 mM CaCl₂ at 5°C for 1 hr. All subsequent steps were performed at 5°C. The extract was centrifuged (48,000 \times g, 30 min) and the resulting supernatant was subjected to precipitation with 20–60% ammonium sulfate saturation. The material that precipitated between these salt concentrations was redissolved and dialyzed against the extraction buffer. The dialyzed extract was filtered using a 0.8- μ m filter and fractionated using affinity chromatography.

A mixture of both high and low pI isozymes of α -amylase was purified by β -cyclodextrin affinity chromatography according to the methods of Lecommandeur et al (1988) with modifications.

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The affinity column (12 × 3 cm) consisted of epoxy-activated Sepharose 6B coupled to cycloheptaamylose (β -cyclodextrin). Sepharose 6B was activated using 1,4-butanedioldiglycidyl ether (Sigma, St. Louis, MO) according to the methods of Sundberg and Porath (1974) and coupled to cycloheptaamylose using the method of Vretblad (1974). α -Amylase was eluted with 20 mM sodium acetate buffer (pH 5.5) containing 1 mM CaCl_2 and 0.5M NaCl and 8 mg/mL of cycloheptaamylose, and dialyzed against 10 mM sodium acetate buffer (pH 5.5) containing 1 mM CaCl_2 , and reduced in volume by ultrafiltration. Total protein was determined

using the Coomassie Blue dye-binding assay (Bradford et al 1976) and the enzyme was stored at 4°C.

Separation of high and low pI isozymes of α -amylase was achieved using the cycloheptaamylose affinity column, followed by an immunoaffinity column prepared according to the methods of Katoh and Terashima (1992) with modifications. The immunoaffinity column (5 × 1 cm) was prepared by coupling a monoclonal antibody (MAb) (specific for the high pI isozyme of amylase in barley and wheat), to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The MAb (1H5.1F12, Queensland Department of Primary Industries, Brisbane, Australia) was coupled to CNBr-Sepharose 4B at a concentration of 7.3 mg/mL. A crude extract of amylase purified using the cycloheptaamylose method was dialyzed against the immunoaffinity column equilibration buffer (50 mM Tris, pH 7.6, containing 5 mM CaCl_2 and 0.5M NaCl). High pI α -amylase was eluted with 2.5M KSCN containing 5 mM CaCl_2 and 0.5M NaCl. The unbound fraction (thought to contain low pI amylase) was collected during loading onto the column. This fraction, along with the eluted enzyme, was dialyzed and concentrated as described above.

Producing MAb and PAb to α -Amylase

MAb to α -amylase were produced by immunizing BALB/c mice with a mixture of both high and low pI isozymes of the enzyme (purified separately from both Janz and Osprey cultivars). Mice were given an initial intraperitoneal injection of 200 μg of total protein mixed 1:1 in Freund's Complete Adjuvant (FCA) (Sigma). This was followed by two intraperitoneal injections (in Freund's Incomplete Adjuvant [FICA]) of 100 μg of protein at two-week intervals. At four weeks after the third injection, mice were given a final intraperitoneal booster of 200 μg of amylase per mouse three days before fusion. The quantity of enzyme used for immunization was optimized in initial experiments in which three groups of mice (two mice per group) were immunized initially with either 200, 40, or 10 μg of total protein, and subsequently with either 100, 20, or 5 μg of total protein. Blood was collected from all mice by tail bleed at 10 days after the third injection and tested for antibodies against α -amylase by indirect ELISA. Hybridomas were produced according to the general methods of Skerritt and Underwood (1986) and supernatants secreted by the resulting hybridoma cells were tested for specificity to α -amylase using indirect ELISA. Positive cell lines were subcloned and antibodies were isotyped using a mouse-typer sub-isotyping kit (BioRad, Hercules, CA) and purified using either gamma bind (Pharmacia) Protein G affinity chromatography (IgG isotypes) (Akerstrom et al 1985) or ammonium sulfate precipitation (IgM isotypes) (Knutson et al 1991).

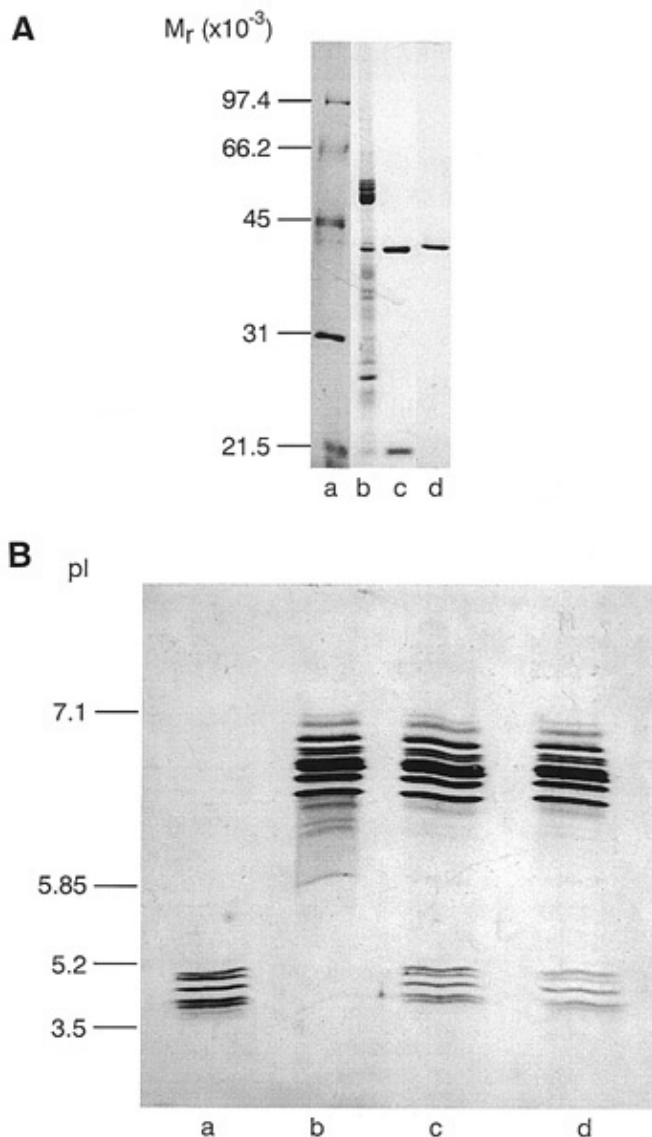


Fig. 1. A. SDS-PAGE of α -amylase purified by affinity chromatography stained with Coomassie Brilliant Blue G-250. Lanes a-d: molecular weight markers (molecular weights indicated); extract of germinated wheat after ammonium sulfate purification steps but before cyclodextrin or immunoaffinity purification steps; polypeptides purified by affinity chromatography on β -cyclodextrin in the absence of NaCl; polypeptides purified by affinity chromatography on β -cyclodextrin in the presence of 0.5M NaCl. **B.** Isoelectric focusing (IEF) of α -amylase after purification by β -cyclodextrin affinity chromatography. Isozymes were separated using a 0.5-mm, 7.5% polyacrylamide gel with a pH gradient of 3–10 and detected by silver staining. Approximate pI was calculated from the position of reference proteins of known pI (indicated). Lanes a-d: low pI group of isozymes (Janz unbound fraction from subsequent immunoaffinity chromatography), high pI group of isozymes (Janz fraction from subsequent immunoaffinity chromatography); high and low pI isozymes (Janz); and high and low pI isozymes (Osprey).

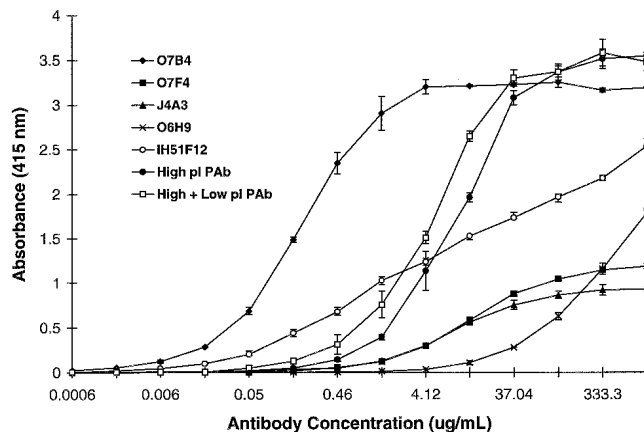


Fig. 2. Titration of monoclonal and polyclonal antibodies with α -amylase using indirect enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with 1 μg of purified amylase per microwell (high and low pI isozymes from Janz). Mean \pm standard deviation of three replicates.

Polyclonal antisera were produced to the high pI amylase isozymes and to a mixture of both the high and low pI isozymes of the enzyme. A total of eight rabbits (four groups with two rabbits per group) were utilized. Groups 1 and 2 were immunized with high pI amylase purified from Janz and Osprey cultivars, respectively, while groups 3 and 4 were immunized with a mixture of high and low pI amylase from Janz and Osprey. Rabbits were given an initial injection of 1 mg of protein (mixed 1:1 in FICA) split between three injection sites in the muscles of both hind legs and subcutaneously in the neck area. Rabbits were boosted 14 and 28 days after the initial injection (500 µg of protein in FICA) and blood was collected 10 days after the third booster. Sera were tested for antibodies to α-amylase using indirect ELISA. Rabbits were boosted thereafter at monthly intervals with 500 µg of protein in FICA. Antibodies were purified from rabbit sera using Gamma Bind IgG affinity chromatography. PAb and MAb were coupled to horseradish peroxidase (HRP) (Boehringer-Mannheim, Germany) using a method modified from Nakane and Kawoi (1974).

SDS-PAGE, Isoelectric Focusing, and Immunoblotting

SDS-PAGE and isoelectric focusing (IEF) were used to check the purity of α-amylase samples prepared by affinity chromatography. For SDS-PAGE, samples of enzyme were run on a 12% T, 2% C polyacrylamide gel at constant voltage for 1,500 Vhr and stained using colloidal Coomassie Blue G-250 (Andrews and Skerritt 1996). IEF was performed on a flat-bed apparatus system (Pharmacia) using 7.5% T, 3% C polyacrylamide gels (0.5 mm) with an ampholyte gradient of pH 3–10. Cathode and anode solutions were 1M sodium hydroxide and 1M phosphoric acid, respectively, and gels were run at 4°C under constant power (8–10 W) for 3 hr. IEF gels were silver-stained using the method of Zhao and Sharp (1996).

MAb and PAb were tested for specificity for α-amylase using semidry immunoblotting of SDS-PAGE gels and passive immunoblotting of IEF gels. SDS-PAGE gels were loaded with a crude extract of α-amylase prepared from whole meal flour. Electrophoresis and immunoblotting (for 4 hr at 250 mA/gel) onto nitrocellulose was done according to Andrews and Skerritt (1996). The IEF gels were loaded with purified α-amylase and focusing. Immediately after focusing, proteins from IEF gels were transferred to nitrocellulose membrane by passive diffusion (1 hr at 20°C) in 10 mM sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂. Membranes from both procedures were blocked with 3% (w/v) bovine serum albumin (BSA) in 50 mM sodium phosphate-buffered saline (PBS), pH 7.2, probed with MAb or PAb, and detected with alkaline phosphatase-labeled second antibodies from Promega (Madison, WI).

Microwell ELISA for Detection of α-Amylase

PAb and MAb (including the MAb to high pI amylase from barley malt) were initially titrated by indirect ELISA before being evaluated for the ability to capture and detect α-amylase in sandwich ELISA. In the first of these assays, 96-well ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated for 16 hr at 20°C with 100 µL of purified α-amylase antigen at 1 µg/well in 50 mM carbonate buffer, pH 9.6. Wells were then washed three times with PBS and 0.05% Tween 20 (PBST), and nonspecific binding sites were blocked with 1% BSA in PBS for 1 hr at room temperature. Microwell-bound antigen was incubated for 90 min with 100 µL of antibody solution diluted in 1% BSA in PBS and then washed three times with PBST. This was followed by a 30-min incubation with either 100 µL/well peroxidase-labeled rabbit anti-mouse or goat anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) diluted 1:2,000 and 1:400, respectively, in 1% BSA in PBS. After four washes, 150 µL of substrate-chromogen (2 mM 2,2'-azino-bis-3-ethylbenzthiazoline sulfonic acid [ABTS] in 0.1M sodium citrate buffer, pH 4.5, containing 0.003% H₂O₂) was added, and plates were incubated for 20 min at room temperature. The enzyme reaction was terminated by the addition of 50 µL of oxalic

acid (3%, w/v). Absorbance values were measured at 414 nm. For sandwich ELISA, plates were coated for 16 hr at 20°C with 100 µL of either purified MAb or PAb at 1 µg/well in 50 mM carbonate buffer, pH 9.6. All subsequent steps were performed at 20°C. The wells were washed three times with PBST and nonspecific binding sites were blocked with 1% BSA in PBS for 1 hr. Purified α-amylase was serially diluted in 1% BSA in PBST, added to the wells (100 µL/well), and incubated for 1 hr. After washing three times with PBST, 100 µL of HRP-labeled MAb or PAb diluted in 1% BSA in PBST was added to all wells and incubated for 30 min. The dilution of the labeled antibody had been previously determined by direct ELISA to provide an absorbance of ≈1.0–1.5. Plates were washed as before, and ABTS substrate was added to all wells. The reaction was stopped after 20 min and absorbance values were measured at 415 nm. Samples were analyzed in triplicate, and the absorbance of blank wells (no addition of α-amylase) was subtracted from the absorbance of each well.

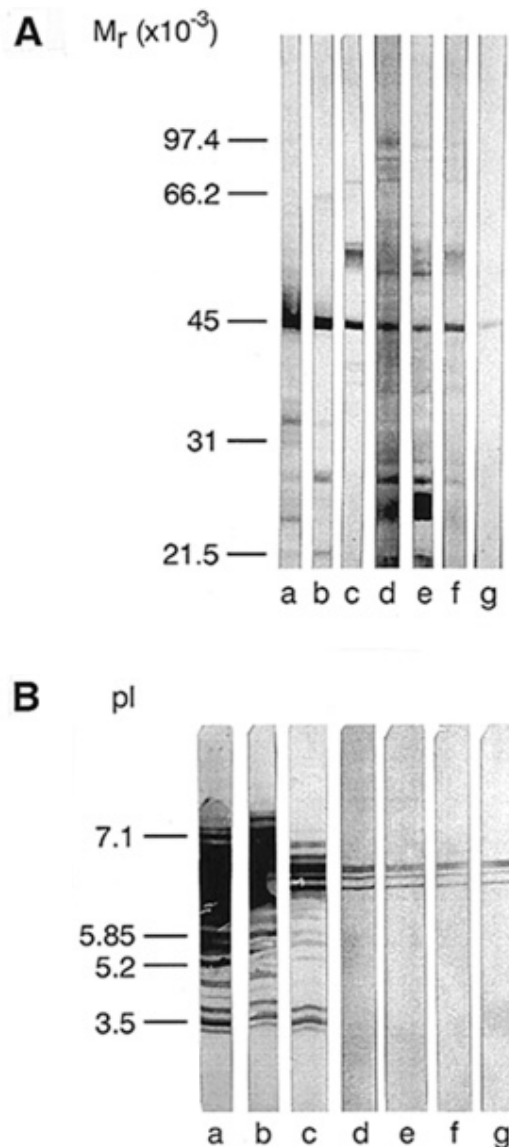


Fig. 3. Immunoblot analyses. **A.** α-Amylases in unprocessed crude wheat extract separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal (MAb) and polyclonal (PAb) antibodies. Lanes a–g: PAb to high and low pI amylase; PAb to high pI amylase; MAb O7B4; MAb O7F4; MAb J4A3; MAb O6H9; MAb 1H5.1F12. **B.** Purified α-amylase separated by isoelectric focusing (IEF), transferred to nitrocellulose membrane, and probed with antibodies. Lanes a–g: PAb to high and low pI amylase; PAb to high pI amylase; MAb O7B4; MAb O7F4; MAb J4A3; MAb O6H9; MAb 1H5.1F12.

Tube Assay for Detection of α -Amylase

Based on the initial performance of the antibodies in the plate ELISA, several tube ELISAs for detection of α -amylase were developed. One assay (the standard assay) was designed to detect the high pI isozyme of amylase and used a PAb specific for the high pI isozymes for both capture and detection. A second assay (the rapid assay) used the PAb to the high and low pI isozymes of α -amylase for both capture and detection. Sets of polystyrene test tubes (12 mm diameter \times 75 mm long) were coated for 16 hr at 20°C with purified capture antibody (5 μ g in 500 μ L of 50 mM sodium carbonate buffer, pH 9.6). Tubes were washed three times with PBST, and nonspecific binding was blocked for 60 min using 1% BSA in PBS. Large batches of tubes could also be prepared by blocking with a proprietary solution for 1 hr (after antibody coating) and then tipping out the contents and freeze-drying the antibodies onto the tube surface for 40 hr.

Initial experimentation was undertaken to establish the tube assay procedure and simplify it. The conventional ELISA format, involving sequential addition of antigen (in this case, wheat extract) and enzyme conjugate, with an intermediate washing step, was compared with an assay protocol involving simultaneous addition of wheat extract and enzyme (with no intermediate washing step). In the sequential assay, 20 g of whole grain was blended in a Waring blender for 2 min at 4°C in 20 mL of 50 mM sodium malate buffer containing 50 mM NaCl and 2 mM CaCl_2 (the extraction buffer used in the Ceralpha enzyme assays of McCleary and Sheenan [1987]), and 500 μ L of undiluted grain extract was added to antibody-coated tubes and incubated for 10 min. The tubes were then washed three times in PBST and 500 μ L/tube of antibody-HRP conjugate (diluted in 1% BSA in PBST) were added and incubated for 5 min. In the simultaneous assay, antibody HRP conjugates at a higher concentration in 1% BSA in PBST were added (50 μ L/tube), followed immediately by the addition of 500 μ L of undiluted grain extract and incubated for 10 min (5 min for rapid assay). Tubes were washed three times with PBST and 500 μ L of substrate (0.6 mg/mL of 3,3',5,5'-tetramethylbenzidine in 0.1M sodium acetate, pH 5, containing hydrogen peroxide) was added to each tube. The reaction was stopped after 5 min (3 min for rapid assay) by the addition of 250 μ L of 1.25M sulfuric acid. The absorbance was measured at 450 nm using a rapid photometric analyzer (RPA-1, Source Scientific, Garden City, CA) and the absorbance of blank tubes (addition of extraction buffer) was subtracted. Analyses were performed in duplicate.

Additional experimentation undertaken to simplify the tube assay procedure included the use of a Jupiter cereal mill (with no screen) to grind grain into whole meal which was then used in a com-

parative study of extractant solutions. In this comparison, 0.5 g of whole meal was extracted for 4 min with intermittent vortexing in 5 mL of 50 mM sodium malate buffer and compared with whole meal extracted in the same way in buffers containing either 50 mM NaCl, 85 mM NaCl, or 50 mM NaCl containing 2 mM CaCl_2 . Establishment of optimal extraction time (4 min with intermittent vortexing vs. 15 sec of hand shaking), the stability of α -amylase in the chosen extractant buffer, and the use of the same buffer for extraction of α -amylase as well as washing the tubes were also investigated.

Field Samples

Twenty grain samples collected from elevators in Western Australia during the 1995 harvest (cultivars Wilgoyne, Amery, and Eradu), as well as 30 additional grain samples from the 1995 harvest in New South Wales, Australia (Suneca, Hartog, Sunstate, and Janz) (FN 62–494 sec) were ground using the Jupiter mill and analyzed in duplicate for α -amylase using the plate sandwich ELISA (detection of high pI and high-low pI amylase isozymes). Another 130 grain samples from eight wheat cultivars (Hartog, Cunningham, Pelsart, Banks, Sunco, Sunstate, Perouse, and Janz) (FN 62–420 sec) were collected from elevators at Miles and Roma in southern Queensland, Australia, during the 1996 harvest. These samples were ground using the Jupiter mill and analyzed for α -amylase using the standard and rapid tube assays. In addition, 108 samples from six cultivars (Hartog, Halberd, Sunland, Tincurrin, Matong, and Vulcan) (FN 101–418 sec) grown at Narrabri, New South Wales, Australia, and subjected to controlled wetting were milled on an FN mill with a 0.8-mm screen and analyzed using the standard and rapid tube assays to further examine the influence of cultivar on the test results. These cultivars contained hard and soft wheat types of diverse protein content (8–15%) and end-use types. All samples were analyzed in duplicate as two separate runs, and raw absorbances were standardized to the assay absorbance produced by a standard of FN 187 included in each run. FN for whole meal (ground with a FN 3100 mill) were determined in duplicate (AACC 1995) except where indicated.

α -Amylase Enzyme Assay

α -Amylase activity was determined using the Ceralpha assay (Megazyme International, Bray, Ireland) utilizing *p*-nitrophenyl-heptanoside as substrate (McCleary and Sheehan 1987). The assay was performed according to manufacturer's instructions with modifications to increase the sensitivity of the assay: 1) 250 μ L of Ceralpha substrate solution was used; 2) 500 μ L of wheat extract was added; and 3) the reaction was terminated by adding 2 mL per

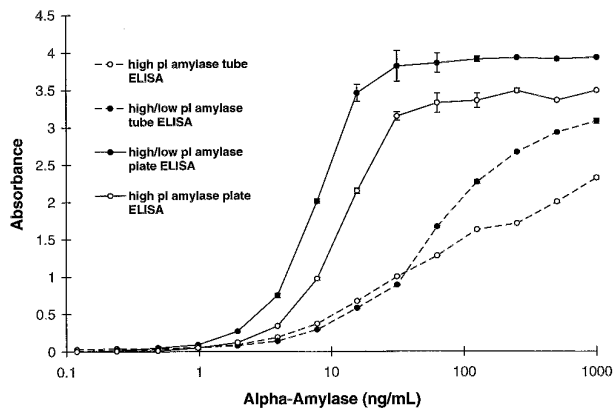


Fig. 4. Standard curves for α -amylase using plate and tube sandwich enzyme-linked immunosorbent assay (ELISA). Serial dilutions of purified high pI α -amylase from Janz (for antiserum to Janz high pI α -amylase) and high and low pI α -amylase from Osprey (for antiserum to Osprey high and low pI α -amylase). Mean \pm standard deviation of three replicates.

TABLE I
Sequential vs. Simultaneous Format in Development
of a Tube Enzyme-Linked Immunosorbent Assay (ELISA)
for Detection of High and Low pI Isozymes^a

Falling Number (sec)	Mean ELISA Absorbance \pm Standard Deviation
Sequential format (incubation for 10, 5, and 5 min) ^b	
187	1.42 \pm 0.04
240	1.13 \pm 0.23
284	0.71 \pm 0.20
351	0.60 \pm 0.10
374	0.43 \pm 0.08
Simultaneous format (incubation for 10 and 5 min) ^c	
187	0.79 \pm 0.11
284	0.30 \pm 0.01
374	0.14 \pm 0.02

^a Wheat extract from whole grain blended in 50 mM sodium malate buffer and tubes washed with phosphate-buffered saline and 0.05% Tween 20 (PBST). Means of two replicates for each test.

^b Washing step between addition of wheat extract (incubation for 10 min) and horseradish peroxidase conjugate (incubation for 5 min).

^c Simultaneous addition of wheat extract and horseradish peroxidase conjugate (incubation for 10 min, no washing step).

tube of 2% (w/v) tris(hydroxymethyl)aminomethane base solution. Initial experiments with a set of 10 sprouted wheat samples indicated the results of the modified method were highly correlated with the standard method but the sensitivity was greater. Samples were analyzed in duplicate.

RESULTS AND DISCUSSION

Purification of α -Amylase

The purity of α -amylase prepared from a crude wheat extract by affinity chromatography was initially assessed using SDS-PAGE (Fig. 1A). A single band was detected with an approximate M_r 44,000, in agreement with previous reports (Hill and MacGregor 1988). IEF of purified α -amylase revealed the presence of approximately six to eight bands at pH 6–7 (high pI isozymes) (Fig. 1B, lanes b–d) and approximately four to six less strongly stained bands at pH 4.5–5.5 (low pI isozymes) (Fig. 1B, lane a). These correspond, respectively, to the products of the *alpha-amy 1* genes on the group 6 chromosomes and the *alpha-amy 2* genes on group 7 (Nishikawa and Nobuhara 1971, Gale 1983). The two groups of isozymes were successfully separated using β -cyclodextrin affinity chromatography followed by immunoaffinity chromatography (Fig. 1B, lanes a and b). Initial attempts to isolate the high pI isozyme by immunoaffinity chromatography (using the MAb to the barley high pI isozyme) without the initial β -cyclodextrin chromatography step produced a complex of high pI isozymes with α -amylase inhibitor (Fig. 1A, lane c). The contaminating polypeptide was identified as the inhibitor (Mundy et al 1984) on the basis of its apparent molecular weight on SDS-PAGE (approximate M_r 22,000) (Weselake et al 1985) and pI 7.1 on IEF (Maeda 1986). Earlier work by Lecommandeur et al (1988) showed that use of β -cyclodextrin affinity chromatography could isolate α -amylases free of the inhibitor if all buffers were made 0.5M NaCl to dissociate the inhibitor and the enzyme. Germinated grain (200 g of both Janz and Osprey cultivars) yielded \approx 24 mg of a mixture of high and low pI amylase and \approx 14 mg of high pI amylase.

Development and Specificity of MAb and PAb to α -Amylase

The fusion products from a total of four fusions were seeded across the wells of 96-well tissue culture plates (Nunc). Of the \approx 500 wells that exhibited growth, five independent cell lines that secreted high-titer antibodies were isolated. The isotypes of the MAb were determined using a commercial isotyping kit: O7F4, O6H9, J1H5, and J4A3 were immunoglobulin M, κ -light chain; and antibody O7B4 was immunoglobulin G₁, κ -light chain. Four of these MAb, as well as the MAb against barley malt high pI amylases (isotype G₁, κ -light chain) and two rabbit PAb (one raised to high pI amylase and the other raised to both isozymes), were assessed for their activity for α -amylase on indirect ELISA, and for specificity on immunoblots of a crude wheat extract separated by SDS-PAGE and on immunoblots following IEF of the purified enzyme. Titration against a purified mixture of high-low

pI isozymes (Fig. 2) indicated that, while all the antibodies showed activity to α -amylase, MAb O7B4 and the two PAb showed the highest activity.

On immunoblots prepared from a crude wheat extract, the polyclonal antiserum to high-low pI amylase showed activity with approximately six polypeptide bands in the M_r 22,000–50,000 range (Fig. 3A, lane a). These included the M_r 44,000 band detected on SDS-PAGE analysis of the purified enzyme (Fig. 1A, lane b). The high pI amylase polyclonal recognized approximately five polypeptide bands in the M_r 21,500–66,000 range, including the M_r 44,000 band (Fig. 3A, lane b). The MAb showed varying reactivity on immunoblots of a crude wheat extract; four out of the five wheat antibodies showed a moderate to strong reaction with the M_r 44,000 band in addition to similar reactivity with other polypeptides in the M_r 21,500–88,000 range (Fig. 3A, lanes c–f). The barley MAb also showed reactivity with the M_r 44,000 band (Fig. 3A, lane g).

The two groups of bands detected on silver staining of IEF gels, were recognized on IEF-immunoblots by both polyclonal antisera (Fig. 3B, lanes a and b) where the reaction of the antiserum raised to high pI amylases was less intense with the low pI isozymes than the corresponding reaction of the high-low pI amylase antibody. Even though the wheat MAb were produced by immunization of mice with a mixture of both high and low pI isozymes of amylase, only one of the five antibodies produced detected both groups of isozymes; the remaining four (as well as the MAb to barley malt amylase) bound the high pI group only (Fig. 3B, lanes d–g).

Development of Tube ELISA

All possible combinations of PAb and MAb were tested initially as capture and detection antibodies in the plate sandwich ELISA using a wide range of concentrations of purified amylase. Each PAb functioned well as either a capture or detection antibody when used in conjunction with either the same antibody or another PAb. The MAb to high pI α -amylases from barley malt also functioned as either a capture or detection antibody when used in conjunction with a PAb. However, the MAb that we prepared did not function as either capture or detection reagents; there was no difference between the absorbance of wells to which amylase had been added and the blank wells which contained no enzyme (data not shown). Although the MAb were specific for α -amylase on immunoblots (Fig. 3A and B) and in indirect ELISA (Fig. 2), they failed to detect amylase in a plate sandwich ELISA and, consequently, were not used in the tube ELISA. The failure of these antibodies to detect amylase in a sandwich ELISA format is probably due to the inability of the MAb, when acting as both capture and detection agents, to simultaneously bind spatially separate epitopes on the amylase proteins. Their failure to function when used in conjunction with the polyclonal antisera is more problematic. While the polyclonal antisera may also recognize the same epitope, other epitopes must also be recognized by these antibodies in order for the polyclonal antisera to simultaneously function as capture and

TABLE II
Comparison of Extraction Buffers in Development
of a Tube Enzyme-Linked Immunosorbent Assay (ELISA)
for Detection of High and Low pI Isozymes^a

Falling Number (sec)	Mean ELISA Absorbance \pm Standard Deviation			
	Sodium Malate Buffer	50 mM NaCl	50 mM NaCl + 20 mM CaCl ₂	85 mM NaCl
187	0.62 \pm 0.03	0.58 \pm 0.01	0.57 \pm 0.01	0.57 \pm 0.01
284	0.32 \pm 0.03	0.30 \pm 0.05	0.31 \pm 0.03	0.30 \pm 0.00
403	0.14 \pm 0.01	0.15 \pm 0.01	0.16 \pm 0.01	0.14 \pm 0.00

^a Simultaneous addition of wheat extract and horseradish peroxidase conjugate (incubation for 10 min, no washing step) and substrate (incubation for 5 min). Tubes washed with phosphate-buffered saline and 0.05% Tween 20 (PBST). Two replicates for each test.

TABLE III
Time and Method of Extraction in Development
of a Tube Enzyme-Linked Immunosorbent Assay (ELISA)
for Detection of High and Low pI Isozymes^a

Falling Number (sec)	Mean ELISA Absorbance \pm Standard Deviation	
	Wrist-Action Shaking for 15 sec	Intermittent Vortexing for 4 min
187	0.53 \pm 0.06	0.63 \pm 0.13
284	0.27 \pm 0.01	0.27 \pm 0.03

^a Simultaneous addition of wheat extract and horseradish peroxidase conjugate (incubation for 10 min, no washing step). Wholemeal flour extracted in 85 mM NaCl. Tubes washed with phosphate-buffered saline and 0.05% Tween 20 (PBST). Two replicates for each test.

detection antibodies. Although the specificity of the barley MAB on immunoblots was similar to the MAB we developed, it must recognize a different epitope from that of the MAB to wheat amylase.

When the PAB were used for both capture and detection in a plate sandwich ELISA, α -amylase was sensitively detected with a limit of detection (absorbance of 0.1 above background) of ≈ 1 ng/mL for either high pI amylase or high-low pI amylase (Fig. 4). These antibodies also functioned in a sandwich assay when coated to polystyrene tubes rather than microwells. The larger volume of the tubes makes them more suitable for field use since reagents can be added using droppers and larger reagent volumes can be used, making the assay more acceptable to nonlaboratory personnel. Two overall assay times were evaluated: a standard assay with a 10-min sample incubation and 5-min color development period; and a rapid assay with a 5-min sample incubation and a 3-min color development. The incubation periods in the standard assay would enable growers to analyze sets of samples, while the rapid assay may be sufficiently fast for elevator use. To compare the performance of reagents with different specificities, we arbitrarily decided to use reagents specific for high pI amylase for the standard assay and reagents detecting both the high and low pI amylase for the rapid assay. The tube assays were less sensitive than the plate assays with detection limits of ≈ 4 ng/mL of amylase (Fig. 4), probably due to the much shorter incubation periods used.

The results of initial experimentation to compare the tube assay in a sequential versus a simultaneous ELISA format are shown in Table I. Results indicate that mean ELISA absorbance (color development) showed a decrease with increasing FN for the tube assay in either format. Although mean ELISA absorbances were lower for the simultaneous format (due to an overall reduction in incubation time), the test still provided discrimination between sprouted and sound wheats. Subsequent use of the simultaneous format in the tube assay removed an intermediate washing step, thus simplifying the procedure. The initial procedure for extraction of amylase (i.e., blending whole grain in sodium malate buffer) could be simplified by vortex mixing whole meal flour (ground in the Jupiter mill) for 4 min in malate buffer. The malate extraction buffer could also be replaced by a simple salt solution (Table II). The use of any of three extraction solutions (85 mM or 50 mM NaCl or 50 mM NaCl plus 20 mM CaCl_2) provided adequate extraction of enzyme and discrimination between sprouted and sound wheat. Thus, an extraction buffer consisting of 85 mM NaCl (which can be made easily under field conditions by dissolving a NaCl tablet in a predetermined volume of water) was used in subsequent experiments. This may also remove effects of variation in water quality in remote situations.

The results of a comparison in which enzyme was extracted from whole meal flour in 85 mM NaCl buffer either by intermittent vortex mixing for 4 min or by wrist-action shaking for 15 sec are shown in Table III. Hand-shaking for 15 sec not only allowed for sufficient extraction of amylase for discrimination between sprouted and sound wheat, it also removed the need for vortex mixing, thus simplifying the tube assay procedure further. In addition, the color produced by α -amylase in the flour extract was stable for up to but not longer than 45 min following a 15-sec

extraction by shaking in 85 mM NaCl buffer (Table IV). This allows adequate time for analysis of a large batch of samples. McCleary and Sheehan (1987) also noted a loss in enzyme activity on extended standing of the extracts in an assay using a dye-labeled substrate and suggested that the effect was due to α -amylase binding to intact and damaged starch granules. A final simplification made to the tube assay was the replacement of the PBST wash buffer with the 85 mM NaCl buffer used for enzyme extraction. Unlike washing with water (data not shown), washing the tubes with 85 mM NaCl did not cause an increase in nonspecific background color. The tube assay still provided good discrimination between wheats with varying degrees of sprouting (Table V).

Detecting Preharvest Sprouting in Wheat Samples

Three diverse sets of wheat samples were analyzed for preharvest sprouting with the aim of establishing the relationship between FN and color development in the ELISA test, as well as establishing whether the relationships noted were substantially independent of the wheat cultivar analyzed. The first set of wheat samples collected from Western Australia and New South Wales ($n = 50$) were analyzed in duplicate for α -amylase using plate sandwich ELISA. For these analyses, mean ELISA absorbance (color development) was strongly negatively correlated with FN ($r = -0.97$, detection of high pI amylase; and $r = -0.98$, detection of high-low pI amylase). The decrease in color with increasing FN was due to a decrease in the amount of amylase in sound wheat. Samples collected from elevators in Queensland ($n = 130$) during the 1996 harvest were analyzed for α -amylase using the standard (detection of high pI isozymes) and rapid tube assays (detection of high-low pI isozymes). The results of these analyses (Fig. 5A and B) indicated that relative ELISA color development was strongly (and negatively) correlated with FN for both tube assays. This curvilinear relationship between α -amylase activity and FN over a wide range of values has been previously reported (Perten 1964, Barnes and Blakeney 1974, Kruger and Tipples 1980) and can be transformed to a linear function by converting FN to liquefaction number (LN): $\text{LN} = 6,000/\text{FN} - 50$ (Hylinka 1968). While the correlation between ELISA absorbance and FN was slightly better for the standard assay (detection of high pI amylase) (Fig. 5A) than for the fast assay (detection of both high and low isozymes) (Fig. 5B), the differing specificities of the two antibodies is probably not important in guiding their use in the tube assay. The slight difference is probably due to the shorter incubation times of the rapid assay, making the assay less suitable for analysis of large batches of samples. There was an excellent correlation ($r = 0.97$) (Fig. 6) between the relative ELISA absorbances obtained with the standard assay versus the rapid assay. This result arises possibly in part from the relatively low proportion of low pI isozyme in weather-damaged wheat but nonetheless suggests that variation in this isozyme also contributes to the FN. Although the low pI amylase isozymes are predominant during grain development, they are also induced by rain at harvest, and the proportions of the high and low pI isozymes reportedly stay constant in sprouting (Battershell and Henry 1990).

TABLE IV
Stability of α -Amylase in 85 mM NaCl Extraction Buffer^a

Falling Number (sec)	Mean ELISA Absorbance \pm Standard Deviation			
	0 min	30 min	45 min	60 min
187	0.52 \pm 0.01	0.51 \pm 0.01	0.51 \pm 0.09	0.44 \pm 0.06
284	0.22 \pm 0.09	0.21 \pm 0.01	0.22 \pm 0.05	0.16 \pm 0.02

^a Simultaneous addition of wheat extract and horseradish peroxidase conjugate (incubation for 10 min, no washing step). Wholemeal flour extracted in 85 mM NaCl. Tubes washed with phosphate-buffered saline and 0.05% Tween 20 (PBST). Two replicates for each test.

TABLE V
Comparison of Tube Washing Solutions^a

Falling Number (sec)	Mean ELISA Absorbance \pm Standard Deviation	
	85 mM NaCl ^b	PBST ^c
187	0.53 \pm 0.03	0.55 \pm 0.01
284	0.23 \pm 0.03	0.27 \pm 0.05
403	0.16 \pm 0.01	0.18 \pm 0.01

^a Simultaneous addition of wheat extract and horseradish peroxidase conjugate (incubation for 10 min, no washing step). Two replicates for each test.

^b Wholemeal flour hand-shaken in 85 mM NaCl for 15 sec.

^c Tubes washed with phosphate-buffered saline and 0.05% Tween 20 (PBST).

The 108 wheat samples subjected to controlled wetting at Narrabri, New South Wales, were analyzed for α -amylase using both standard and rapid versions of the tube assay. As with the previous two sets of samples, relative ELISA absorbance was strongly negatively correlated with FN for detection of the high pI isozyme of amylase ($r = 0.95$) and detection of both high and low pI isozymes ($r = 0.98$). While the previous two sets of wheats (and the wheats used initially in the development of the assay) were ground using the Jupiter mill (with no screen), this set from Narrabri was milled using a standard FN mill with a 0.8-mm screen. Although a direct comparison of the two grinding methods was not performed, results of the tube assay analyses suggest that use of the Jupiter mill did not affect the ability of the tube assay to differentiate sprouted from sound wheats.

The varietal independence of the relationship between relative ELISA absorbance (standard assay) and FN for the 130 Queensland grain samples is shown in Fig. 7A where the eight different wheat cultivars among the samples are plotted along with a number of samples of unknown cultivar. There was no tendency for samples of one cultivar to cluster to one side of the regression curve. Instead, wheat samples of different cultivars with similar FN values gave similar absorbance readings in the tube ELISA. This result was further examined by analysis of 108 wheat samples of six more genetically diverse cultivars (including soft and hard grained cultivars) that were subjected to controlled wetting. In this set, wheat samples of different cultivar with similar FN values gave similar but, in some cases, not identical absorbance readings in both rapid and standard tube ELISA (Fig. 7B). Starch

pasting analysis of some of this set using the Rapid ViscoAnalyser and silver ions to inhibit amylase activity revealed that some of the samples differed in paste viscosity. For example, Vulcan samples with a slightly low FN for a given ELISA color had low starch paste viscosities, whereas Halberd samples with a slightly high FN for a given ELISA color had high starch paste viscosities. Finney (1985) and Moss (1987) have also shown that different cultivars differed in FN for a given amylase content or visual degree of sprouting.

Relationship Between Enzyme Activity and ELISA Results

Ceralpha enzyme activity was determined for the 130 Queensland samples. The correlation between Ceralpha enzyme activity (units/g of flour) and FN ($r = 0.93$) was similar in magnitude to the correlation between ELISA absorbance (standard assay) and FN ($r = -0.91$). Approximately half of the samples tested (FN > 250 sec) were clustered in a narrow band of absorbance values and were not significantly different from one another. Although the modifications made to the enzyme assay increased its sensitivity, it still appeared to be less sensitive than the FN test or the tube ELISA by failing to clearly discriminate wheat samples with FN > 250 sec (data not shown). While the correlation between ELISA absorbance (standard assay) and Ceralpha enzyme activity for the 130 Queensland wheat samples was good ($r = 0.91$) (Fig. 8), there was a large cluster of points of low enzyme activity due to the lower sensitivity of the enzyme assay. The results suggest that the ELISA tube assay only detects active enzyme. The relationship between Ceralpha enzyme activity and ELISA absorbance was fitted linearly (Fig. 8).

Precision of Tube ELISA

Analyses of the precision of both standard and rapid versions of the tube ELISA are shown in Table VII. In this study, eight whole meal samples from the 1997 Narrabri set were analyzed six times in both tube ELISAs. Calculated FN for these samples were estimated by comparing their mean absorbance values with the absorbance values of standards of known FN units included in each run. FN for the same set of flour samples were measured three times using FN test apparatus. Precision estimates obtained were in keeping with earlier studies (Greenaway and Neustadt 1967). Both tube ELISAs displayed close relationships between the measured and calculated FN. The precision of the ELISA assays was equal or better than that of FN test apparatus. The practical implication of this study is that the sprouted and sound samples could be discriminated at critical industry cutoff values. For example, Australian Hard wheat must have FN ≥ 300 sec. An unacceptable sample with a mean FN of 277 gave calculated FN values of 278–290 in the standard ELISA, while an acceptable sample with mean FN of 329 gave calculated FN values of 322–332 in the standard ELISA.

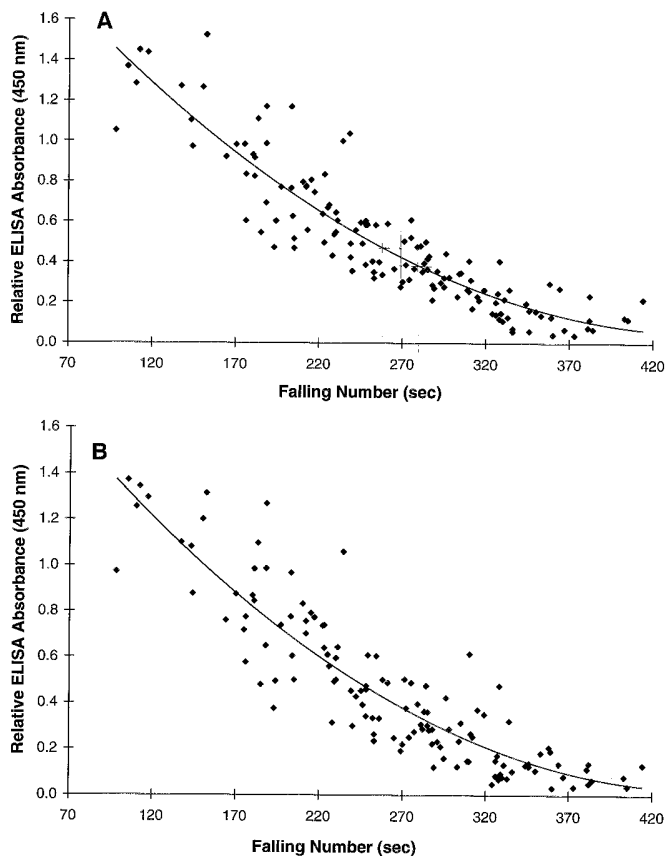


Fig. 5. Relationship between relative enzyme-linked immunosorbent assay (ELISA) absorbance and falling number (FN) for 130 grain samples from elevators in Queensland analyzed using standard tube ELISA (detection of high pI isozymes) (A) and rapid tube ELISA (detection of high and low pI isozymes) (B). Samples were analyzed in duplicate in two separate runs and raw absorbances standardized to the absorbance of a standard of FN 187 included in each run. Coefficients of determination for a parabolic fit for A and B, respectively: $r^2 = 0.83$ and 0.81 .

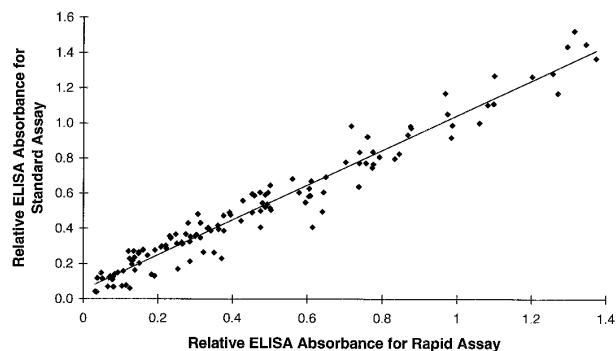


Fig. 6. Relationship between relative enzyme-linked immunosorbent assay (ELISA) absorbance for standard tube assay (detection of high pI amylase) and relative ELISA absorbance of rapid tube assay (detection of high and low pI amylase) for grain samples from elevators in Queensland (linear correlation $r = 0.97$, $n = 130$).

TABLE VI
Precision of Preharvest Sprouting Screening Using Falling Number (FN) Apparatus
and Standard and Rapid Enzyme-Linked Immunosorbent Assay (ELISA)

Sample	FN Apparatus		Standard ELISA		Rapid ELISA		Standard ELISA		Rapid ELISA	
	Measured FN (sec) ^a	Range	Calculated FN (sec) ^b	Range	Calculated FN (sec)	Range	Absorbance (450 nm) ^c	Range	Absorbance (450 nm)	Range
A	217 ± 8.0	211–225	217 ± 3.0	212–220	241 ± 2.0	238–242	0.75 ± 0.01	0.75–0.77	0.67 ± 0.02	0.65–0.70
B	210 ± 8.0	203–219	228 ± 4.0	224–236	241 ± 2.0	238–242	0.71 ± 0.02	0.69–0.73	0.66 ± 0.01	0.64–0.68
C	262 ± 13.0	249–274	278 ± 9.0	260–284	275 ± 2.0	272–278	0.52 ± 0.04	0.49–0.60	0.49 ± 0.01	0.48–0.51
D	277 ± 4.0	274–282	283 ± 4.0	278–290	288 ± 2.0	288–290	0.50 ± 0.01	0.48–0.52	0.43 ± 0.01	0.40–0.44
E	298 ± 27.0	268–319	299 ± 2.0	296–300	299 ± 2.0	296–302	0.43 ± 0.01	0.42–0.45	0.37 ± 0.01	0.36–0.39
F	329 ± 6.0	323–334	328 ± 5.0	322–332	330 ± 1.0	328–330	0.32 ± 0.01	0.30–0.34	0.26 ± 0.01	0.25–0.28
G	406 ± 4.0	402–409	391 ± 7.0	382–400	400 ± 1.0	398–400	0.08 ± 0.02	0.06–0.11	0.02 ± 0.01	0.00–0.04
H	393 ± 16.0	377–408	396 ± 3.0	392–400	399 ± 1.0	398–400	0.05 ± 0.02	0.03–0.08	0.02 ± 0.01	0.01–0.04

^a Mean ± standard deviation ($n = 3$).

^b FN calculated by comparing absorbance of samples with absorbance of standards with known FN values analyzed using standard and rapid tube ELISA. Mean ± standard deviation ($n = 6$).

^c Absorbance readings measured with standard and rapid tube ELISA to estimate calculated FN. Mean ± standard deviation ($n = 6$).

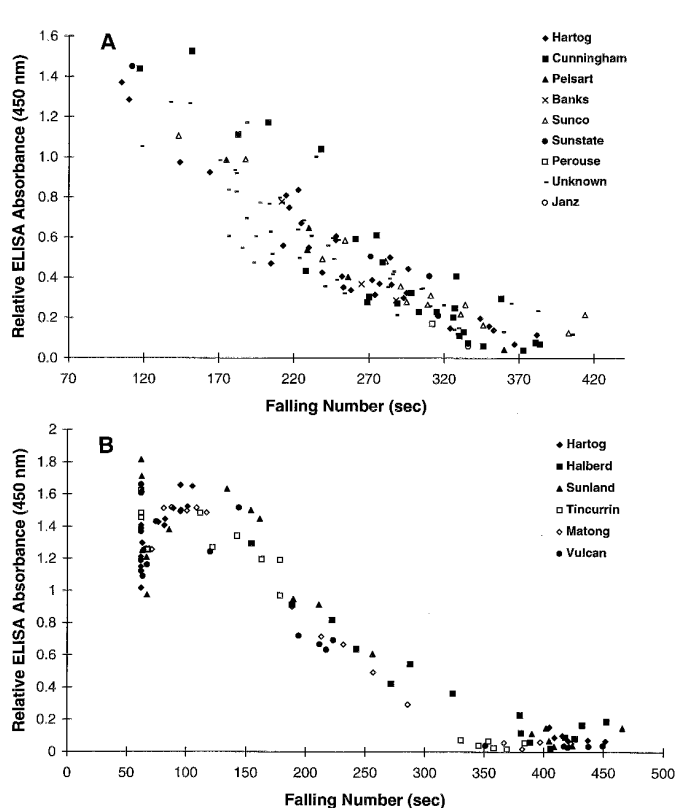


Fig. 7. Varietal independence of the relationship between relative enzyme-linked immunosorbent assay (ELISA) absorbance (standard assay) and falling number for wheat grain samples from elevators in Queensland (A) (linear correlation coefficient $r = 0.91$, $n = 130$) and artificially wetted grain samples from Narrabri, New South Wales (B) (linear correlation coefficient $r = 0.95$, $n = 108$).

CONCLUSIONS

A tube sandwich ELISA that can be used for detection of preharvest sprouting in wheat has been developed. The assay is based on the detection of α -amylase enzymes and uses PAB to the enzyme as both capture and detection agents. α -Amylases were considered to be the most appropriate targets for the test because they are relatively abundant, they are synthesized early in the preharvest sprouting sequence (Corder and Henry 1989), they are responsible for many of the quality defects that occur when end products are prepared from sprouted wheat, and the basis of the measurements in the industry standard test (FN) is a change in the

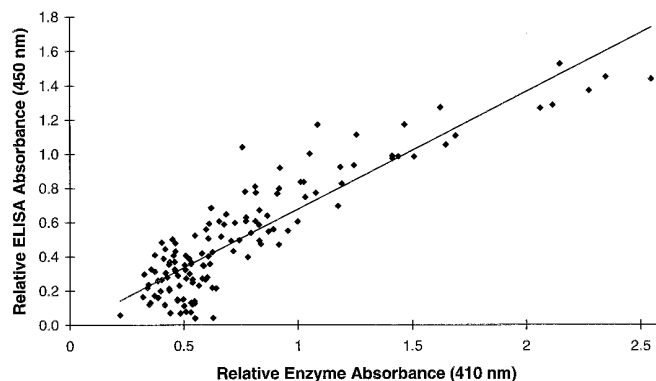


Fig. 8. Relationship between relative enzyme-linked immunosorbent assay (ELISA) absorbance (standard assay) and Ceralpha enzyme absorbance for grain samples from elevators in Queensland (linear correlation coefficient $r = 0.91$, $n = 130$).

viscosity of a whole meal and water slurry due to the presence of carbohydrate-degrading enzymes such as amylases. Enzymes such as amylases can also be quantified directly by various enzyme assays. The results from the tube ELISAs that we have developed showed a strong negative correlation with FN for three diverse sets of wheats and a positive correlation with the Ceralpha enzyme assay. The ELISA is simple and easy to use and is reproducible over a wide range of FN values. Although the tube sandwich ELISA was less sensitive than the plate ELISA, it still had the capacity to detect FN < 350 sec (the critical industry cutoff point). At >350 sec, it is difficult to establish close correlations between FN and α -amylase activity (Mares 1989).

The tube assay in the form of a simple kit ELISA has the potential for on-farm use by individual growers, allowing identification of areas of sprouting before harvest, thus preventing contamination of sound wheat by wheat that is weather damaged. Currently, growers may harvest grain across their whole property, and tests on elevator receipt are done on the whole parcel of grain. However, except in very bad harvests, the extent and presence of preharvest sprouting can vary quite markedly between and within fields, being dependent on the rate of drying of the crop after rainfall has occurred (affected by field aspect and drainage), on the wheat cultivar sown, and on the time of sowing. Growers usually have an intimate knowledge of the behavior of different parts of their own property. If they were able to test the grain from different paddocks and parts of paddocks before harvest, it should be possible to harvest the damaged grain separately from sound grain and avoid the financial losses that result from downgrading the whole crop.

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