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Article

Proteomics and Metabolomics Reveal that an Abundant α-Glucosidase Drives Sorghum Fermentability for Beer Brewing

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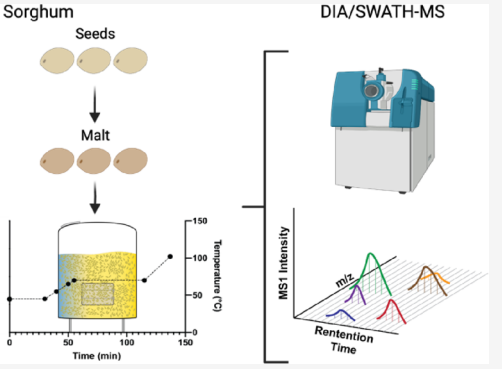
ABSTRACT: Sorghum (*Sorghum bicolor*), a grass native to Africa, is a popular alternative to barley for brewing beer. The importance of sorghum to beer brewing is increasing because it is a naturally gluten-free cereal, and climate change is expected to cause a reduction in the production of barley over the coming decades. However, there are challenges associated with the use of sorghum instead of barley in beer brewing. Here, we used proteomics and metabolomics to gain insights into the sorghum brewing process to advise processes for efficient beer production from sorghum. We found that during malting, sorghum synthesizes the amylases and proteases necessary for brewing. Proteomics revealed that mashing with sorghum malt required higher temperatures than barley malt for efficient protein solubilization. Both α- and β-amylase were considerably less abundant in sorghum wort than in barley wort, correlating with lower maltose concentrations in sorghum wort. However, metabolomics revealed higher glucose concentrations in sorghum wort than in barley wort, consistent with the presence of an abundant α-glucosidase detected by proteomics in sorghum malt. Our results indicate that sorghum can be a viable grain for industrial fermented beverage production, but that its use requires careful process optimization for efficient production of fermentable wort and high-quality beer.



KEYWORDS: *sorghum, barley, malting, mashing, alpha-glucosidase, fermentation, beer, proteomics, metabolomics*

INTRODUCTION

Beer is one of the most popular beverages worldwide, with ∼1.95 billion hectoliters produced annually.[1] Typically, beer is made from malted barley seeds (*Hordeum vulgare*L. subsp. *vulgare*). While barley is central to current industrial brewing, sorghum (*Sorghum bicolor*), a grass native to Africa and the fifth most produced cereal worldwide,[2] is a popular alternative to barley for brewing for several reasons. Fermented beverages produced from sorghum can have a different flavor profile to those made from grains such as barley. Depending on its eventual severity, climate change over the coming decades is expected to cause a reduction in the production of barley by 3−17% globally.[3] This will have severe ramifications for beer production, price, and consumption. Sorghum is adapted to hot, drought-prone, semiarid environments with low rainfall, making it well-suited for a warming climate. Global production of sorghum is therefore set to increase, making it an interesting alternative grain to barley for beer production.[4] In addition, barley, wheat, rye, and oats are generally classified as gluten-containing cereals due to their prolamin storage proteins. Celiac disease is caused by an inappropriate immune response triggered by the ingestion of gluten. Approximately 1% of the global population suffers from celiac disease,[5,6] with this number increasing.[7,8] Many people are also gluten intolerant or choose to avoid gluten. Sorghum is inherently a gluten-free cereal, as its kafirin (prolamin) storage proteins do not trigger an immune response as in celiac disease.

In many African countries, sorghum is a staple food and is the main grain used to make fermented beverages.[9] However, there are substantial shortfalls in the suitability of sorghum compared to barley for beer production. For the malting and brewing industries, the differences between sorghum and barley start at malting. Barley seeds are malted in three stages: steeping, germination, and kilning. Steeping is the process of repeatedly soaking the grains followed by periods of air-rest to drain the water, usually at 14−16 °C over 2−4 days,[10] which increases grain moisture and stimulates metabolic activity. Excess water is removed and germination commences, allowing the synthesis of starch- and protein-degrading enzymes, usually over 3−6 days between 16 and 20 °C.[10,11] Seeds are then kilned to prevent excessive degradation of nutrients by the newly synthesized enzymes.[12−16] Malting of sorghum requires different conditions than barley, and malting parameters can

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impact sorghum malt quality. Amylases and proteases are lower in abundance in sorghum malt compared to barley malt,[9,17] but malting conditions can overcome these limitations. For example, in sorghum malting, lowering the degree of steeping (the percentage moisture content of the grain) to 41% compared to the standard ∼45% has been shown to increase free amino nitrogen (FAN).[18,19] Barley is usually steeped for 24−48 h, while steeping sorghum for only 20 h improved α-amylase activity regardless of cultivar.[9,17,20] Barley seeds are germinated anywhere from 3 to 7 days at 17−20 °C for malt production.[20,21] Sorghum germination of 4 days at 30 °C or 5 days at 26 °C results in the highest α- and β-amylase activity.[17,19] Germination temperatures of 20 and 25 °C have also been reported to result in similar amounts of reducing sugars and fermentable extracts.[22] In summary, different malting conditions are needed for barley and sorghum, with the parameters for sorghum not yet standardized or fully optimized.

Malt is milled to open the grains and combined with warm water in the process of mashing to solubilize starch (gelatinization) and proteins (solubilization) and allow enzymes to degrade these macromolecules into fermentable sugars and FAN.[13,15,16,23] The liquid fraction, wort, is separated from the spent grain and boiled with the addition of hops (*Humulus lupulus*). This hopped wort is cooled, fermented by yeast, which utilizes the soluble and consumable nutrients, matured, packaged, and sold. Mashing with sorghum requires 68−78 °C for gelatinization, significantly higher than 51−60 °C used for barley.[18,24] The higher gelatinization temperature of sorghum is likely due to its starch polymer structure[25,26] and reduces the accessibility of enzymes to their starch substrate during mashing. Gelatinization temperature is a key reason that different mashing temperatures are used to mash sorghum malt and barley malt. The challenges of sorghum's high gelatinization temperature can be partially overcome by decantation style mashing, which involves boiling a part of the wort and adding it back to the main mash, resulting in higher extraction and fermentability than a standard infusion (single temperature) mash.[22,27] Brewing with sorghum requires optimization of the malting and mashing process parameters, and even with optimized processes, the wort produced will likely be less fermentable than barley wort.

Although sorghum proteins and starch have high solubilization temperatures, its enzymes are comparatively heat sensitive.[28] This unfortunate combination has a substantial impact on the quality of the sorghum wort. Here, we used our established workflow for brewing proteomics[29] to gain insights into the potential deficiencies in the sorghum proteome compared to barley and to guide malting and mashing process design to increase the efficiency of wort production from sorghum.

METHODS

Barley and Sorghum Malting

Micromalting was carried out in a Phoenix automated micromalting unit (Queensland Department of Agriculture, Leslie Research Facility, Toowoomba, Australia) to make fully modified malt as follows: 200 g of barley grain (cultivar: Commander; donation from Barrett Burston Malting Company) was first steeped with water for 8 h, then 8 h air rest followed by another 8 h steeping, all at 17 °C. Following steeping, germination was allowed to occur by leaving samples in the dark at 17 °C for 4 days, turning barley grains every hour to avoid hot spots. Sorghum grain (200 g; cultivar; Liberty; donation from Pacific Seeds) was steeped with water for 10 h, then 8 h air rest followed by another 8 h steeping, all at 21 °C. Following steeping, germination was allowed to occur by leaving at 21 °C for 4 days, turning sorghum grains every hour to avoid hot spots. For both barley and sorghum, germination was ceased by kilning: the grain was slowly heated to 50 °C to remove excess moisture and then further heated to 80 °C for 24 h to suspend enzyme activity and to reduce the malt moisture to around 4%.

Mash and Boil Experimental Design

A small-scale mash was performed for both barley and sorghum malt in triplicate in Schott bottles incubated using an IEC programmable mash bath (IEC Melbourne, Australia). The mash method followed the Congress mashing program: 45 °C for 30 min and 70 °C for 60 min.[30] Samples were taken at the start and end of both stages and at 55 and 65 °C. Following mashing, wort was filtered and incubated at 102 °C for 5 min after which a sample was taken.

Proteomic Sample Preparation

Seed and malt samples were prepared as previously described.[21,31] Briefly, 10 mg of barley malt, 50 mg of sorghum seeds, and 50 mg of sorghum malt were ground and solubilized in 600 μL of 50 mM Tris-HCl buffer pH 8, 6 M guanidine hydrochloride, and 10 mM dithiothreitol (DTT) and incubated at 37 °C for 30 min with shaking. Cysteines were alkylated by the addition of acrylamide to a final concentration of 30 mM and incubation at 37 °C for 1 h with shaking. Excess acrylamide was quenched by addition of an additional 10 mM DTT, samples were centrifuged at 18,000 rcf for 10 min, and proteins in 10 μL of the supernatants were precipitated by the addition of 100 μL of 1:1 methanol:acetone and incubation at −20 °C overnight. Samples taken during the mash or boil were clarified by centrifugation at 18,000 rcf for 1 min and prepared as previously described.[29] Briefly, proteins from 10 μL of barley wort or 30 μL of sorghum wort were precipitated by addition of 4 volumes 1:1 methanol/acetone and incubation overnight at −20 °C. Precipitated proteins were resuspended in 100 μL of 100 mM ammonium acetate and 10 mM DTT with 0.5 μg of trypsin (Proteomics grade, Sigma) and digested at 37 °C for 16 h with shaking.

Proteomic Mass Spectrometry

Peptides were desalted with C18 ZipTips (Millipore) and measured by data-dependent acquisition (DDA) and data-independent acquisition (DIA) LC-ESI-MS/MS using a Prominence NanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (SCIEX) as previously described.[32] In brief, peptides were separated with buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile with 0.1% formic acid) with a gradient of 10−60% buffer B over 24 min. Gas and voltage settings were adjusted as required. For DDA, an MS TOF scan from m/z of 350−1800 was performed for 0.5 s followed by DDA of MS/MS with automated CE selection of the top 20 peptides from m/z of 40−1800 for 0.05 s per spectrum. Identical LC conditions were used for DIA/SWATH-MS, with an MS-TOF scan from an m/z of 350−1800 for 0.05 s followed by high-sensitivity DIA with 34 m/z isolation windows with 1 m/z window overlap each for 0.1 s across

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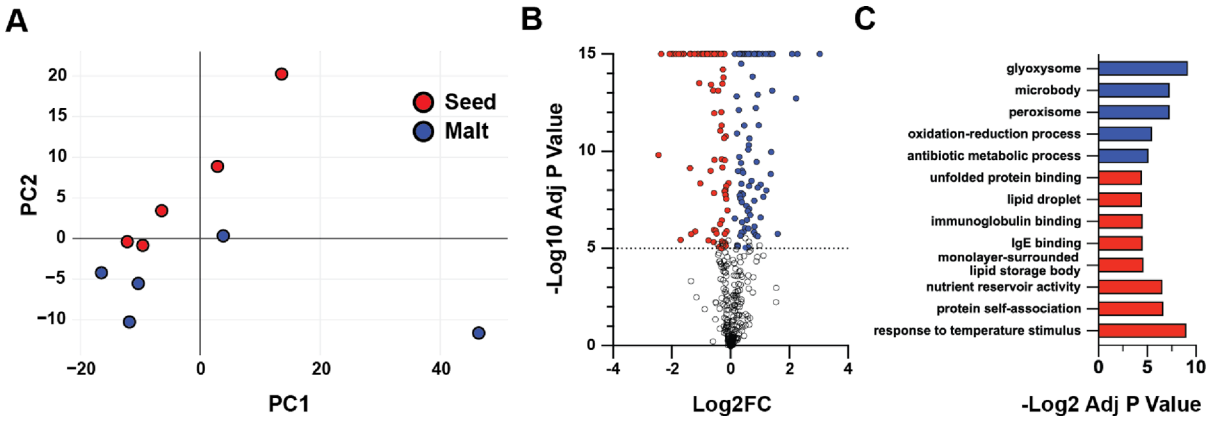

Figure 1. Impact of malting on the sorghum seed proteome. (A) PCA of protein abundance normalized to the abundance of trypsin in each sample (biological replicates). The first component (x-axis) accounted for 61.13% of the total variance and the second (y-axis) 15.30%. (B) Volcano plot of the comparison of mature sorghum seeds and sorghum malt. Gray, not significantly different; red, significantly ($p < 10^{-5}$) less abundant in malt; blue, significantly ($p < 10^{-5}$) more abundant in malt. (C) Significantly enriched GO terms for proteins with significant differences in abundance comparing mature seeds and malt. Values are shown as $-\log_2$ of Bonferroni-corrected p-values for GO terms, which were significantly enriched ($p < 0.05$) in proteins that were significantly more abundant in malt, blue or significantly less abundant in malt, red.

an m/z range of 400−1250. Collision energy was automatically assigned by the Analyst software (SCIEX) based on m/z window ranges.

Data Analysis

Peptides and proteins were identified using ProteinPilot version 5.0.1 (SCIEX). Sorghum samples were searched against all predicted proteins from *S. bicolor*v3.1.1 (PRJNA38691, downloaded 29 May 2017; 47,121 proteins), and barley samples were searched against all high-confidence proteins from transcripts from *H. vulgare*L. subsp. *vulgare* (Uniprot UP000011116, downloaded 20 December 2019; 189,799 proteins). Both search databases also included contaminant proteins. Search settings were as follows: sample type, identification; cysteine alkylation, acrylamide for grain samples and none for wort samples; instrument, TripleTof 5600; species, none; ID focus, biological modifications; enzyme, trypsin; search effort, thorough ID.

The abundance of peptide fragments, peptides, and proteins was determined using PeakView 2.2 (SCIEX) with the following settings: shared peptides, allowed; peptide confidence threshold, 99%; false discovery rate, 1%; XIC extraction window, 6 min; XIC width, 75 ppm. To make protein names human readable and to provide GO terms for downstream analysis, identified barley and sorghum proteins were matched against UniProtKB (downloaded 2 December 2017; 555,318 total entries) using BLAST+ to find the best matching annotated entry, as previously described.[21] Protein-centric analyses was performed as previously described,[33] and protein abundances were recalculated using a strict 1% FDR cutoff.[21] Normalization was performed to either the total protein abundance in each sample or to the abundance of trypsin self-digest peptides, as previously described.[29] Principal component analysis (PCA) was performed using Python, the machine learning library Scikit-learn (0.19.1), and the data visualization package Plotly (1.12.2). Protein and sample clustering was performed using Cluster 3.0,[34] implementing a hierarchical, uncentered correlation, and complete linkage. Proteins that were able to be measured in at least one sample in either mature sorghum seeds or sorghum malt, but not both, were defined as uniquely measured in that sample type. For statistical analysis, PeakView output was reformatted as previously described[21] and significant differences in protein abundance were determined using MSstats (2.4)[35] in R, with a significance threshold of $p = 10^{-5}$.[36] Gene ontology (GO) term enrichment was performed using GOstats (2.39.1)[37] in R, with a significance threshold of $p = 0.05$.[36]

Sugar and Amino Acid Quantification by Multiple Reaction Monitoring

Postboil samples were filtered, diluted 1:1000 in H_2O, and measured by multiple reaction monitoring (MRM) with UPLC-MS/MS as previously described.[38] Sugars (glucose, maltose, and maltotriose) and amino acids (L-serine, L-proline, L-valine, L-threonine, L-leucine, L-isoleucine, L-aspartic acid, L-lysine, L-glutamic acid, L-methionine, L-histidine, L-phenylalanine, L-arginine, L-tyrosine, and L-cystine) were quantified using external calibration to multipoint standard curves with R^2 values >0.99.

Fermentation Assay

Fermentation of boiled wort was performed for both sorghum and barley wort using two brewing yeast strains: US-05 (Fermentis, American ale yeast) and M20 (Mangrove Jacks, Bavarian wheat yeast). Fermentations were tracked by weight loss as previously described,[39] and samples were collected for ethanol quantification once ferments were complete.

Ethanol Quantification by Headspace GC-MS/MS

Samples were diluted 1:50 in H_2O, with 0.05% isopropyl alcohol added as an internal standard. Ethanol was quantified using external calibration to multipoint standard curves with R^2 values >0.98. Chromatography was performed with a Rxi-624Sil 3.0 μm, 30 m × 0.53 mm column (Restek). Headspace GC-MS was performed as previously described,[40] with a total run time of 20 min per sample.

Data Availability

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[41] with the data set identifier PXD034981, http://www.ebi.ac.uk/pride/archive/projects/PXD034981.

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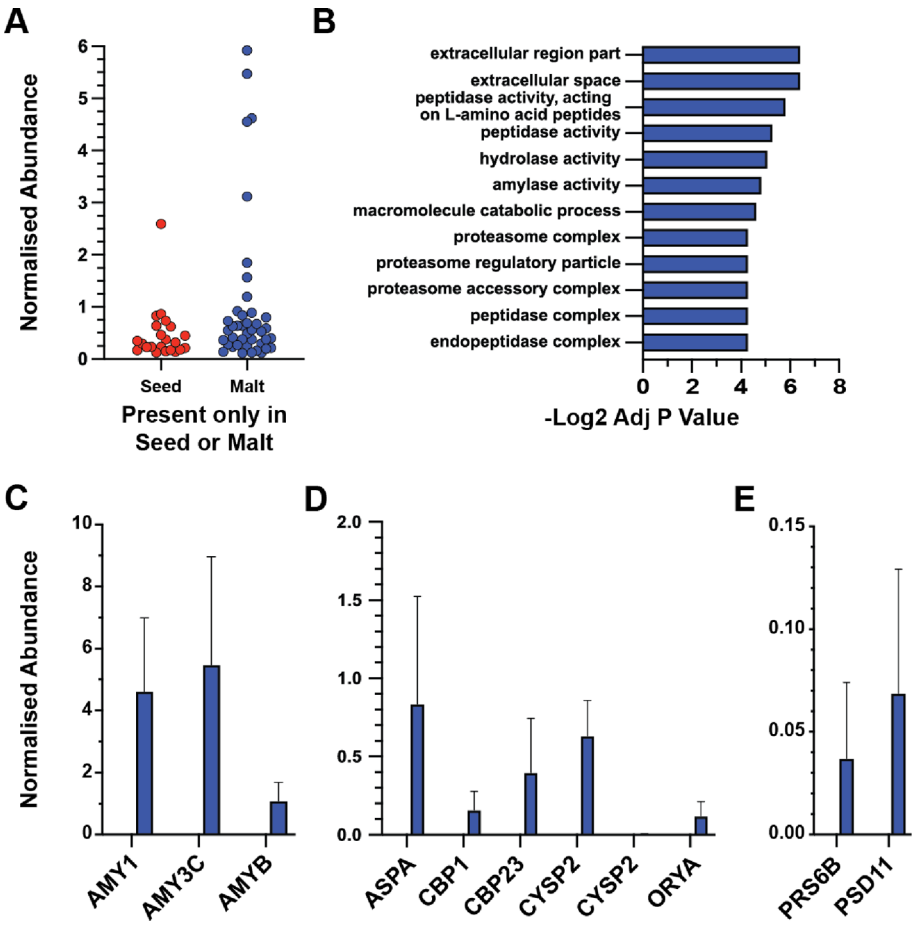

Figure 2. Sorghum germination/malting triggers synthesis of key brewing enzymes. (A) Abundance of proteins measured only in either malt or seed. Values show mean of biological replicates, $n = 3$. Protein abundance normalized to the abundance of trypsin in each sample. (B) Significantly enriched GO terms in proteins unique to malt. Values are shown as $-\log_2$ of Bonferroni-corrected p-values for GO terms, which were significantly enriched ($p < 0.05$) in proteins that were only measured in malt. No GO terms were significantly enriched in proteins unique to seed. (C) Abundance of two α-amylase proteins, AMY1 and AMY3C, and a β-amylase protein, AMYB. (D) Abundance of peptidases: aspartyl protease (ASPA), serine carboxypeptidase 1 (CBP1), serine carboxypeptidase II-3 (CBP23), two forms of cysteine proteinase EP-B 2 (CYSP2), and oryzain alpha chain (ORYA). (E) Abundance of proteasome proteins: 26S proteasome regulatory subunit 6B homologue (PRS6B) and 26S proteasome non-ATPase regulatory subunit 11 homologue (PSD11). Normalized protein abundance is relative to trypsin: values show mean, $n = 3$; error bars, SEM; seed, red (no values shown); malt, blue.

RESULTS AND DISCUSSION

Proteome of Malting Sorghum Mirrors Malting Barley

The malting process is essential to the brewing process, as it facilitates the synthesis of enzymes involved in degrading starch into fermentable sugars and protein into FAN. We aimed to investigate the biochemical changes that occur during malting of sorghum seeds with a focus on the synthesis and abundance of key proteins important for the brewing process. To achieve this, we examined the proteomes of mature sorghum seeds and sorghum malt. DDA analyses identified 583 proteins at 1% global FDR (Table S1) of which 533 proteins were quantifiable by DIA/SWATH (Table S2). Principal component analysis (PCA) of the proteomic variance revealed that the proteomes of mature seeds and malt were clearly separated, indicating substantial proteomic changes during malting (Figure 1A). We next performed statistical analysis to determine which proteins were significantly ($p < 10^{-5}$) different in abundance between sorghum seeds and sorghum malt (Figure 1B and Table S3). We found 95 proteins that were significantly more abundant and 106 that were significantly less abundant in sorghum malt than in mature seed (Figure 1B and Table S3).

Following a statistical comparison between sorghum malt and mature sorghum seeds, we performed GO term enrichment analysis on proteins that were significantly different in abundance between malt and mature seeds (Figure 1C and Table S4). A suite of GO terms was enriched in proteins significantly more abundant in sorghum malt than in mature sorghum seeds: "glyoxysome", "microbody", "peroxisome", "oxidation-reduction process", and "antibiotic metabolic process" (Figure 1C). The enrichment of "glyoxysome", "microbody", and "peroxisome" was particularly interesting as they are all involved in the conversion of stored lipids into acetyl-CoA and then carbohydrates, which is common during germination (CATA1, CYSZ, MASY, MDHG, MFP2, and THIK2; Figure S1A).[42,43] As malt is a partially germinated seed, this is consistent with an enrichment of "glyoxysome", "microbody", and "peroxisome" in the malt proteome. A set of GO terms were enriched in proteins significantly less abundant in sorghum malt than in mature sorghum seeds: "response to temperature stimulus", "protein self-association", "nutrient reservoir activity", "monolayer-surrounded lipid storage

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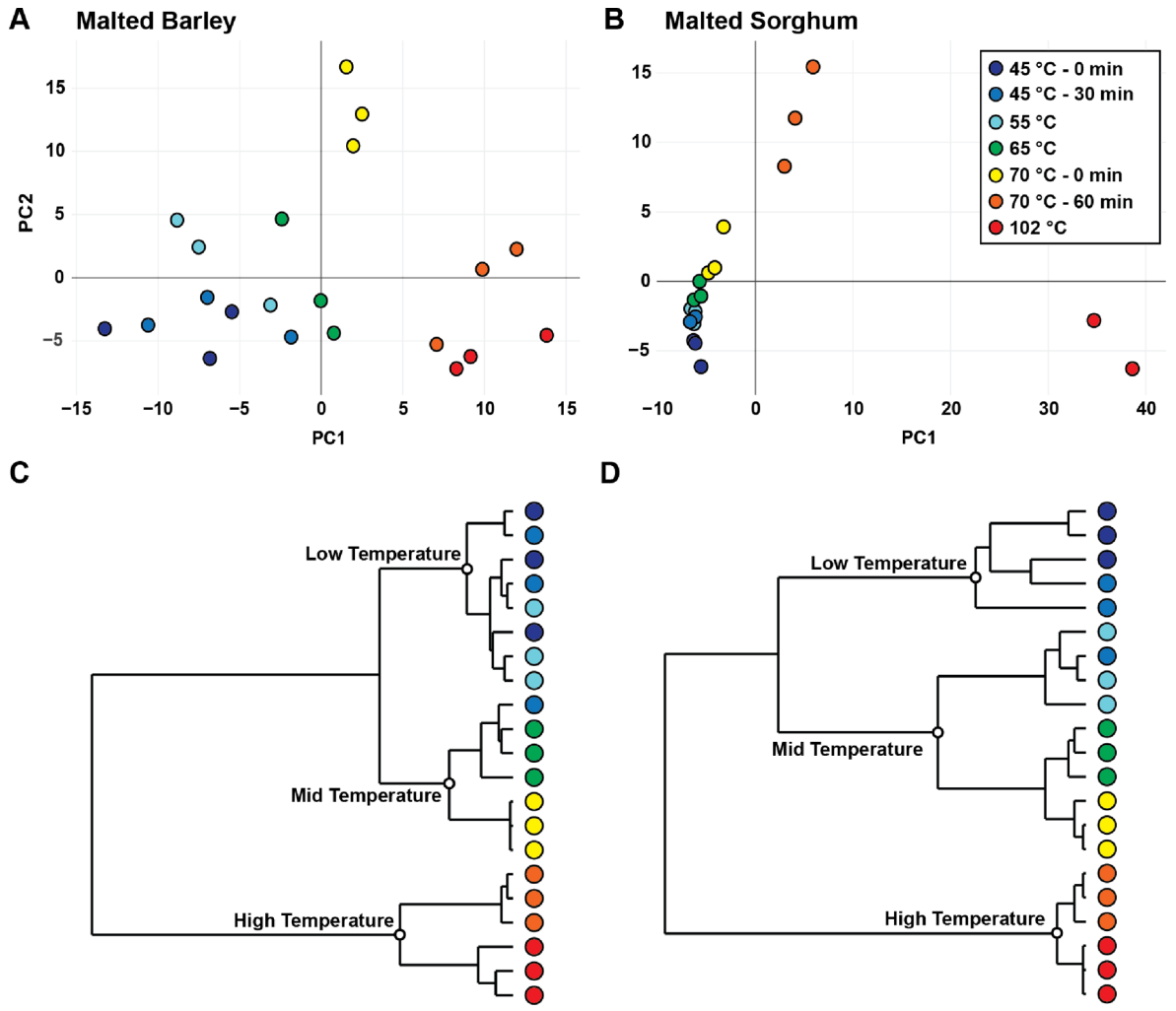

Figure 3. Delayed proteomic changes in wort in sorghum brewing compared to barley brewing. (A) PCA of barley mash and boil. The first component (*x*-axis) accounted for 36.36% of the total variance and the second (*y*-axis) for 26.28%. (B) PCA of sorghum mash and boil. The first component (*x*-axis) accounted for 47.72% of the total variance and the second (*y*-axis) for 9.13%. A single outlying data point for 102 °C has been removed from this PCA. Dendrogram of (C) barley mash and boil and (D) sorghum mash and boil. Values were calculated from protein abundances normalized to the abundance of trypsin in each sample, clustered, and shown as both a PCA and a dendrogram. Clades visually noted within the dendrograms were highlighted by white circles and labels.

body", "IgE binding", "immunoglobulin binding", lipid droplet", and "unfolded protein binding" (Figure 1C). Proteins contributing to "response to temperature stimulus", "protein self-association", and "unfolded protein binding" were heat shock proteins (HS177, HS181, HS232, HS23M, HS26P, HSP19, and HSP21) (Figure S1B), which have been previously shown to be abundant in mature barley seeds and which reduce in abundance after imbibition into germination in barley.[21] Proteins, which contributed to the GO terms "nutrient reservoir activity", "IgE binding", and "immunoglobulin binding", were seed storage proteins (11S2, CUCIN, GL19, GLB1, GLUB5, VCL21, VCL22, and ZEG1) (Figure S1C), highlighting the abundance of seed storage proteins in mature sorghum seeds ready to be hydrolyzed during germination. The reduction in abundance of seed storage proteins in sorghum malt aligns with the process of germination in barley, where seed storage proteins decrease in abundance during germination as they are digested by proteases resulting in smaller peptides and FAN.[21] Proteins that contributed to "lipid droplet" and "monolayer-surrounded lipid storage body" GO terms were two oleosin proteins (OLE16 and OLEO3), an oil body-associated protein 1A (OBP1A), and a peroxygenase (PXG) (Figure S1D). Oleosins are known to help regulate oil body size and localization,[44] and oil body-associated proteins are known to be involved in stabilizing lipid bodies during desiccation,[45] while peroxygenases are involved in the degradation of storage lipid in oil bodies during germination.[46]

We found that some proteins were only able to be measured in either mature sorghum seeds or sorghum malt but not both, indicating a dramatic change in protein abundance as a result of malting (indicated by "NA", Table S3). Twenty-two proteins were unique to mature sorghum seeds, and 37 proteins were unique to sorghum malt (Figure 2A). We performed GO term enrichment on these groups of uniquely measured proteins and found several GO terms enriched in proteins only present in sorghum malt (Figure 2B). The most notable of these GO terms was "amylase activity", which was contributed by two α-amylases (AMY1 and AMY3C) and a β-amylase (AMYB). These amylases were not measured in mature seeds but were highly abundant in malt, consistent with their synthesis during the malting process (Figure 2C). The

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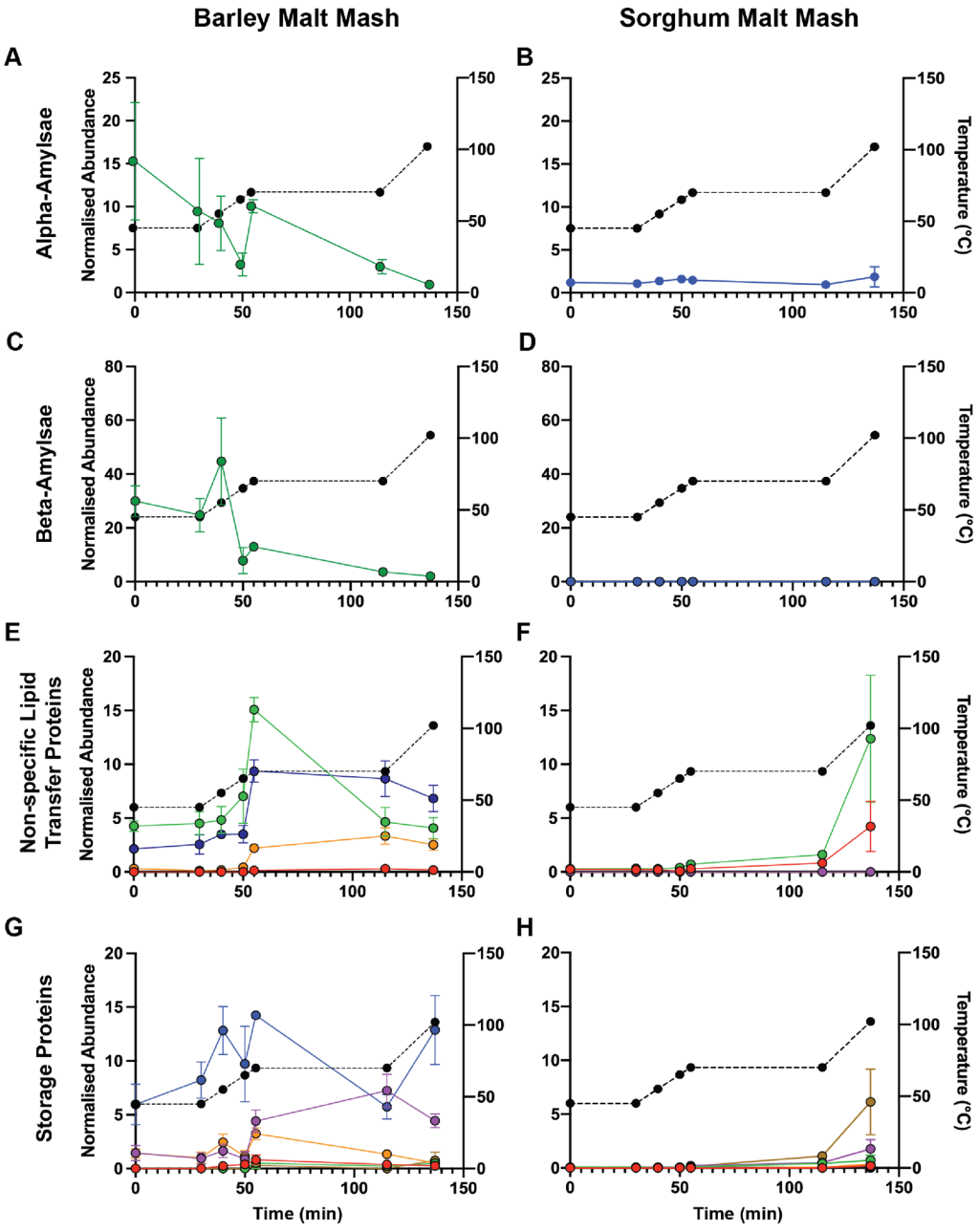

Figure 4. Dynamics of the abundance of key brewing proteins during the mash and boil for sorghum and barley. Abundance of summed forms of α-amylase in (A) barley and (B) sorghum. Abundance of summed forms of β-amylase in (C) barley and (D) sorghum. (E) Abundance of nonspecific lipid transfer proteins in barley: NLTPX, red; NLTP2, green; NLTP1, purple; NLTP2, orange; NLTP4, blue. (F) Abundance of nonspecific lipid transfer proteins in sorghum: NLT2G, red; NLTL1, green; NLTPX, light purple; NLTP2, orange; NLTP7, black; LTPG1, gold; NLTP2, blue; NLTP1, dark purple. (G) Abundance of Cupincin (CUCIN), red; Globulin-1 (GLB1), green; Vicilin-like seed storage protein At2g18540 (VCL21), purple; Vicilin-like seed storage protein At2g28490 (VCL22), orange; B3-hordein (HOR3), blue; Gamma-hordein-1 (HOG1), gold. (H) Abundance of 11S globulin seed storage protein 2 (11S2), red; Cupincin (CUCIN), green; 19 kDa globulin (GL19), light purple; Globulin-1 (GLB1), orange; Glutelin type-B 5 (GLUB5), black; Vicilin-like seed storage protein At2g18540 (VCL21), gold; Vicilin-like seed storage protein At2g28490 (VCL22), blue; 50 kDa gamma-zein (ZEG1), dark purple. (A−H): Abundance (a.u.: arbitrary units) of each protein normalized to trypsin. Values show mean, n = 3. Error bars show SEM. Mash temperature profile is shown on the right y-axis.

appearance of amylases in malt is very promising for sorghum malting and mashing as amylases are essential for producing fermentable sugars during mashing, which is needed for efficient downstream yeast fermentation.

The enrichment of "peptidase activity, acting on L-amino acid peptides" and "peptidase activity" in proteins unique to malt was associated with proteases (ASPA, CBP1, CBP23, CYSP2, CYSP2, and ORYA), which were only found in sorghum malt (Figure 2B,D). The increased abundance of proteases in sorghum malt aligned with the decrease in abundance of seed storage proteins, the main substrates of these proteases.[13,47] "Proteasome complex", "proteasome regulatory particle", and "proteasome accessory complex" were all associated with two proteasome regulatory subunits, PRS6B and PSD11 (Figure 2B,E). Proteasomes are protease complexes, which assist in the degradation and recycling of

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specific proteins.[48] Altered proteasome regulation may be associated with protein degradation to generate FAN or with regulating the abundance of key proteins involved in germination. As with amylases, the synthesis of proteases during malting is promising for the performance of sorghum in malting and brewing due to the requirement for yeast to be supplied with sufficient FAN for efficient fermentation. We noted generally large variances in the abundance of proteins that were only detected in sorghum malt (Figure 2C−E). As these are analyses of biological replicates, this suggests that there may be substantial variation in the strength or timing of the proteomic response to germination between individual sorghum seeds.

The increase in abundance of amylases, proteases, and proteasome proteins along with the decrease in abundance of lipid droplet proteins, seed storage proteins, and heat shock proteins in sorghum malt compared to mature sorghum seeds is consistent with published proteomic changes in malted/germinating barley seeds.[21] The conserved changes to the proteome of sorghum as it undergoes malting that we measured here indicate that sorghum malting shows all the hallmarks of standard industry barley germination.[21]

Nuanced Differences in the Dynamic Mash Proteome between Sorghum and Barley

Our proteomic analysis of mature sorghum seeds and sorghum malt showed the presence of the nutrient proteins and enzymes needed for malting and mashing (Figures 1 and 2). To better understand how sorghum malt performed in brewing, we performed side-by-side mash and boil of sorghum malt and barley malt and investigated the proteome of the soluble wort fractions throughout the mash and boil (Tables S1, S2, S5, and S6). Previously, we have shown that the barley mash proteome was dynamic and complex.[29] We found that proteins increased in abundance early in the mash as they were extracted from the malt and then rapidly decreased in abundance at higher temperatures due to protein denaturation, aggregation, and precipitation due to lack of thermostability.[29] Consistent with these processes, PCA of the wort proteome throughout the barley mash and boil showed a clear proteome shift as the mash progressed (Figure 3A). In contrast, PCA of the wort proteome throughout the sorghum mash and boil showed a small shift in proteome through the early stages of mashing and larger changes at higher temperatures (70 °C − 60 min and 102 °C) (Figure 3B), probably due to the high protein solubilization temperature in sorghum due to differences in starch polymer structure.[18,24]

To identify the relationships between each stage of the barley and sorghum mash and boil, we next performed clustering analyses of the respective proteomes (Figure 3C,D). In the barley mash, we noted three clades: low temperature (45 and 55 °C), mid temperature (65 and 70 °C − 0 min), and high temperature (70 °C − 60 min and 102 °C) (highlighted in Figure 3C). Proteomes within the "low-temperature" clade did not cluster by replicate, highlighting the limited overall proteomic changes occurring at low temperatures (Figure 3C). The rapid change in proteomes at 65 °C and above was highlighted by the separation of the "mid-temperature" clade from the "low-temperature" clade (Figure 3C). The rapid shift in proteome from "low temperature" to "mid temperature" highlighted efficient protein extraction from barley malt as the mash temperature increased. Finally, the separation of the "high-temperature" clade from the other clades indicated a large shift in protein abundance as temperature increased and proteins began to denature, aggregate, and precipitate (Figure 3C).[29] In the sorghum mash, as with barley, we identified three major clades: low temperature (45 °C), mid temperature (55 °C, 65 °C, and 70 °C − 0 min), and high temperature (70 °C − 60 min and 102 °C) (Figure 3D). Within the "low-temperature" and "mid-temperature" clades, we saw a large overlap of replicates, suggesting a lack of substantial changes in the mash proteome during these stages (Figure 3D). Toward the higher end of temperatures within the "mid-temperature" clade and into the "high-temperature" clade, we saw stronger clustering of replicates. This clearer separation and clustering of "mid temperatures" indicated the start of protein extraction from sorghum malt, supported by the proteome variance in the PCA (Figure 3B,D). The separation of the "high-temperature" clade (Figure 3D) that was also apparent by PCA (Figure 3B) indicated a substantial shift in protein abundance as temperatures continued to increase and proteins potentially began to denature. Overall proteome dynamics throughout the mash and boil were similar for barley and sorghum malt, with the main apparent difference being that higher temperatures were required for protein extraction from sorghum malt.

The analysis of the overall variance in sorghum and barley brewing proteomes showed that while both showed qualitatively similar dynamics, the changes during the sorghum mash were muted in comparison to the substantial changes observed in the barley mash. The limited changes throughout the sorghum mash were potentially due to lower overall protein levels or slower solubilization from sorghum malt compared to barley. To better understand these differences, we compared the abundance of key proteins important to brewing performance: α- and β-amylase, seed storage proteins, and nonspecific lipid transfer proteins.[12,13,49] We found that in the barley mash, α-amylase behaved as expected, decreasing in abundance when the temperature exceeded ∼65 °C due to unfolding and aggregation[29] (Figure 4A). The abundance of α-amylase was substantially different between mashes, with the barley mash having an order of magnitude more α-amylase than the sorghum mash (Figure 4A,B). The abundance profile of α-amylase in the sorghum mash was also different from the barley mash, only increasing in abundance at 55 °C and then decreasing at 70 °C (Figure 4B). β-Amylase in the barley mash increased in abundance at a mash temperature of 55 °C, followed by a substantial decrease in abundance at 65 °C, and eventually, a complete loss as the mash temperature increased further (Figure 4C). Although we detected β-amylase in sorghum malt, it was not detected at any stage of the mash (Figures 2C and 4D). The combined lower abundance of α- and β- amylase in the sorghum mash would likely affect overall brewing performance by severely limiting the production of fermentable sugars, causing reduced downstream fermentation efficiency, and lower alcohol production (Figure 4A−D).

Lipid transfer proteins (LTPs) are important in beer as they are positively associated with foam formation and stability.[50−52] In the barley mash, the abundance of LTPs increased at 55 °C until the start of 70 °C and then decreased during the 70 °C stage (Figure 4E), consistent with previous reports.[29] In the sorghum mash, the abundance of LTPs began to slowly increase only at 70 °C and continued to increase throughout the remainder of the mash (Figure 4F).

We next explored the dynamics of the abundance throughout the mash of seed storage proteins, which contain storage reserves of nitrogen, carbon, and sulfur for developing

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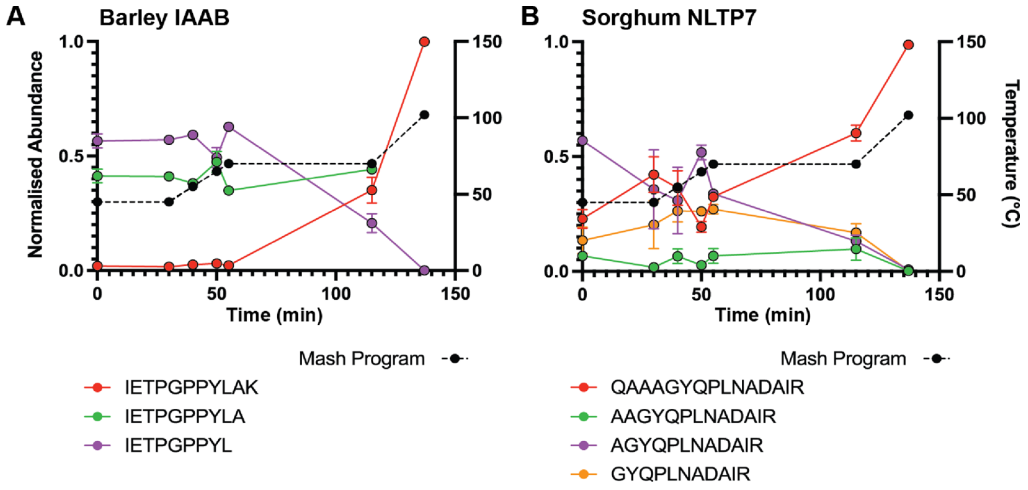

Figure 5. Abundant physiological proteolysis during the mash in both barley and sorghum. (A) Normalized abundance (a.u.: arbitrary units) of full- and semitryptic peptides corresponding to R-I_{55}ETPGPPYLAK_{65}-Q from IAAB (α-amylase/trypsin inhibitor CMb) in barley. (B) Normalized abundance (a.u: arbitrary units) of full- and semitryptic peptides corresponding to K-Q_{87}AAAGYQPLNADAIR_{101}-D from NLTP7 (nonspecific lipid-transfer protein 7) in sorghum. Values show mean, n = 3. Error bars show SEM. Mash temperature profile is shown on the right y-axis.

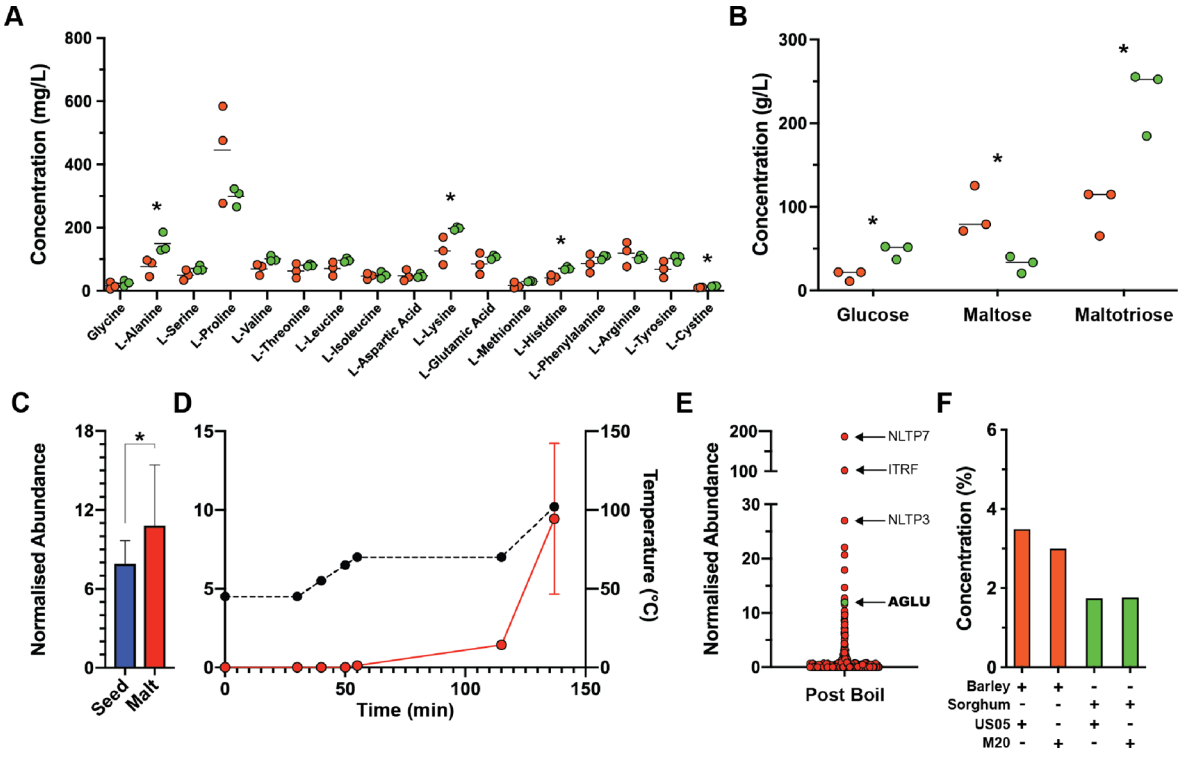

Figure 6. Fermentation performance of wort produced from barley malt and sorghum malt. (A) Amino acids quantified in wort produced from barley malt (orange) and sorghum malt (green). (B) Glucose, maltose, and maltotriose quantified in wort produced from barley malt (orange) and sorghum malt (green). Bars show mean, n = 3. *, statistically significant differences between barley malt and sorghum malt ($p < 0.05$). Abundance of probable α-glucosidase (AGLU) in (C) sorghum seeds (blue) and malt (red) and (D) during the mash and boil. Values, mean; Error bars, SEM; *, $p < 10^{-5}$. (E) Abundance of proteins post boil. Values, mean. Proteins labeled: NLTP7 (nonspecific lipid-transfer protein 7), ITRF (trypsin/factor XIIA inhibitor), NLTP3 (nonspecific lipid-transfer protein 3), and AGLU (probable alpha-glucosidase). (F) Concentration of ethanol produced in ferments from barley wort (orange) and sorghum wort (green), n = 1.

seeds.[53] In brewing, FAN from seed storage proteins acts as nutrients for yeast instead of developing seeds, and mobilization of these nutrients in the mash is critical for efficient yeast growth during fermentation.[13,54] In the barley mash, seed storage proteins increased in abundance at 55 °C and then began to decrease in abundance at 70 °C (Figure 4G), as expected due to solubilization and denaturation. In the sorghum mash, seed storage proteins began to increase slowly in abundance only at 70 °C, reflecting either protein solubilization or starch gelatinization only occurring at this high temperature. The low abundance of seed storage proteins throughout most of the sorghum mash (Figure 4H), in contrast to the high abundance of this class of protein in the barley mash (Figure 4G) might limit their accessibility to digestion by proteases and result in low FAN production. Furthermore, barley seed storage proteins increased in

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abundance early in the mash but then decreased in abundance at higher temperatures (Figure 4G) due to the relatively low thermal stability of proteolytically clipped forms.[29] This decrease in abundance was not observed in the sorghum mash (Figure 4H), also suggesting that protease activity was low throughout the sorghum malting and mashing. To determine if we could detect direct evidence of protease activity in the sorghum mash, we inspected our peptide-level proteomic data for evidence of nontryptic physiological proteolysis. We could indeed identify such physiological proteolytic events during the early stages of the mash (Figure 5 and Tables S7 and S8). At lower mash temperatures in both barley and sorghum mashing, semitryptic peptides representing physiological proteolysis events were more abundant than full tryptic peptides representing unproteolytically clipped protein (Figure 5). As temperatures in the mash increased, the full tryptic peptides became the dominant form, reflecting the unfolding and aggregation of the proteolytically clipped protein forms (Figure 5).[29,55] The abundance of semitryptic peptides in both sorghum and barley mashing suggested that FAN was being created in either malting or the early stages of the mash in both cereals.

Comparable Nutrient Concentrations in Sorghum and Barley Wort

Our proteomic analyses showed that while sorghum malt does contain the enzymes necessary for an efficient mash, they are present in low abundance and are only solubilized at much higher temperatures than from barley malt. We also found that wort produced with sorghum had a lower abundance of amylases and storage proteins, potentially due to sorghum malt having intrinsically lower levels of these proteins or requiring a higher temperature for protein and starch solubilization during mashing (Figure 4). This may limit the fermentability of the final worts produced from sorghum. To determine the consequences of a lower abundance of amylases and other enzymes on sorghum wort quality, we used metabolomics to measure the amount of FAN and fermentable sugars present in barley and sorghum wort (Figure 6A,B and Table S9). Our proteomic data showed that in the sorghum mash seed storage, proteins that are critical sources of FAN were not highly abundant until late in the mash, likely compromising proteolysis and FAN production (Figure 4F). However, we also found evidence of proteolysis in the early stages of mashing (Figure 5B), which was consistent with the presence of at least some FAN-producing proteolytic activity. Using metabolomics, we measured the concentrations of 17 amino acids in sorghum and barley wort and found significantly higher concentrations of L-alanine, L-lysine, L-histidine, and L-cystine in sorghum wort compared to barley wort, with no significant differences in the concentrations of the remaining 13 amino acids (Figure 6A). This surprising result was consistent with our proteomic results, which showed proteolysis of seed storage proteins in sorghum malting or early in the mash, even though these proteins were not efficiently solubilized until very late in the mash. This is also consistent with previous studies of total FAN, which showed that regardless of protein abundance, both sorghum and barley wort have similar levels of FAN.[56,57]

We next compared the concentrations of fermentable sugars present in wort from the sorghum and barley mashes. We found that there was significantly less maltose in sorghum wort (33.6 g/L) than in barley wort (79.1 g/L) (Figure 6B). The low amount of maltose in sorghum wort was likely due to the limited abundance of α- and β-amylases in the mash (Figure 5B,D). In contrast, the much higher level of maltose in barley wort was consistent with the abundant levels of α- and β-amylase throughout the mash (Figure 5A,C). This difference in amylase abundance between barley and sorghum has been reported[28] and is likely a result of the inherent biology of barley as well as decades of breeding selecting for brewing performance. Despite the low abundance of α-amylase, we found that maltotriose concentrations in sorghum wort (252.5 g/L) were significantly higher than those in barley wort (114.7 g/L) (Figure 6B). Finally, and somewhat surprisingly, we found that there was significantly more glucose in sorghum wort (51.7 g/L) than in barley wort (21.8 g/L; Figure 6B). This relatively high glucose concentration is consistent with previous reports.[56,57] We therefore inspected our proteomic data to investigate whether there was an enzyme present in the sorghum mash that could produce free glucose. We were able to identify a probable α-glucosidase (AGLU) that was not only abundant in sorghum malt but that was solubilized at later stages of the mash (Figure 6C−E). In the final sorghum wort after the boil, AGLU was the 11th most confidently identified protein, consistent with high activity during mashing and potentially even during fermentation (Figure 6E). This abundant α-glucosidase is likely the cause of the significantly higher concentrations of glucose and maltotriose in sorghum wort compared to barley wort (Figure 6B).

While our proteomic analyses showed a low abundance of enzymes and seed storage proteins throughout the sorghum mash, our metabolomics analyses showed that sorghum wort had equivalent FAN and fermentable sugar profiles to barley wort. To functionally validate the suitability of sorghum and barley wort, we fermented the sorghum and barley wort with two commercial brewing strains of yeast (US05 and M20) and compared their fermentability (Figure 6F and Table S10). We measured the extent of fermentation by tracking the weight loss caused by consumption of glucose and production of CO_2 and ethanol.[39] Barley wort inoculated with US05 or M20 produced 3.48 or 2.99% ethanol (v/v), respectively (Figure 6F). In contrast, fermentation of sorghum wort with US05 or M20 produced only 1.73 or 1.76% ethanol (v/v), respectively (Figure 6F). Together with our mashing proteome data, this demonstrated that wort produced from sorghum malt using standard malting and mashing parameters was not as fermentable as wort produced from barley malt but nonetheless resulted in efficient fermentation comparable to mid-strength beer.

CONCLUSIONS AND FUTURE DIRECTIONS

In this study, we have shown that when it is malted, sorghum synthesizes the enzymes needed for brewing, specifically amylases that degrade starch to fermentable sugars and proteases that digest seed storage proteins to produce FAN.[21] However, our proteomic analyses of the dynamic sorghum mashing proteome highlighted that mashing with sorghum malt required higher temperatures for efficient protein solubilization and that the key enzymes α- and β-amylase were considerably less abundant in sorghum wort than in barley wort. This correlated with a lower amount of maltose in the sorghum wort. Surprisingly, metabolomics analyses detected more glucose in sorghum wort than in barley wort, consistent with the presence of an abundant α-glucosidase in sorghum malt, AGLU. Finally, we showed that the

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fermentation of barley and sorghum wort was consistent with their FAN and fermentable sugar content with efficient fermentation of barley wort and moderate fermentation of sorghum wort. Importantly, our results provide a molecular basis for previous descriptions of peculiarities and inefficiencies in sorghum wort production.[9,28]

Our results indicated that while sorghum mashing does not produce wort that is as fermentable as wort produced from barley mashing, it is still potentially viable for industrial beverage production. Our data showed that sorghum α-glucosidase AGLU is relatively stable at high temperatures and contributed to the high glucose concentrations in sorghum wort. The behavior of this sorghum α-glucosidase and the high concentrations of maltotriose suggest that manipulation of sorghum mash parameters to allow α-glucosidase access to gelatinized starch for a longer period would produce wort that could support efficient fermentation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00436.

Figure S1. Abundance of malting related proteins in sorghum seeds and sorghum malt (PDF)

Table S1. Sorghum Identification; Table S2. Sorghum protein abundance; Table S3. Sorghum malting MSstats; Table S4. Sorghum malting GOstats; Table S5. Barley identification; Table S6. Barley protein abundances; Table S7. Sorghum proteolysis; Table S8. Barley proteolysis; Table S9. Amino acids and sugar; Table S10. Fermentation (XLSX)

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Notes

The authors declare no competing financial interest.

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