Evaluating Sugar and Enzyme Accumulation Patterns in Laboratory Germinated and Micromalted Barley Grains

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ABSTRACT

Grains of the malting barley cultivar Legacy were micromalted (MM) or laboratory germinated (LG) and sampled daily from 0 to 5 days after imbibition/steeping (DAI). α-Amylase and β-amylase activities and protein levels along with starch, osmolyte concentration (OC), and sugar (glucose, sucrose, fructose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) concentrations were determined. MM grains had a larger increase in α-amylase activity than LG grains earlier in germination. Additionally, MM grains had higher activity at every germination stage. Two α-amylase isoforms were detected in LG and MM grains. In LG grains, the larger isoform was the predominant isoform early in germination and as germination proceeded was degraded to the smaller isoform. In MM grains, the smaller isoform was the predominant form and increased as germination proceeded. β-Amylase activity remained constant throughout both LG and MM germination procedures. However, LG grain β-amylase underwent more proteolytic processing than MM grains. Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, total sugars, and OC all accumulated one day later in LG grains. Differences in imbibition/steeping time appear to explain the delay in sugar accumulation.

INTRODUCTION

One of the reasons quiescent barley grain is malted is to induce the production of starch degrading hydrolytic enzymes. Three of the four main starch degrading enzymes in the endosperm are synthesized *de novo* during the germination stage of malting. α-Amylase, α-glucosidase, and limit dextrinase are all synthesized *de novo*. α-Amylase is an endohydrolase that hydrolyzes α-1,4-D-glucosidic bonds in starch and other maltodextrins and can attack native starch granules alone. Starch degradation by α-amylase produces substrates for α-glucosidase, β-amylase, and limit dextrinase. Expression of α-amylase in the germinating barley grain has been extensively studied. Briefly, upon imbibition the embryo begins to produce gibberellic acid, which migrates to the aleurone layer and stimulates the expression of α-amylase and other catabolic enzymes. α-Amylase is then secreted into the starchy endosperm and begins hydrolyzing starch.

Barley endosperm-specific β-amylase (*Bmy1*) is not synthesized *de novo* during germination like α-amylase, α-glucosidase, and limit dextrinase but is expressed and stored during the development of the barley grain and is found in the quiescent grain in two states: bound (insoluble) and free (soluble) (1-7). β-Amylase is bound to the periphery of starch granules during the desiccation phase (free to bound) (2). In the quiescent grain, between 45 and 60 percent of β-amylase is bound (i.e. insoluble) (8,9).

The release of β-amylase from bound to free (insoluble to soluble) occurs during the germination stage of malting. Bound β-amylase must be released from the bound form before becoming fully active. The majority of the bound β-amylase is released between 1 and 3 days of germination (9). The release of β-amylase is accomplished *in planta* by cysteine endoproteinases, such as MEP-1 (malt endopeptidase), and can be released *in vitro* with reducing agents such as DTT or β-mercaptoethanol (8-11). β-Amylase released by the cysteine protease papain results in a 5 kDa removal at the C-terminus (9). Lundgard and Svensson (12) have reported that the proteolytic cleavage of β-amylase occurs in a stepwise manner beginning at the C-terminal end. They identified four major β-amylase forms, which are formed by the degradation of the full protein. The degradation occurs in a stepwise fashion yielding intermediate isoforms ranging from the full 60 kDa protein to the predominant 54 kDa isoform.

β-Amylase is very important to the malting and brewing industry. β-Amylase hydrolytically cleaves α-1,4-D-gluosidic bonds from the reducing end of a variety of maltodextrins (polymers of three and up) releasing β-maltose. It has the highest activity of the four predominant starch degrading enzymes (α-amylase, α-glucosidase, β-amylase, and limit dextrinase) involved in fermentable sugar production during malting and mashing (13-15). β-Amylase has been shown to be the primary contributor to diastatic power (DP) and is positively and highly correlated with DP (15-18).

This research was conducted to identify differences between the accumulation of sugars and two enzymes that are imperative to the production of sugars and to determine if grains treated to a laboratory germinated procedure would be as effective at studying malting quality as the more common method of micromalting grains.

RESULTS & DISCUSSION

Malting is the controlled germination of barley grains such that sufficient enzymes are produced and the grain is adequately modified without excessive malting loss (i.e. seedling growth). Industrial malting uses numerous methods to accomplish this. One historical method that is still in practice is the labor-intensive floor malting. Researchers interested in studying the malting process and/or germination must rely on laboratory protocols to imitate malting or germination. One common method is micromalting (MM), which uses large specialized equipment, such as the Joe White Micromalting system, which is used at the Cereal Crops Research Unit in Madison, WI for malting samples from U.S. barley breeders. However, the MM method requires a lot of sample (60 g) and is generally not available to most researchers. Laboratory germination (LG) does not require specialized equipment and can be accomplished in most labs with an incubator, Petri dish, germination paper, and water. Most of germination dogma has been elucidated using one laboratory method or another and this may pose a problem when trying to extrapolate laboratory germinated data to the malting world. Our research aimed to determine how much difference there was between a common laboratory germination procedure and a micromalting procedure commonly performed in the U.S.

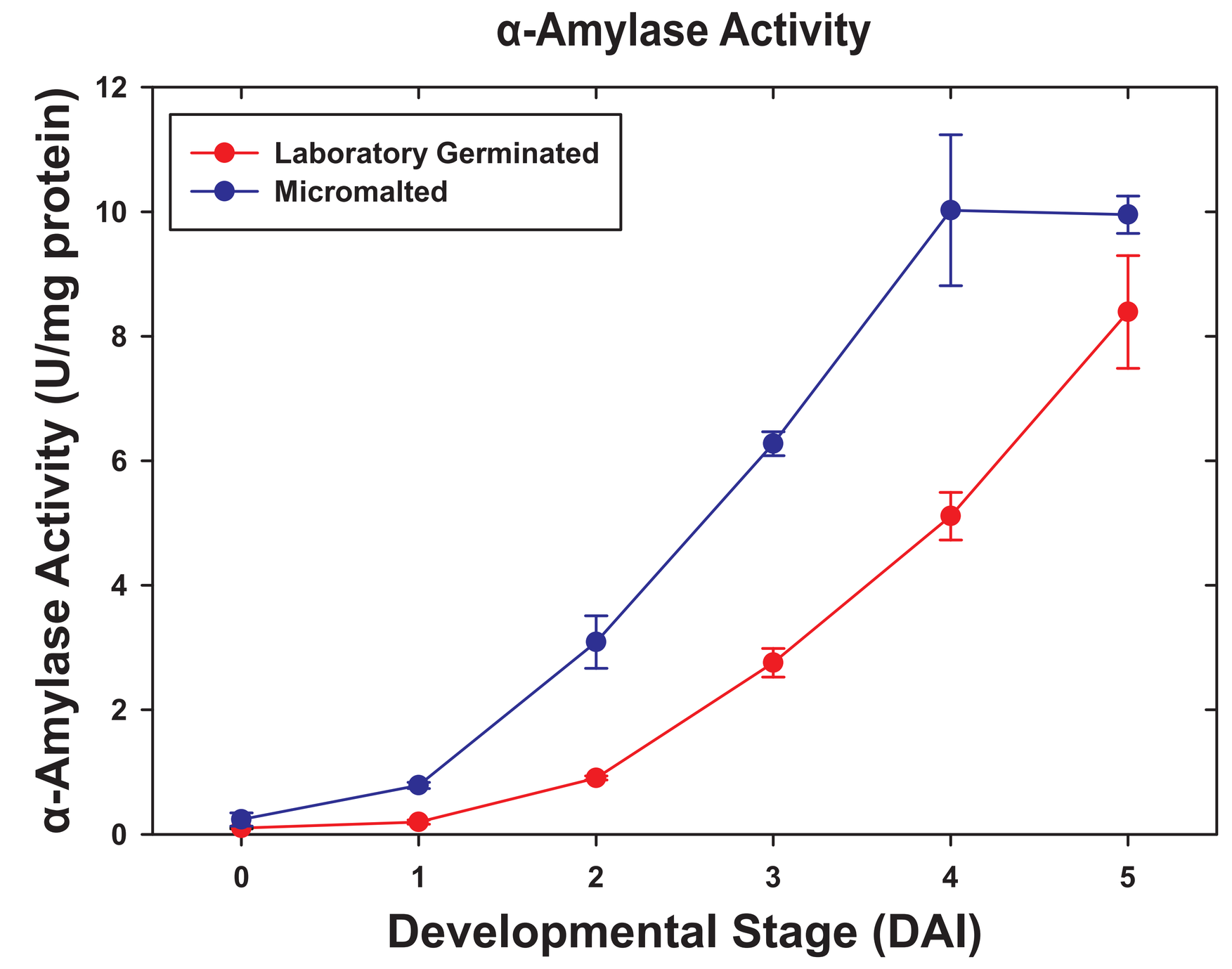

Figure 1: α-Amylase activity (U/mg protein) in laboratory germinated (LG) and micromalted (MM) grains at 0-5 days after imbibition/steeping (DAI). Error bars represent standard error.

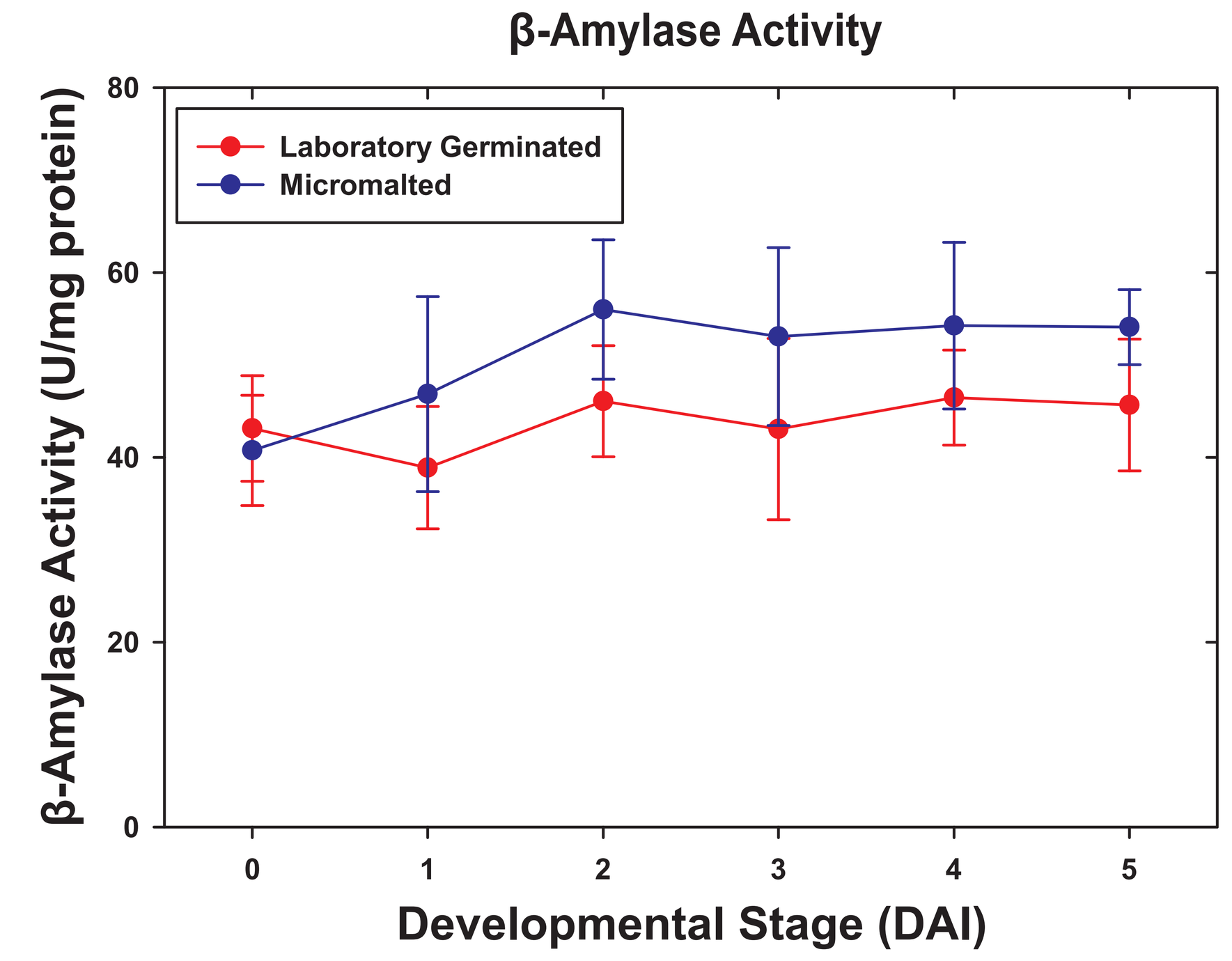

Figure 2: β-Amylase activity (U/mg protein) in laboratory germinated (LG) and micromalted (MM) grains at 0-5 days after imbibition/steeping (DAI). Error bars represent standard error.

Synthesis and processing of enzymes occur at different rates in LG and MM grains. α-Amylase activity is consistently lower in LG grains than MM grains until 5 DAI (Figure 1) and appearance of the α-amylase protein at appreciable levels occurs earlier in MM grains (Figure 3B and C). β-Amylase activity levels are higher in MM grains than LG grains although not significantly different at any of the germination stages (Figure 2). β-Amylase is not synthesized *de novo* during germination but is accumulated and stored in the developing grain (1,7). β-Amylase is processed during germination to fully release and activate the insoluble fraction (8-12). β-Amylase processing occurs more rapidly in LG grains than MM grains (Figure 3A). LG grains lose the entire C-terminal region containing the epitope used to create the antibodies by 4 DAI, whereas MM grains continue to have small amounts of β-amylase that has not undergone proteolytic loss of the epitope in the C-terminus. LG grain α-amylase also undergoes more intense processing than MM grains. In LG grains, α-amylase first appears as a ~50kDa protein before being degraded to form and accumulate the ~40 kDa isoform, which is the predominant form in MM grains. The discrepancy in α-amylase processing could be the reason for the discrepancy in α-amylase activity levels. MM grains have higher α-amylase activity from 0-4 DAI but by 5 DAI the activity levels are not significantly different. Interestingly, the ~40 kDa α-amylase isoform is the predominant isoform in MM grains whereas the ~40 kDA isoform appears later in germination in LG grains and is the predominant isoform at 5 DAI, which is the same time that the α-amylase activity levels catch up to MM grains.

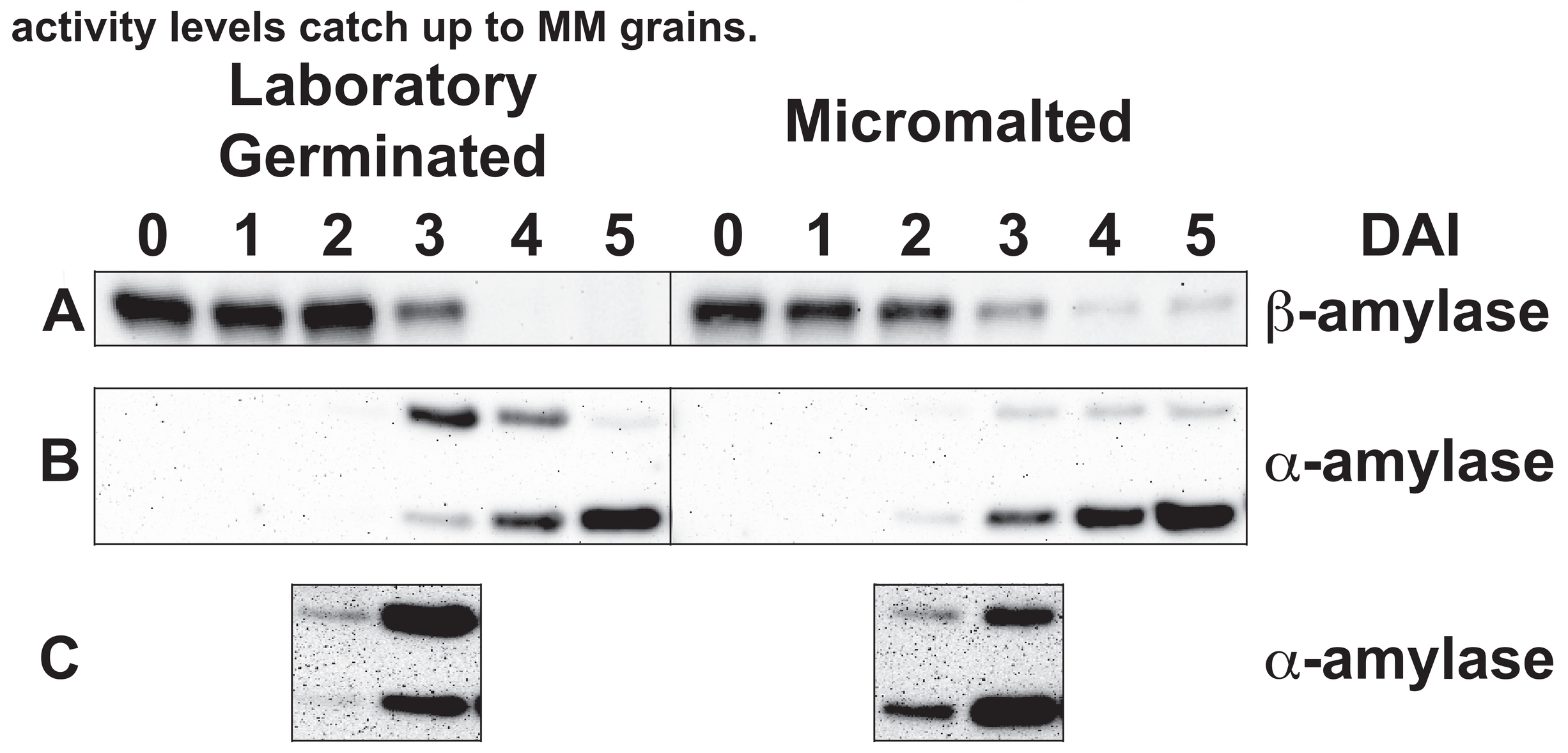

Figure 3: Immunoblot of crude protein extract from laboratory germinated (LG) and micromalted (MM) grains at 0-5 days after imbibition/steeping (DAI). Each sample lane contains 5 µg of total protein. A.) β-Amylase immunoblot B.) α-Amylase immunoblot C.) Overexposed image of 2 and 3 DAI from the same immunoblot shown in panel B.

Accumulation of maltoheptaose, fructose, and glucose along with starch degradation (0-4 DAI) all occur in a similar manner in both LG and MM grains (Data not shown and Figure 6) although the majority of sugar accumulation begins earlier in MM grains. Maltoheptaose and fructose occur in very small amounts in both LG and MM grains and were not present in early germination (Fructose) or later germination (Maltoheptaose). Glucose levels are higher in LG grains later in germination (3-5 DAI), albeit not significantly higher (Figure 6). LG grains have substantially more shoot and root growth than MM grains. The shoots and roots were removed from the LG grains near the embryo. However, Ishida et al. (19) did not observe any sugars farther than 7 mm away from the embryo. It is possible that a few mm of the shoots or roots were still present after dissection. Perhaps more sucrose is processed into glucose for the growing embryonic axis in LG grains causing slightly higher levels of glucose. Sucrose levels are higher in MM grains from 2 to 5 DAI despite LG grains needing more sucrose as the transport sugar for the growing seedling. More processing of sucrose in LG grains for the growing seedling would explain the higher glucose levels and lower sucrose levels in LG grains. Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and total sugars all begin to increase in accumulation one day earlier in MM grains than in LG grains (Figure 6). The larger chain maltodextrins exhibit a pronounced one day delay in accumulation (Figure 6). The delayed accumulation of sugars is most likely caused by the delay in enzyme synthesis and difference in enzyme processing, which in turn appears to be caused by the difference in imbibition/steeping time.

Malting consists of steeping, germination, and drying. Germination, as defined by seed physiologists, is a different term than malting germination. The emergence of the embryonic axis from the seed coat is the strict definition of germination, whereas malting germination is where modification of the barley grain occurs. In malting, when the grain is done steeping the barley is usually "chitted," meaning the rootlets have emerged. Therefore, barley grains are technically already germinated when it reaches the germination stage. Laboratory germination consists of imbibition and incubation. Common imbibition times are between 3 and 4 hours, whereas steeping times range from 24 to 48 hours. Our micromalting procedure used a 24-hour steep. The American Society of Brewing Chemists and European Brewing Convention germination energy methods do not contain an imbibition step.

The biggest difference between LG and MM grains occurred during the imbibition/steeping step. MM grains at 0 DAI had already chitted (i.e. germinated) whereas LG grains did not germinate until 2 DAI. Kleinwächter *et al.* (20) compared four malting/steeping procedures and

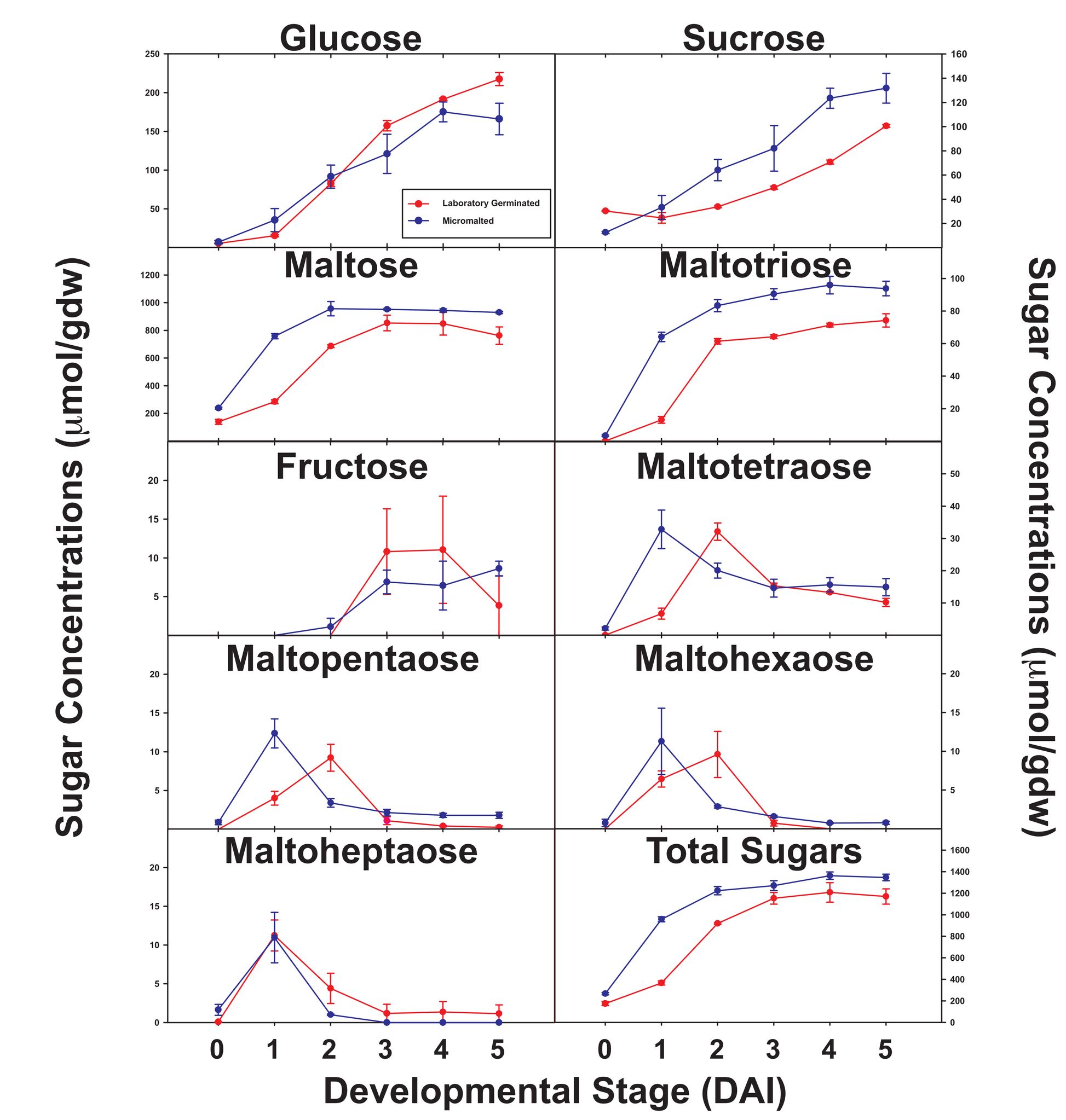

Figure 6: Sugar concentrations from laboratory germinated (LG) and micromalted (MM) grains at 0-5 days after imbibition/steeping (DAI). Error bars represent standard error.

determined the most important step is steeping. Steep aeration, similar to the steeping step the MM grains underwent, is known to induce quick germination and enhanced modification but can be considered undesirable as it is known to increase malting loss (21). Kleinwächter *et al.* (20) observed no difference between β-amylase activity similar to this research despite differences in malting procedure or germination progression. Researchers should be cautious when extrapolating germination data from non-malting researchers. The process is very similar but the stages may be delayed due to differences in steeping/imbibition. Schmitt *et al.* (22) compared LG grains to MM grains in a recent report and found similar gene expression profiles which is most likely explained by their 8 h imbibition step in their LG protocol. If malting researchers do not have access to a micromalter or commercial maltster then extreme care should be employed during the imbibition step to best represent the micromalter or commercial malting treatment.

MATERIALS & METHODS

Each germination treatment was replicated three times using three different biological repetitions of the malting barley cultivar Legacy. The USDA-ARS Cereal Crops Research Unit produced the MM grains by following their standard procedure. For LG grains, four milliliters of MilliQ water was added to each Petri dish. Petri dishes were sealed with Parafilm and placed in the dark at 20°C. Grains were sampled after steeping/imbibition and each subsequent day after (0-5 DAI).

CelLytic P and a protease inhibitor cocktail were used to prepare crude extracts. Crude extracts were dialyzed (MWCO 2.5-5 kDa).

Coomassie Plus Assay Reagent was used to determine the protein concentrations following the manufacturer's instructions.

β-Amylase and α-amylase assays were conducted using the Betamyl and Ceralpha Method following the manufacturer's protocol.

Sugars were extracted in five volumes of water for 30 min at 70°C. Sugars were determined using a method described by Duke *et al.* (23).

Polyclonal α-amylase antibodies were purchased from Agrisera. Antibodies were created using an α-amylase from *Oryza sativa* and are reported to detect all α-amylase isoforms from rice, barley, and other cereals.

Polyclonal β-amylase antibodies were created using a short peptide sequence from the C-terminal end of "endosperm-specific" β-amylase and is gene specific (6).

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