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TECHNICAL NOTE

Streamlined alpha-amylase assays for wheat preharvest sprouting and late maturity alpha-amylase detection

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Abstract

Late maturity alpha-amylase (LMA) and preharvest sprouting (PHS) lead to elevated alpha-amylase in wheat (*Triticum aestivum* L.) grain. Risk of poor end-product quality due to elevated alpha-amylase is detected in the wheat industry using the Hagberg–Perten falling number (FN) method. In breeding programs, selection for PHS and LMA tolerance requires higher throughput methods requiring a smaller sample size than the 7 g required for the FN method. Specifically, LMA can only be screened only using detection of alpha-amylase activity or protein after cold treatment of individual wheat spikes at a specific stage of grain development resulting in very small samples (≤1 g). This study developed and evaluated a high throughput 96-well method for the Phadebas alpha-amylase enzyme assay for small wheat grain samples and compared this method to FN and the Megazyme Alpha-Amylase SD (Sprout Damage) Assay Kit performed on the automated Awareness Technology ChemWell-T Analyzer. In parallel, the efficacy of low-cost small-scale milling methods was evaluated relative to traditional larger scale mills. The Phadebas enzyme activity was highly reproducible and showed a strong correlation to the SD enzyme assay and FN method regardless of which mill was used to process the grain. The SD assay offers simpler standardization and calculation of enzyme activity, whereas the Phadebas assay offers higher sensitivity and lower expense. Both the 96-well Phadebas and automated Megazyme SD assays are suitable for alpha-amylase detection from small samples, and the use of low-cost coffee grinders to process small samples did not significantly impact assay performance.

Abbreviations: ANOVA, analysis of variance; Au, absorbance units; CORR, correlation; CV, coefficient of variance; FN, falling number; GLM, general linear model; LMA, late maturity alpha-amylase; PHS, preharvest sprouting; rpm, rotations per minute.

1 | INTRODUCTION

Higher-throughput α-amylase enzyme assays are an important selection tool for lowering mature-grain enzyme activity in wheat (*Triticum aestivum* L.)-breeding programs (Brown et al., 2018; Derkx & Mares, 2020; Henry, 1989; Liu et al., 2021b). The wheat industry detects elevated α-amylase in wheat meal or flour using the Hagberg–Perten falling number (FN) method in which a lower FN, measured in seconds, is associated with higher α-amylase activity (Hu et al., 2022; Perten, 1964; Ross & Bettge, 2009). The FN method detects α-amylase in wheat meal or flour based on reduced viscosity resulting from loss of pasting capacity with enzyme digestion of starch. Higher α-amylase results in a lower FN, which is the time in seconds required for a stirrer to fall through a heated meal/water slurry. Because a low FN/high α-amylase level in grain is associated with higher risk of poor end-product quality, farmers receive substantial discounts for grain with a FN below 300 s. Breeding for resistance to low FN can reduce farmers' financial risk. Unfortunately, the FN method is impractical for early generation selection in breeding programs because it requires a large (>14 g) grain sample, expensive equipment (including a FN machine), and skilled personnel who can only evaluate two samples every 10 min. Consequently, in this study two higher throughput α-amylase enzyme assays were evaluated as FN alternatives for breeding and genetic research, a Phadebas assay adapted to 96-well plates (developed in this study) and the Megazyme SD assay using the ChemWell-T analyzer (Barnes & Blakely, 1974; McKie & McCleary, 2015).

There are two major, genetically distinct, causes of elevated α-amylase in wheat grain, preharvest sprouting (PHS) and late maturity alpha-amylase (LMA) (reviewed by Mares & Mrva, 2014; Cannon et al., 2022). LMA is the induction of α-amylase expression in the aleurone layer in response to cool conditions during the late grain maturation stage of development (Barrero et al., 2013; Derkx et al., 2020). PHS is the induction of grain germination or sprouting by rain before harvest while still on the mother plant. During germination, α-amylase levels can rise before the grain visibly sprouts, necessitating the use of FN or enzyme assays to detect sprout damage (Henry, 1989). As the genetic tendency towards low FN is poorly linked to visible sprouting in wheat, selection solely for lack of visible sprouting may not suffice to obtain higher FN (Barnard et al., 2005; Martinez et al., 2018). Thus, there is a need to use more sensitive, selection methods, such as α-amylase enzyme assays or immunoassays to detect α-amylase proteins (Barrero et al., 2013; Farrell & Kettlewell, 2008). Because the only α-amylase immunoassay available to date is not sold outside Australia, researchers use α-amylase enzyme assays to detect LMA induction (Kondhare et al., 2012; Liu et al., 2021a, Liu et al., 2021b). Sensitive colorimetric enzyme assays are needed because, for practical reasons, PHS and LMA screening in breeding programs needs to be performed on single spikes (≤1 g of grain). Alpha-amylase induction by PHS can be screened by placing five individual field-grown spikes under a misting system, and then screening for activity in each single spike replicate (Brown et al., 2018). LMA is induced by cold treatment of single wheat spikes at 21–28 d past anthesis, followed by α-amylase enzyme assays of individual spikes (Liu et al., 2021b; Mrva & Mares, 2001). Brown et al. (2018) performed such a screen using the Megazyme Ceralpha α-amylase enzyme assay performed on protein extracts from single spikes in glass tubes, but the low-throughput and expense of this approach has limited its adoption in breeding programs. The FN method is not applicable to such greenhouse experiments that use single spikes because a minimum of 7 g of meal is required.

Alpha-amylase enzyme activity can be measured using one of several different chromogenic substrates. Hormonal regulation of α-amylase in the barley (*Hordeum vulgare* L.) aleurone was investigated using an iodine-based α-amylase enzyme assay that cannot differentiate between α- and β-amylase activity (reviewed by Jacobsen et al., 1995). Alpha-amylase catalyzes the hydrolysis of α-D-1,4- and α-D-1,6-glucosidic bonds in starch to yield shorter polysaccharides, and finally the disaccharides maltose and maltodextrin (Ju et al., 2019; reviewed by Mieog et al., 2017). Modern assays are specific to α-amylase, the enzyme induced during LMA and PHS (Barrero et al., 2013). Beta-amylase is an exohydrolase catalyzing the cleavage of the outermost glucosidic linkages, whereas α-amylase is an endohydrolase that catalyzes internal cleavage. Assays, such as the Phadebas and Megazyme assays, specifically measure α-amylase activity by placing a chromophore at the end of a starch molecule that cannot be

Core Ideas

- High throughput 96-well Phadebas enzyme assays provided data in the linear range of detection for small samples.
- The 96-well Phadebas assay run in plasticware had similar sensitivity to the original glass tube method.
- The Phadebas assay generated highly reproducible data over time and when performed by multiple users.
- There were strong positive correlations between Phadebas and SD enzyme assays.
- There were strong correlations between FN and enzyme activity regardless of milling method or assay chemistry.

cleaved by β-amylase but that is solubilized by α-amylase α-glycosidic bond cleavage (Barnes & Blakeney, 1974; Cornaggia et al., 2016; McCleary & Sheehan, 1987; McKie & McCleary, 2015).

The Phadebas Amylase Test was originally developed to measure α-amylase activity in biological fluids such as human salivary and pancreatic fluids, and subsequently adapted for use with protein extractions from grain (Barnes & Blakeney, 1974; Hsu & Varriano-Mariston, 1982). The Phadebas tablets are composed of interlinked starch polymers that form globular microspheres. These microspheres are covalently linked with a blue dye and are insoluble in aqueous solution until cleaved by α-amylase. Cleavage liberates the blue dye into the aqueous fraction, leading to increasing blue color with increasing enzyme activity.

The two α-amylase enzyme assays from Megazyme, Ceralpha and SD, rely on a two-step enzymatic cleavage of *p*-nitrophenyl monosaccharide by α-amylase and α-glucosidase to liberate a *p*-nitrophenolate ion resulting in a yellow color (McCleary & Sheehan, 1987). The SD assay uses a conjugated ethylidene substrate instead of the conjugated benzylidene substrate used by the Ceralpha assay, resulting in a faster, less expensive assay that has been adapted for use on the robotic ChemWell-T platform (Mangan et al., 2016; McKie & McCleary, 2015).

The current study developed a higher throughput Phadebas assay adapted for 96-well plates for use with protein extracts from wheat meal and compared this approach to the FN method and to the SD assay performed on the ChemWell-T platform. A major limitation in performing enzyme assays using smaller single-spike sample sizes has been the lack of small-scale milling methods. Thus, this study also compared the use of a small commercial coffee mill to mills requiring larger samples.

2 | MATERIALS AND METHODS

2.1 | Sample acquisition and preparation

Field samples in Supplemental Table S1 were obtained from the Washington State University (WSU) Cereal Variety Testing program in 2013 and 2019 (http://smallgrains.wsu.edu/variety/; Neely et al., 2020). Samples that had low FN due to PHS or LMA were identified based on examination of weather data and using half-grain enzyme assays to differentiate LMA and PHS (Mrva & Mares, 1996; Sjoberg et al., 2020). Samples with a wide range of FN are described in Supplemental Table S1 (steberlab.org; Sjoberg et al., 2020). Grain was combine-harvested at harvest maturity, cleaned, and stored at −15 °C in the dark to reduce the gradual decrease in FN over time (Ji & Baik, 2016). Samples known to have experienced LMA, or a PHS-inducing rain event are indicated in Supplemental Table S1.

An additional 65 samples were generated using field LMA induction in cultivars and breeding lines grown at Spillman Agronomy Farm, Pullman, WA, in 2020 (Liu et al., 2021b). Note that LMA induction of α-amylase occurs in response to cool temperatures during grain filling without rain. Briefly, 20 spikes per genotype were cut from the field at the soft dough stage (Zadoks' stage 85) of grain development and subjected to 7 d of cool temperature shock in a growth chamber with 16 h of light and an 18 °C day/7.5 °C night cycle (Zadok et al., 1974). Samples were then moved outside and allowed to dry, then threshed and milled with a UDY Cyclone Mill before performing FN and 96-well Phadebas assays.

2.2 | Falling Number method

The FN method was performed using a Perten FN machine (model 1700) on all 2013 and 2019 field samples using the AACC Method 56–81.03A (1999) in 2013 (as in Martinez et al., 2018) and the FGIS Directive 9180.3 (5/2019) method in 2019 with the exception that sample weight was adjusted to obtain the same dry weight as 7 g of meal at 14% moisture. Field samples from 2013 were milled using a UDY Cyclone Mill (UDY Corp) fitted with a 0.5-mm screen, and 2019 field samples were milled using a Perten 3100 Mill (0.8-mm particle size). The FN were corrected using the altitude correction table at 762 m (2,500 ft) in 2013 (Directive 9180.38, 5/2013) and using barometric pressure correction in 2019 (Delwiche et al., 2018).

2.3 | Milling methods

The Perten 3100 Mill was used to mill 200 g of grain and the UDY Cyclone Mill 20 g of grain for each of the 17 standard samples (no. 15–no. 31, Supplemental Table S1) in preparation for FN testing and enzyme assays. Mills were cleaned after every sample, first with a brush and then a vacuum. The UDY mill was allowed to cool for 5 min after every sixth sample. Experiments used meal milled on a Perten 3100 unless otherwise indicated.

A coffee grinder was used to mill small grain samples of 0.5–2 g (Liu et al., 2021b). A Krups F203 blade-type coffee grinder (www.krupsusa.com) was modified to improve the contact of the blade with grain by reducing the chamber size from 335 to 75 ml with the addition of a sheet metal false lid fabricated to fit the interior chamber of the coffee grinder, resting on the interior metal chamber bottom (Supplemental Figure S1). Samples were ground using three coffee grinders that are replaced annually to avoid variability due to

dulling blades (approximately 300 samples of hard wheat, 500 samples of soft were milled in total). For FN testing a total of 15 g of grain was ground in aliquots of 1–2 g. Each sample aliquot was ground for 10 s and then added to a glass jar until the entire sample was ground. The grinding chamber, blades, and false lid were cleaned thoroughly using a dry brush or Kimwipe (Kimberly-Clark) to remove residual flour and then with 70% ethanol and a Kimwipe in between each sample. The use of three coffee grinders allowed the instruments to air dry and cool between samples.

2.4 | Phadebas glass tube method

Wholemeal samples were analyzed for α-amylase enzyme activity using a glass tube method developed for the Phadebas Amylase Test (Liu et al., 2021b; Tuttle et al., 2015) that was based on the previously published methods (Phadebas AB catalogue no. 1302) (Barnes & Blakely, 1974; Mares et al., 1994). The 2013 variety trial samples milled on a UDY cyclone mill were analyzed. Phadebas substrate tablets were ground with a mortar and pestle and suspended at a ratio of 1 Phadebas tablet per 4 ml of extraction buffer (100 mM sodium malate, pH 6.0, 5 mM calcium chloride) consistent with the manufacturer's protocol. A magnetic stirrer was used to keep the particles uniformly suspended. Meal was shaken to ensure uniformity before weighing 0.2 g or indicated quantity into 16 by 100 mm glass test tubes. Meal was vortexed for 5 s to prevent clumping immediately before adding 2 ml of extraction buffer. Extractions were vortexed, incubated in a 50 ˚C water bath for 10 min, and then centrifuged at 2,800 rpm for 10 min at 22 ˚C. Two technical replicates were assayed per extraction by transferring 200 µl of supernatant to two 13- by 100-mm glass tubes and incubating with 1 ml of Phadebas suspension in a 50 ˚C water bath for 30 min. Enzyme reactions were stopped with 1 ml of 0.5 M NaOH, briefly vortexed, then centrifuged at 2,800 rpm for 10 min. After 0.2 ml of supernatant was transferred to a 96-well optical plate (Corning 351172), absorbance was measured at 620 nm using a BioTek SYNERGY 2 microplate reader (2006-2015 BioTek Instruments) and BioTek Gen5 software (version: 2.06.10). The 50 ˚C incubation temperature gave a stronger signal than the 37 ˚C indicated by the Phadebas Amylase Test protocol (Barnes & Blakely, 1974).

2.5 | 96-well Phadebas method

A high throughput 96-well method was developed for measuring wholemeal wheat α-amylase activity using the Phadebas Amylase Test reagent (Phadebas AB; Liu et al., 2021a). Low-protein-binding plasticware was used to avoid loss of activity from the α-amylase enzyme sticking to the tube or well. Extraction: Wholemeal was shaken, then 0.2 g weighed into a 2 ml Lo-Bind microfuge tube (MSP 15–1216). Dry samples were vortexed for 5 s to reduce clumping, and then 1 ml of extraction buffer (100 mM sodium malate, pH 6.0, 5 mM calcium chloride) added using a HandyStep repeating pipette (BrandTech; www.sigmaaldrich.com) with a 50-ml tip. Samples were briefly vortexed, then incubated in a 50 ˚C water bath for 10 min with shaking every 2 min. Tubes were placed in an open tube rack to allow good exposure to the water bath (BrandTech 4340060; Colepalmer.com). After incubation, samples were vortexed, then centrifuged at 2,800 rpm for 10 min at 22 ˚C. There appears to be a gradient of α-amylase activity in the supernatant after centrifugation with higher activity closer to the bottom of the tube. This problem can be eliminated using the optional step outlined below or by always pipetting the supernatant from the same point in the tube; in our case determined by setting a ridge on the pipette tip against the edge of the 2-ml tube. [Optional Step: Pipette 0.5 ml of supernatant from each tube into a well of a 2-ml deep-well, 96-well plate and mix by pipetting up and down twice.] Extractions were stored on ice until addition of the Phadebas substrate. Enzyme Assay (Supplemental Figure S2): Two technical replicates were prepared by pipetting 40 µl of supernatant into two separate 96-deep-well 2-ml plates (Eppendorf Protein LoBind plates, EPP951032905). If the coefficient of variance (CV) between technical replicates is higher than .05, then perform the optional step. Phadebas substrate is supplied as 0.2-g tablets intended for use in a 4-ml reaction. To obtain this concentration, 10 ground Phadebas tablets (2.0 g) were suspended in 40 ml of extraction buffer in a 50-ml beaker. A magnetic stirrer was used to keep the substrate in suspension without creating bubbles. A 5-ml HandyStep repeating pipette was used to quickly add 0.2 ml of Phadebas substrate suspension to each 40 µl aliquot of protein extract while avoiding bubbles and settling of substrate.

After adding substrate, plates were incubated in a 50 ˚C water bath for 45 min (Barnes & Blakeney, 1974). After incubation, 0.2 ml of stop solution (0.5 M sodium hydroxide) was added to each well using a 5-ml repeater pipette, and plates were centrifuged at 2,800 rpm for 5 min at 22 ˚C. Using a multichannel pipette, 0.2 ml of supernatant from each sample was transferred to a 96-well flat-bottomed optical plate (250 µl; Corning 351172). Absorbance was read at 620 nm using a BioTek Synergy HTX plate reader as above. This study was conducted using Eppendorf Protein LoBind (EPP951032905) 96-deep-well plates and tubes. It is possible to substitute VWR 96-well deep-well plates (75870-790) without significant loss of sample or activity (Supplemental Figure S3).

2.6 | Normalizing Phadebas reagent batch variation

The Phadebas package insert provides an equation for calculating enzyme units L^{-1} : $(e^N) \times 60$, where $N = A + \sqrt{(B + C \times \ln(A_{620})}$. A_{620} is the experimentally determined sample absorbance, and the values for A, B, and C are unique to each batch of Phadebas Amylase Test reagent providing a correction for reagent batch differences. We converted from units L^{-1} to units g^{-1} by multiplying all units L^{-1} by 0.055. This is based on using 0.2 g of meal per sample extraction, and 40 µl of supernatant from each extracted sample in the Phadebas 96-well method as described in 96-well Phadebas method: (units × 0.44 ml)/(1,000 ml) (0.008 g) = units g^{-1}.

2.7 | The Megazyme SD assay using a ChemWell-T robot

The α-amylase activity of wholemeal samples was analyzed using the high throughput Megazyme SD assay chemistry (K-AMYLSD) on the ChemWell-T Automated Chemistry Analyzer, Model-4620 series according to the Megazyme K-AMYLSD_DATA booklet (04/2019) (McKie & McCleary, 2015). For each sample, 0.2 g of wholemeal was weighed into a 2 ml low-bind tube (MSP 15–1216). To each tube 1.5 ml of 1X Amylase extraction buffer (50 mM of sodium malate pH 5.4, 50 mM of sodium chloride, 2 mM of calcium chloride, 0.00045% of sodium azide) was added with a HandyStep repeating pipette (50-ml tip), the tube vortexed for 10 s until the wholemeal was suspended, and then the samples were incubated in a 40 ˚C water bath for 20 min with vortexing every 5 min. After centrifugation at 11,000 rpm for 2 min, 1 ml of the supernatant was pipetted into a 2-ml screw top tube. Reactions were performed using the "K-AMYLSD(Calc)" program on the ChemWell-T Analyzer. Briefly, 0.25 ml of 1X Amylase SD-reagent (3.73 mM ethylidene-end blocked *p*-nitrophenyl maltoheptaoside and 3.9 units ml^{-1} of thermostable α-glucosidase) was mixed with 75 µl of sample in separate cuvettes. Samples were incubated at 37 ˚C for 5 min, and then the reactions terminated by addition of 0.1 ml of 500 mM sodium carbonate buffer, pH 11. The absorbance of the *p*-nitrophenolate ion released during the SD assay was measured at 405 nm. The program utilizes a separate blank standard for every protein extraction because pigment in wheat protein extractions can contribute to absorbance at 405 nm. The blank standard is a sample where the stop solution is added to the protein extract before the SD reagent. The program calculates α-amylase activity in SD units g^{-1}:

$$= \frac{\Delta E405}{\text{IncubationTime}} \times \frac{\text{TotalVolumeinCell}}{\text{AliquotAssayed}} \times \frac{1}{\varepsilon\text{mM}} \times \frac{\text{ExtractVol.}}{\text{SampleWeight}} \times \text{Dilution}$$

where ΔE405 is the Absorbance, Incubation Time is 10 min, Total volume in cell is 1.5 mL, Aliquot assayed is 0.4 mL, ε_{mM} of the *p*-nitrophenol at 405 nm is 18.1, Extraction volume is 8 mL per 0.5 g sample, and Dilution is the dilution of the extract if needed.

2.8 | Statistical analysis

Analysis of variance was used to identify statistically significant differences in α-amylase activity. The ANOVAs were performed using the PROC GLM function and were compared using a Tukey's all pairwise comparison in SAS version 9.4 (SAS Institute). Pearson correlations between methods were performed using the PROC CORR function in SAS version 9.4. Variables examined as fixed effects included "sound" vs. "sprouted" grain, glass tubes vs. 96-well plates, the different mills used, and "USER1" vs. "USER2" who were two people performing multiple independent experiments on the same samples. For all experiments described *p* values ≤.05 were considered significant.

3 | RESULTS AND DISCUSSION

3.1 | Evaluation of a 96-well α-amylase enzyme assay using the Phadebas chemistry

This study developed a rapid 96-well Phadebas method enabling evaluation of a large number of samples limited to 0.5–3 g of grain. The glass tube based Phadebas method was used to determine the linear range of the enzyme assay for "sound" wheat grain with low activity (FN 300 s) and for sprouted wheat grain with higher activity (FN 171 s) (samples 1 and 2, Supplemental Table S1) (Mares et al., 1994; Liu et al., 2021a). Extractions were performed using 0.09–0.9 g of wholemeal. The α-amylase activity measured in Absorbance units (Au) at 620 nm increased proportionally to the grams of meal extracted between 0.09 and 0.63 g (Figure 1a). Based on an ANOVA, differences in sample type (sound vs. sprouted; *p* value < .0001) and grams of meal extracted (*p* value < .0001), but not experimental replicate (*p* value .06), had significant effects on enzyme activity (Au g^{-1}) (Supplemental Table S2). Within the linear range of the assay, every extraction estimated the same enzyme activity per gram of sample (Au g^{-1} in Figure 1b). Because reactions containing 0.09 and 0.9 g of meal showed significant variation, they are outside the linear range (Figure 1b; Supplemental Tables S3 and S4). The 0.2 g of meal per milliliter extraction (0.4 g 2 ml^{-1} in Figure 1) used in the 96-well Phadebas method is within the linear range of the assay. This mass can be obtained from as few as five wheat kernels. Enzyme activity in the Phadebas assay is given in Au at 620 nm because all experiments used 0.2 g of meal. If

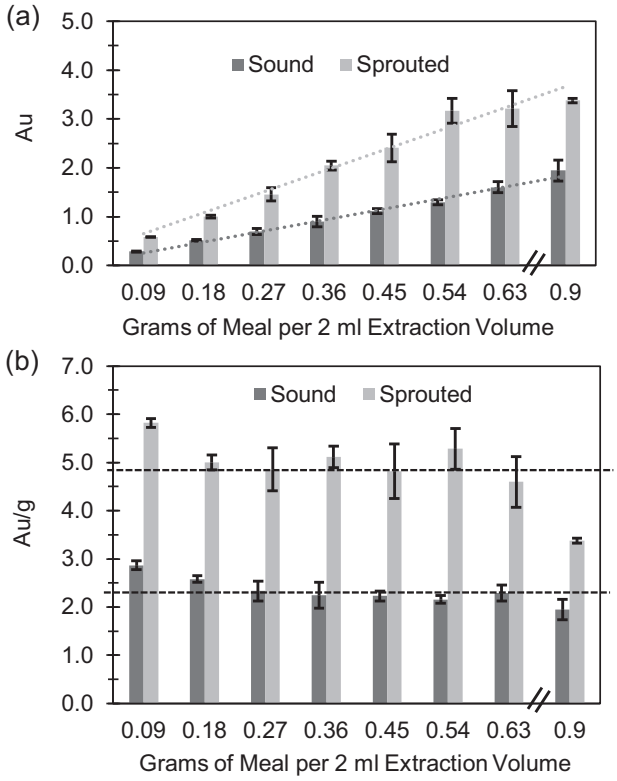

FIGURE 1 The effect of sample mass on α-amylase enzyme activity determined by the Phadebas glass tube assay. Varying amounts of sound (FN = 300 s) and sprouted meal (FN = 171 s) were extracted in 2-ml volumes. (a) Alpha-amylase enzyme activity was measured in absorbance units (Au) at 620 nm. The dashed lines reflect the linear equations for the sound ($y = 0.2267x + 0.0244$) and sprouted ($y = 0.4301x + 0.2213$) samples. (b) Alpha-amylase enzyme activity (Au) per g meal for each reaction. Dashed horizontal bars indicate the average Au g^{-1} for the sound and for the sprouted sample. Error bars = SD, $n = 4$

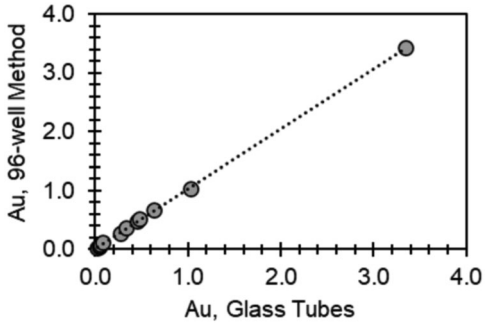

FIGURE 2 Comparison of α-amylase enzyme activity measured using the Phadebas glass tube and 96-well methods. Enzyme activity in absorbance 620 nm units was measured in samples 3–14 (Supplemental Table S1). $N = 3$, $R^2 = .98$, $r = .99$, p value ≤ .0001, and $y = 1.0213x + 0.0137$. Au, absorbance units

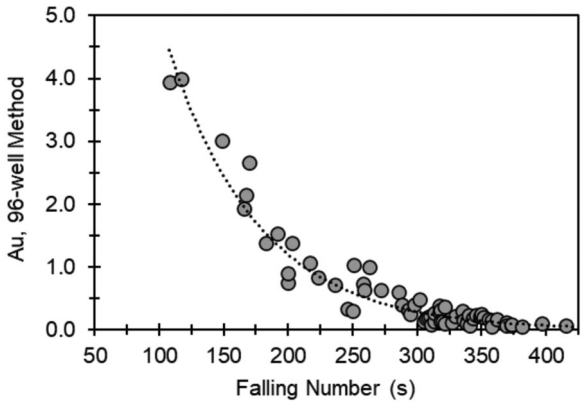

FIGURE 3 Comparison of FN and α-amylase activity (absorbance units [Au] at 620 nm) measured using the Phadebas 96-well method for 65 soft white wheat lines (Supplemental Table S7). $R^2 = .9434$, $r = -.85$, p value ≤ .0001, and $y = 20.68e^{-0.014x}$

different gram quantities per assay are used, enzyme activity should be expressed in Au g^{-1}.

A comparison was made between the enzyme activity of field samples 3–14 with FN ranging from 140 to 339 s measured using the glass tube and the 96-well Phadebas methods (Supplemental Table S1; Supplemental Table S5; Figure 2). Three independent field replicates and two technical replicates were assayed for each cultivar. The enzymatic activity assayed using the 96-well Phadebas method was highly correlated with that determined by the glass tube method based on Pearson's correlation ($r = .99$; p value ≤ .0001). Further, Tukey's pairwise comparison found no significant difference between enzyme activity measured by the two methods (Supplemental Figure S4; Supplemental Table S6).

The relationship of FN and α-amylase activity was examined using a larger set of 65 samples that had been subjected to LMA induction in the field followed by the 96-well Phadebas assay (Supplemental Table S7). Whereas the greenhouse LMA induction typical of genetic studies represents a single genotype with three to four single spikes, it was necessary to bulk-harvest 15–20 spikes in order to obtain enough grain to detect α-amylase using the FN method (Derkx et al., 2020; Farrell & Kettlewell, 2008; Liu et al., 2021b). Consistent with previous studies, FN and Au showed an exponential relationship (Figure 3; $R^2 = .73$) (Barnard et al., 2005; Graybosh et al., 1999; Liu et al., 2021a). The significant negative correlation between FN and Au ($r = -.85$; p value ≤ .0001) indicates that the 96-well method can replace FN in experiments requiring the small single-spike sample size. Given that the substrate is in suspension, variation in substrate concentration could lead to variability between independent experiments and users. To examine this, USER1 and USER2 performed the 96-well Phadebas assay using the same regents for samples 15–31 with FN ranging from 102 to 335 s (Supplemental Table S1). Each user performed the assay on three different days (replicates 1, 2, and 3). Neither user nor replication resulted in significant variation in the results (Figure 4)

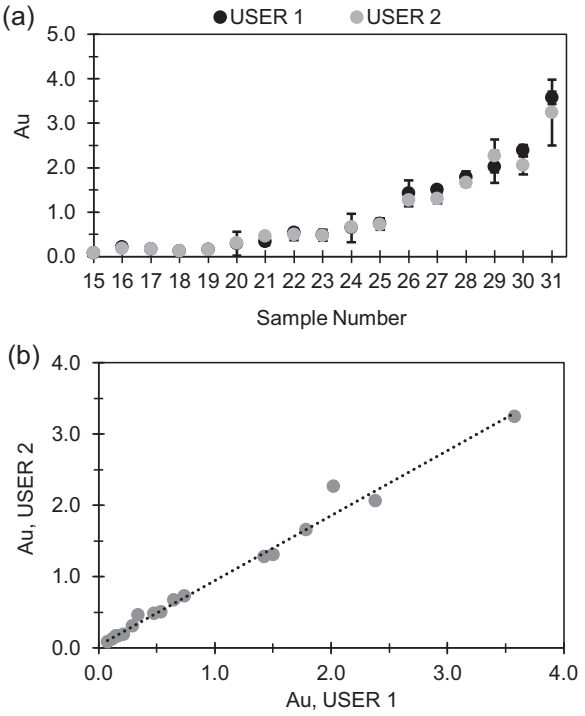

FIGURE 4 The Phadebas 96-well method performed by two users (a) for samples 15 to 31. (b) Correlation of enzyme activity measured by two independent users, $R^2 = .97$, $r = .98$, p value $\leq .0001$, $y = 0.9111x + 0.0361$. $N = 3$ for each user. Samples were milled on a Perten 3100. Au, absorbance units

suggesting that the substrate concentration is high enough to prevent variability due to the use of a suspension.

There are small differences in Au measurements made with different batches of Phadebas substrate. For this reason, experiments must use a single batch of tablets or employ a normalization method. Normalization can be achieved either using the Phadebas equation (see Methods, and also found in the package insert to calculate activity [www.phadebas.com]), or using linear regression of a standard curve (Liu et al., 2021b). The Phadebas equation calculates enzyme units per liter (units L^{-1}) using absorbance and constants A, B, and C to correct for reagent batch variation. Enzyme units L^{-1} can be converted to units g^{-1} for dry samples. Although this correction can be applied to the wheat 96-well method, it does not result in universal enzyme units per gram because the wheat assays are performed 50 ˚C instead of 37 ˚C and use longer reaction times. Future work may be able to obtain universal units by determining the relationship of enzyme activity to both temperature and reaction time. We also compensated for differences between Phadebas reagent batches using linear regression of a standard curve. The enzyme activity of six samples was measured using two different batches of Phadebas reagents (B1 and B2 in Supplemental Figure S5). The values for both substrate batches were corrected using the Phadebas equation. Then the absorbance values for B2 were corrected to units g^{-1} by linear regression using a standard curve in units g^{-1} derived using B1. Both correction approaches resulted in similar batch values. Using linear regression of a standard curve provided excellent correction of B2 vs. B1 in samples with FN of 300 s through 184 s. The Phadebas equation appeared to underestimate and linear regression overestimate activity in samples with very high α-amylase activity and low FN. If greater accuracy is needed in this range, then a nonlinear curve could be fitted to the standards. Researchers can choose the most practical method for their application.

3.2 | Comparison of the 96-well Phadebas to the Megazyme SD assay

Alpha-amylase enzyme activity measured using the Phadebas assay in Au was compared with activity measured using the Megazyme SD assay that generates α-amylase enzyme activity in SD units g^{-1}. The Megazyme SD assay is an established method for higher throughput analysis of α-amylase enzyme activity using a ChemWell-T robot (McKie & McCleary, 2015). The α-amylase activity of wholemeal samples 15–31 (FN of 102–335 s) milled on a Perten 3100 was assayed. Three independent assays were performed per sample, with two technical replicates per assay. The enzyme activity determined by both methods showed a similar negative relationship and correlation to FN (Figure 5a and 5b; $r = .98$, p value $\leq .0001$).

The 96-well Phadebas and ChemWell-T SD assays are highly correlated and provide specific, rapid, and reproducible results (Figures 3, 4, and 5b). Although both methods work well, there are several differences. The Phadebas assay appeared to have greater sensitivity and a broader linear range, especially between a FN of 225 and 300 s (Figure 5a). Hydrolysis of the Phadebas substrate produces a blue product (A_{620} nm) whereas the SD assay produces a yellow product (A405 nm). Because the SD assay product is a yellow color similar to pigments found in wheat protein extractions, it is necessary to have a separate blank for each protein extraction – especially when there is variation in red kernel color (McKie & McCleary et al., 2015). This doubles the number of reaction tubes needed. The ChemWell-T SD program automatically dilutes samples with more than 0.7 SD units g^{-1} (a FN of approximately 175 s). This is an advantage when working with a smaller number of samples but makes it challenging to keep the robot supplied with sufficient reagents and tubes when working at full capacity. To avoid wasting tubes and reagent, people performing large numbers of SD assays may need to perform an initial 96-well Phadebas assay to identify high-activity samples, so that they can be diluted with extraction buffer before performing the automated SD assay. Unlike the Phadebas assay, the Megazyme SD reagent

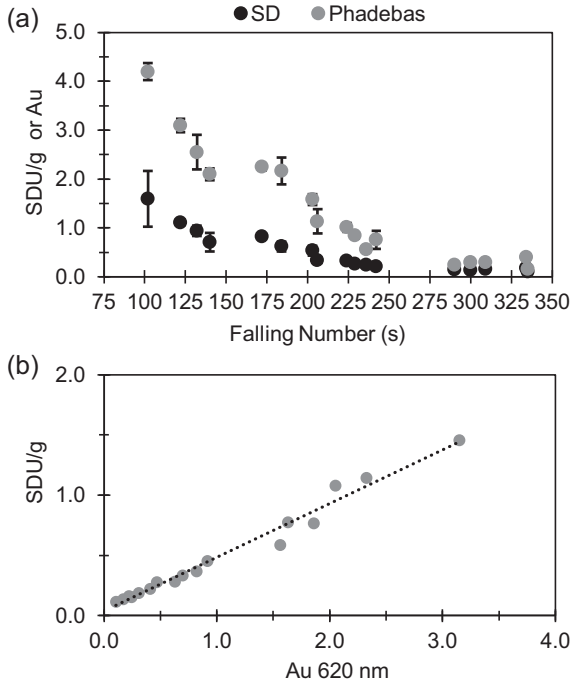

FIGURE 5 Comparison of the Phadebas and SD α-amylase enzyme assays. (a) Enzyme activity of samples 15–31 was determined using the Megazyme SD ($3.82e^{-0.011x}$) and Phadebas 96-well ($y = 16.15e^{-0.013x}$) methods. (b) Correlation between enzyme activity determined with the 96-well Phadebas and SD assays. $N = 3$, $R^2 = .96$, $r = .98$, p value $\leq .0001$, $y = 0.4467x + 0.0366$. Au, absorbance units

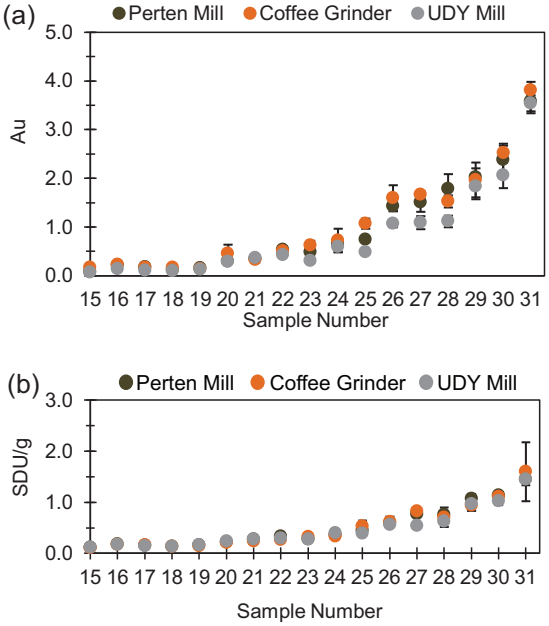

FIGURE 6 The effect of milling method on α-amylase enzyme activity measured using (a) the Phadebas 96-well and (b) the SD assay. Samples 15–31 were milled in using a Perten 3100 mill, a UDY Cyclone mill, or Krups coffee grinder. $N = 3$, error bars = SD. Au, absorbance units

is standardized, requiring no control for reagent batch variation. Moreover, the SD assay generates defined enzyme units g^{-1} and is an industry-accepted method (AACC Method 22-01.01). Prior to the pandemic, the reagents and plasticware required for the 96-well Phadebas method were considerably less expensive than those for the SD assay. However, if the current plasticware shortages persist, the cost per reaction of the two methods may be more similar.

3.3 | Comparison of sample grinding methods

High-throughput LMA and PHS testing platforms require milling of small samples (≤1 g grain) that cannot be ground in mid-capacity mills used for FN research, like the Perten 3100 (minimum 40 g grain) or the UDY Cyclone (minimum 15 g grain) (Brown et al., 2018; Martinez et al., 2018; Mrva & Mares, 2001; Sjoberg et al., 2020). Note that the industry uses 250–300 g of grain samples for FN (FGIS Directive 9180.3, 5/2019). Preliminary experiments found that small-scale mills like the Tecator Cemotec 1090 and a Fritsch Ball mill resulted in inconsistent and coarsely ground samples, whereas a Krups coffee grinder adapted for smaller samples (Supplemental Figure S1) gave finely ground samples. Thus, the use of the coffee grinder was further investigated. The enzyme activity of small samples ground in the coffee grinder was similar to the activity measured when larger samples were processed in Perten 3100 or UDY Cyclone mills. Samples 15–31 were ground in a Perten 3100, a UDY Cyclone, or a modified Krups F203 blade-type coffee grinder (Supplemental Figure S1; Supplemental Table S1), and α-amylase enzyme activity measured using the 96-well Phadebas and SD assays (Figure 6a and 6b). The ANOVA of Phadebas assays only found a small, but significant difference between coffee grinder milled and other methods in sample 25, whereas the enzyme activity in the UDY-milled samples 26–28 were significantly lower than in either the coffee grinder or Perten-milled samples (Figure 6a, p value $\leq .05$). The ANOVA of SD assays found a significantly lower enzyme activity only in UDY-milled sample 27 compared with coffee and Perten milled samples (Figure 6b, p value $\leq .05$). These results suggest that the coffee grinder is an acceptable method for processing small samples. Moreover, these results suggest that the Phadebas assay is more sensitive to small differences resulting from the milling method. Variation between milling methods may result from differences in particle size or in temperature during the milling process (Doblado-Maldonado et al., 2012).

Pearson's correlations demonstrated that enzyme activity measured both by Phadebas and SD assay was highly correlated regardless of milling method (Supplemental Figures S6

and S7). Finally, coffee-grinder samples showed a relationship to FN similar to that observed with Perten-milled samples in Figure 4 (Supplemental Figure S8A, 8B, $r = .99$, p value $\leq .0001$). Thus, a coffee grinder can process the small grain samples typical of greenhouse PHS and LMA experiments without introducing serious error into α-amylase measurements (Brown et al., 2018; Liu et al., 2021b). In addition to enabling the use of small samples, the coffee grinder is inexpensive and portable. The use of multiple grinders at once allows time for cooling and cleaning/drying between samples. A disadvantage to the coffee grinder is the lack of control over particle size, the blade may dull over time, possibly leading to larger or more variable particle size. To avoid this, we recommend use of a single model of coffee grinder and annual replacement. When larger samples are available, the Perten 3100 mill may be a better choice.

4 | CONCLUSIONS

Both the novel 96-well Phadebas and the published ChemWell-T SD assays examined here provide rapid and sensitive methods to detect elevated kernel α-amylase from PHS and LMA events, and hence, are a useful tool for breeding and genetics. The use of a coffee grinder makes it possible to process small samples without a significant change in the activity measured. Detailed methods and practical considerations for method choice have been evaluated and are provided.

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AUTHOR CONTRIBUTIONS

Amber L. Hauvermale: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – original draft; Writing – review & editing. Rehana S. Parveen: Data curation; Investigation; Methodology; Validation. Tracy J. Harris: Conceptulization; Data curation; Formal analysis; Methodology; Validation; Writing – original draft; Writing – review & editing. Keiko M. Tuttle: Conceptualization; Methodology; Writing – review & editing. Galina Mikhaylenko: Investigation; Methodology; Writing – review & editing. Sindhu Nair: Conceptualization; Investigation; Methodology. Andrew G. McCubbin: Methodology; Writing – review & editing. Michael O. Pumphrey: Funding acquisition; Project administration; Resources; Supervision; Writing – review & editing. Camille M. Steber: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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