Late Maturity Amylase versus Pre-Harvest Sprouting - differentiation through enzyme activity testing

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INTRODUCTION

The primary quality assessor of wheat grain produced globally is the Hagberg Falling Number (FN) method, which dates from 1960.[1] This is essentially a viscometric test that measures the ability of α-amylase in wheat extract to depolymerise a starch based slurry. FN results for a given wheat sample set the price paid to the grower. Unfortunately, α-amylase expression is elevated in both pre-harvest sprouted (PHS) and late-maturity amylase (LMA - primarily TaAMY1)[2] affected samples and so the FN test is unable to differentiate between these conditions. Crucially, studies suggest that unlike PHS, LMA affected wheat does not have a major impact on wheat baking properties[3] and is therefore erroneously downgraded to feed quality and penalised financially - between 2014 and 2016, LMA events cost wheat growers in the Pacific North West region ~$300m. Previous reports[4,5] have described the increase in *exo*- and *endo*-acting glycosyl hydrolases (GHs) and protease activities during sprouting and the current study describes our initial efforts to identify a selective enzyme activity marker to allow differentiation of LMA and PHS.

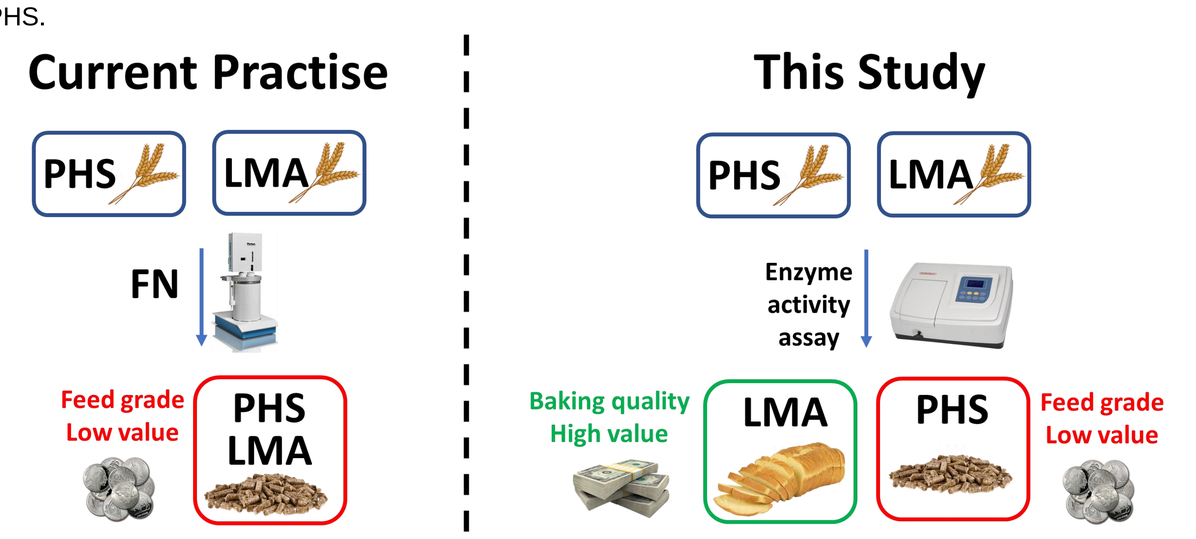

Figure 1: Standard industrial grain quality assessment practise versus the objective of the current study.

RESULTS & DISCUSSION

In phase 1, a set of 3 wheat samples were produced by CSIRO comprising 1) sprouted (FN = 62), 2) unsprouted but LMA affected (FN = 64) and 3) sound (FN = 433) wheat. The sprouted sample was first screened for measurable activities using a broad panel of enzyme substrates following a modified version of the recently reported "one-for-all" extraction procedure[6] to reduce the substrate panel prior to screening all 3 representative wheat samples. Results are shown in Table 1.

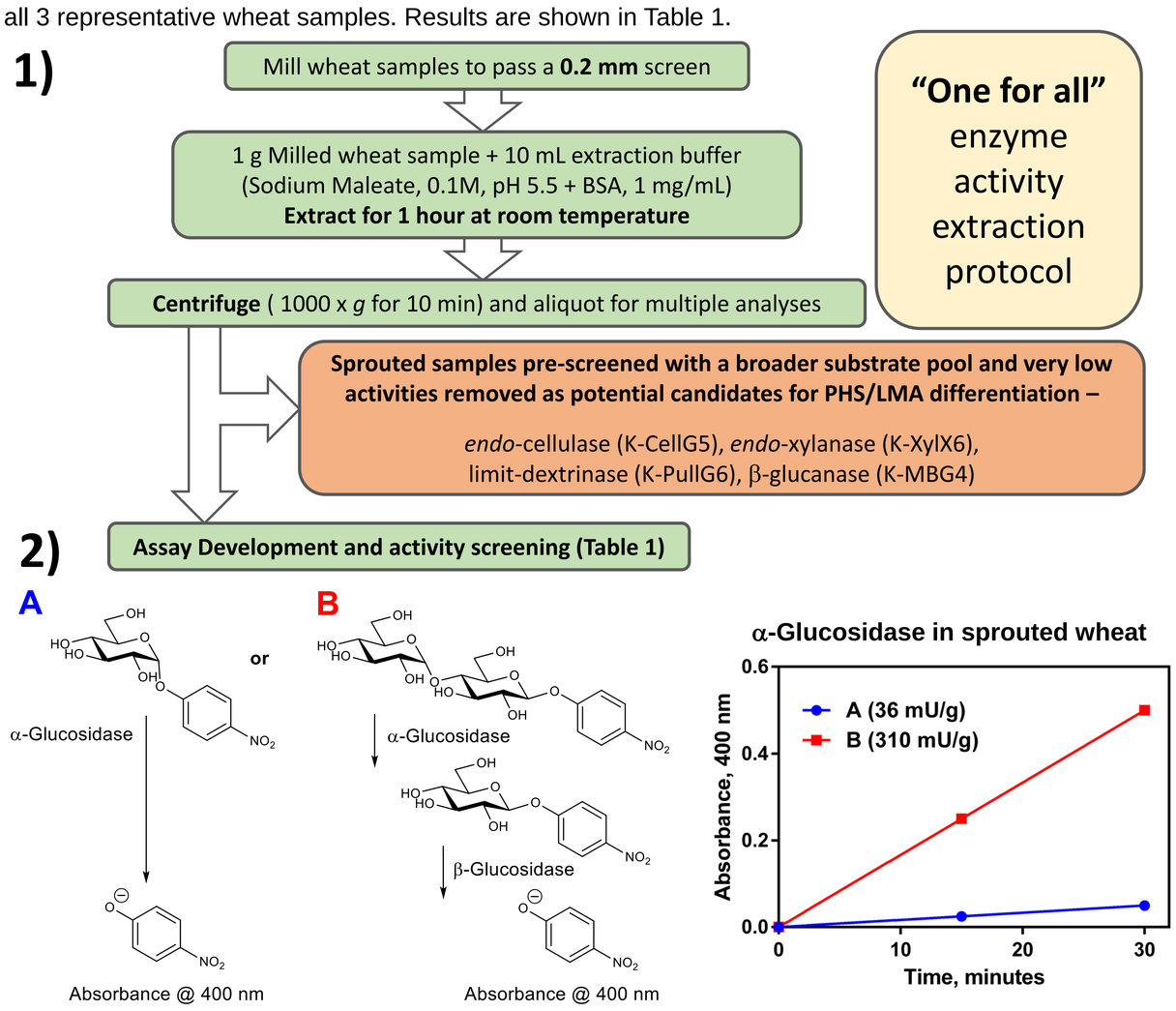

Figure 2: 1) Wheat extraction and clarification procedure. 2) Substrate preference for α-glucosidase: The commonly employed 4-nitrophenyl-α-glucopyranoside (A) was hydrolysed by wheat α-glucosidase at a rate ~9X slower than the β-glucosidase coupled substrate, R-AMGR3 (4-nitrophenyl-β-maltoside).

Megazyme's Ceralpha substrate (K-CERA) for the measurement of α-amylase and Betamyl substrate (K-BETA3) for the measurement of beta-amylase have been used in the cereals industry for over 20 years. Azocasein (S-AZCAS) is a proprietary, high-sensitivity version of the well known non-specific sulfanilamide dyed casein substrate. Ac-PLVQ-AMC is a synthetic fluorimetric substrate that was previously designed by analysing fragments produced by the triticain-α mediated hydrolysis of collagen.[7,8] α-Glucosidase was assayed using both the traditional colourimetric substrate, PNP-α-Glc, but also using an enzyme coupled substrate (R-AMGR3, see **Figure 2 B**). The latter was found to greatly improve sensitivity – a significant boon for the intended application.

	Enzyme Activity	Sample	Substrate	Activity U/g	Activity Rel. %	Sprouted/ Sound
Glycosyl Hydrolase	α-Amylase	Sprouted (FN = 62)	K-CERA	32.90	100	50
		LMA affected (FN = 64)		11.61	35	
		Sound (FN = 433)		0.56	2	
	β-Amylase	Sprouted (FN = 62)	K-BETA3	31.08	100	1
		LMA affected (FN = 64)		26.30	85	
		Sound (FN = 433)		30.96	99.6	
	α-Glucosidase	Sprouted (FN = 62)	R-AMGR3	0.327	**100**	**10**
		LMA affected (FN = 64)		0.025	**8**	
		Sound (FN = 433)		0.031	**10**	
	β-Glucosidase	Sprouted (FN = 62)	O-PNPBG	1.35	100	1.5
		LMA affected (FN = 64)		0.73	54	
		Sound (FN = 433)		0.89	66	
Protease	*endo*-Protease	Sprouted (FN = 62)	S-AZCAS	N/A	100	1.7
		LMA affected (FN = 64)			62	
		Sound (FN = 433)			58	
	Triticain-α	Sprouted (FN = 62)	Ac-PLVQ-AMC	N/A	**100**	**7.1**
		LMA affected (FN = 64)			**40**	
		Sound (FN = 433)			**14**	

Table 1: Enzyme activity screening to identify potential candidates to replace α-amylase as a selective PHS determination tool. All substrates except Ac-PLVQ-AMC were obtained from Megazyme.

In comparing the various activities assayed and reported in **Table 1**, it is evident that α-glucosidase, while not providing the same resolution for PHS versus sound samples as α-amylase, did not display the "false positive" behaviour observed with α-amylase when comparing LMA affected and sound wheat samples. In phase 2, a second sample set using the commercially popular MACE line, was produced by CSIRO to investigate the change in α-glucosidase activity over the course of germination. Triticain-α, previously identified as an upregulated protein during germination[5] was also brought forward to this second phase for further investigation and α-amylase was assayed in all samples as a control. Results are shown in **Figure 3**.

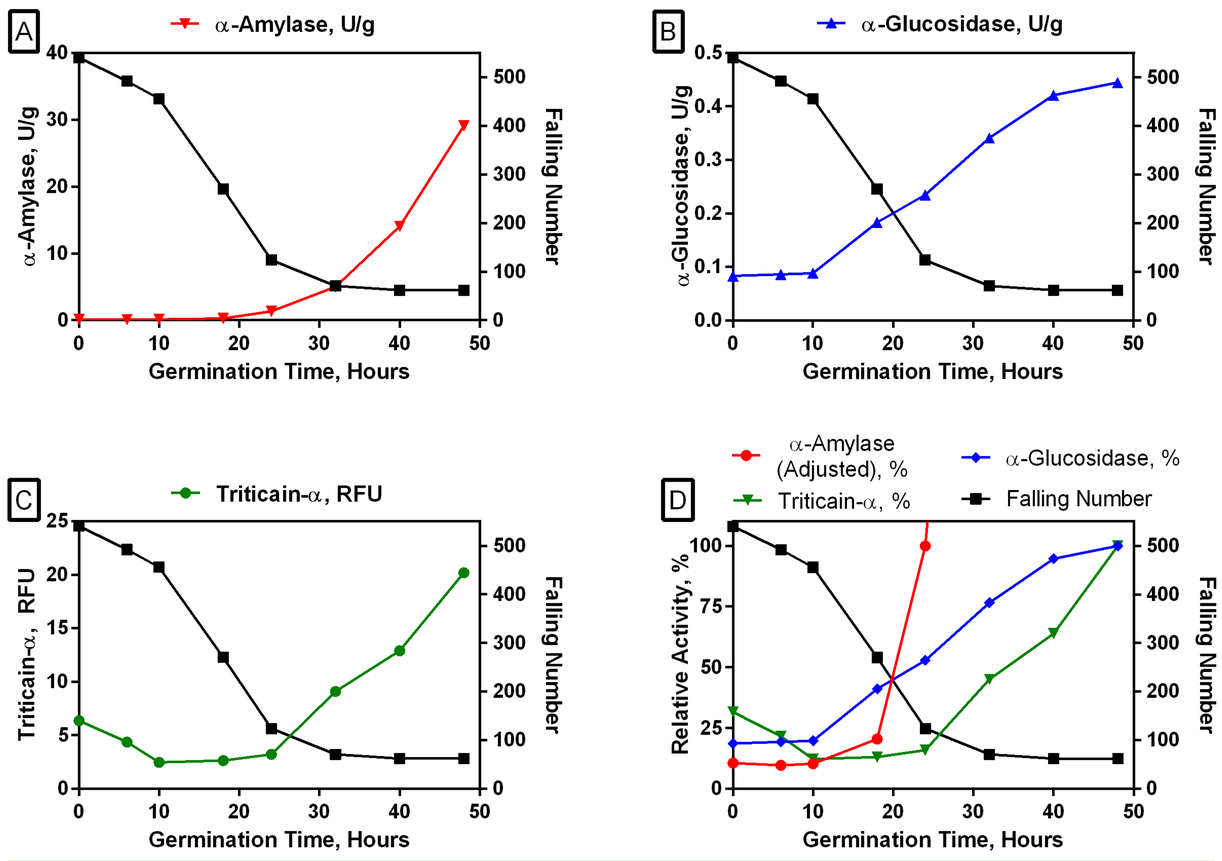

Figure 3: Observed enzyme activity as a function of time following imbibition in MACE wheat line for A) α-amylase, B) α-glucosidase and C) triticain-α. The composite activity profiles are presented in D) with the highest activity measured for each of α-amylase, α-glucosidase and triticain-α set at 100%. In the case of α-amylase (adjusted), the activity measured at 24 hours was arbitrarily set at 100% in order to allow for comparison at similar baseline activity levels across the three enzymes.

Figure 3B/D shows the detectable increase in α-glucosidase activity from 18 hours post germination. Whether the differentiation afforded by this increased enzyme activity versus the baseline activity in sound wheat can compete with the FN method for the purpose of grain quality assessment needs to be further investigated.

CONCLUSION

- ✓ **The FN test (measuring α-amylase activity) is not an optimal metric for wheat grain quality due to its inability to differentiate PHS from LMA affected wheat, costing US growers ~$300m in 2014-2016**
- ✓ **α-Glucosidase is significantly expressed in PHS affected but not in LMA affected wheat, making it a potentially useful wheat quality metric**
- ✓ **The enzyme-coupled assay substrate R-AMGR3 allows for the sensitive assay of α-glucosidase**
- ✓ **Triticain-α lags α-glucosidase expression during germination and is a less valuable indicator**

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