Application of Traditional Methods of Analysis and Modern Proteomics to Evaluate the Malting and Brewing Potential of the Novel Grain Kernza (*Thinopyrum intermedium*)

By

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## Abstract

As the environmental impacts of beer is of increasing concern to maltsters, brewers and consumers, perennial cereal crops offer a more sustainable solution. Their extended lifespans have been shown to result in reduced water use, decreased added nutrient requirements, and lower overall environmental cost when compared to traditional annual cereals like barley and wheat. One new species of interest to the brewing industry is intermediate wheatgrass (*Thinopyrum intermedium* var. *intermedium*), developed by The Land Institute, in Salina, Kansas, USA, which has been recently released under the branded name Kernza®. By malting and later mashing this grain under varied conditions, the usefulness of this novel species in brewing was compared against barley (*Hordeum vulgare* L.), the global industry benchmark for malt. Here, the malted Kernza showed a significant increase in the amount of available fermentable sugars (extract) and free amino nitrogen (FAN) after mashing when compared to the raw Kernza grain. Furthermore, the Kernza malts produced wort that conformed to industry standards for low concentrations of β-glucan, and enough extract and FAN to support healthy fermentation. Extract and FAN in the Kernza malts were lower than in the barley reference, however, and the Kernza worts exhibited higher levels of undesirable soluble protein and haze. Kernza malts were also shown to perform better under multi-temperature step mash conditions than in an isothermal 65 °C infusion mash, comparable to the barley malt reference. In addition to industry standard methods applied to analyze the grains, malt, and wort here, modern mass-spectrometry-based proteomics methods were utilized to evaluate the changes to the protein makeup of these grains during different stages of the malting and brewing process. In the finished Kernza malt, several important proteins for brewing were identified, including mash-active enzymes such as α- and β-amylases, carboxypeptidase, and foam-positive proteins such as

serine proteinase inhibitors (serpins) and lipid transfer proteins (LTPs). Furthermore, structural prolamin-type proteins, including multiple forms of gliadin and glutenin, were identified in the Kernza grains prior to malting, and which reduced in abundance during malting at rates similar to the hordeins in the barley reference, indicating that these malts were modified to degrees similar to commercial malts. Many of these proteins were also found in worts produced under different mash conditions, and their abundances followed similar trajectories to those produced with the barley malt. Additionally, for both Kernza and barley, multi-rest step mashes retained many important enzymes in the wort longer than the higher temperature infusion mashes. These analyses show that not only is Kernza a strong candidate to replace 100 % of the barley malt in the grist, but our insights into the Kernza proteome provide an indication for future breeding of this grain to encourage its wider acceptance as malt by focusing on increasing production of the diverse hydrolyzing enzymes utilized in the mash and reducing the abundance of storage proteins.

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## 1. Introduction

The beer brewing industry is global and massive, producing an estimated 1.91 billion hectoliters of beer globally [1], and includes over 8,000 companies in the US alone [2]. Much of this growth has been driven by craft brewers, defined as independently-owned, and producing fewer than 6 million barrels (700 million liters) annually [3]. In the US, craft breweries amassed $28.5 billion in annual sales in 2019, accounting for nearly a quarter of the $116 billion total US beer sales. This is the result of a greater than five-fold increase in craft beer produced in the US in the last 15 years [2]. As this market share has increased, many of these craft breweries have sought to distinguish themselves with unique products by responding to consumer demands for not only novelty and flavor, but also localness and environmental sustainability [4].

Upstream from the brewery, producers of two of the three main ingredients in beer have followed suit by expanding the range of products for commercial brewers. A nearly three-fold increase in the total volume of hops (*Humulus lupulus* L.) produced in the US was measured from 2001 to 2021 to a current estimated stock of 108 million pounds [5], with a parallel expansion in cultivar variety [6]. Similar interest has been paid to the diversification of the available microorganisms to ferment beer beyond the ubiquitous ale (*Saccharomyces cerevisiae*) and lager (*S. pastorianus*) yeasts [7–10]. However, the third and arguably most important component, malt, has been slower to evolve, with the brewing industry almost myopically reliant on barley (*Hordeum vulgare* L.) as the primary raw material for the production of malt.

Barley was one of the first cereal crops to be domesticated by humans, and archeological evidence indicates that it has been under cultivation since 8,000 BCE in Western Asia, or what is modern-day Syria and Iraq [11]. Today, barley is the fourth most abundant cereal crop globally after corn, rice, and wheat by harvest volume, with $3.69 \times 10^9$ kg harvested in 2019 alone [12]. In

Europe, the annual harvest of barley is second only to wheat, and in the US, barley is fifth behind the global top three and sorghum [12]. Globally, roughly two-thirds of barley is grown for animal feed, while the most widely value-added use of the remainder is to produce malt. Today, barley grown for malt production accounts for roughly 25 % of all the barley grown in the US [13], and 13 % globally [14]. Of the malt produced in the US, 14 % is used for distilled alcohol products and 6 % for other human food products, but the overwhelming majority – roughly 80 %– is destined for beer brewing [13]. Globally, this number may be as high 90 % [14].

Historically, beer has been produced with a wide variety of cereals and starches, but barley was likely one of the first used, as archeological finds have even identified malted barley in ancient Egyptian breweries dated to 4,000 BCE [15]. Barley malt has since sustained as the principal raw material for beer, most notably in its inclusion in the Bavarian beer purity law of 1516 CE known as the Reinheitsgebot [16]. There, brewers were mandated to brew beer only using water, hops, and barley (as yeast was not yet discovered). Though this law is no longer in effect, the global malt and beer industries have been slow to diversify and are still focused on barley for malt production. While many cereal grains can be used to produce malt, barley still accounts for the vast majority, to the extent that the word "malt" is most often assumed to mean "barley malt" [17].

Regrettably, the cultivation of barley and the production of malt encompasses some of the highest environmental costs associated with beer production. Cultivation of conventional barley relies on synthetic fertilizers that can lead to freshwater and marine eutrophication [18], and malt production requires vast amounts of water and electricity [19]. Conversely, the future of barley and malt is also in question due to potential impacts of climate change, such as increased local climate variability, increased extreme weather events, and decreased water availability [20].

By expanding the acceptance of barley alternatives for malt production, the malt and beer industries have the potential to reduce their environmental impact while simultaneously engaging consumers with novel products. Kernza, a recently-domesticated perennial cereal grass, presents a novel opportunity to utilize a new grain for malt and beer production. In this work, raw Kernza was malted and evaluated using traditional malt and beer methods to compare its potential as a barley malt replacement for wort production. Additionally, its malting and beer brewing efficacy was also investigated using recently-developed mass spectrometry-based proteomics methodologies, described below.

## 1.1 Kernza

Kernza (*Thinopyrum intermedium* subsp. *intermedium*) presents a novel opportunity to alleviate some of the environmental costs imposed by barley production. A domestication program of *T. intermedium* has only been in place for the past few decades, and while the primary focus thus far has been on replacing wheat in baking applications, many aspects of the grain show potential for malting and beer brewing. *T. intermedium* shares a number of physiological and biological aspects with its close relatives –wheat, barley, and rye– but is notable in its perennial nature, which has implications for increased environmental sustainability.

*T. intermedium* is a cool-season perennial grass species first introduced to the US in the early 20$^{th}$ century, where it has historically been grown in the Great Plains and Intermountain West mainly as a forage crop and for erosion control. In the 1980s, it was selected by the Rodale Research Center (Kutztown, PA, USA) as an promising candidate for domestication as an edible seed crop from among nearly 100 other species based on its alignment with agricultural technologies, growth and yield potential, and seed palatability [21]. After only a few rounds of selective breeding, the germplasm was transferred in 2002 to the Land Institute (Salina, Kansas,

USA). There, it was trademarked with the trade name Kernza®, and the improvement work continued in tandem with market development and research into its utility to the food industry with numerous partners in North America [22]. The Land Institute reports that Kernza is currently cultivated by more than 100 farmers on over 2,000 acres across the globe [23]. Though improvement breeding for improvement continues, a cultivar known as 'MN-Clearwater' developed at the University of Minnesota, St. Paul, MN was recently approved for sale to the public, with human food as its primary intended use [24].

As a grass species in the Triticeae tribe, *T. intermedium* has been shown to share substantial genetic similarity to domesticated wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and somewhat less so to barley (*Hordeum vulgare* L.) [25] (***Figure 1***). The complete genome has been sequenced and identified *T. intermedium* an allohexaploid ($2n = 6x = 52$) [24], meaning it has six copies of each chromosome pair like wheat, but many more than the diploid barley [26]. Its seeds have been shown to grow similarly to wheat and barley on two-rowed spikes and, unlike barley, yield a hulled kernel after threshing. Similar to wheat, this hull may be removed mechanically, but is not as easily separated from the seed due to its smaller size, making "naked seed" yield an area in need of improvement [22]. Kernza plants produce seeds with an individual mass of roughly 10 mg [24], much smaller than the typical 40 mg per seed weight for wheat or 30 – 50 mg for barley [27].

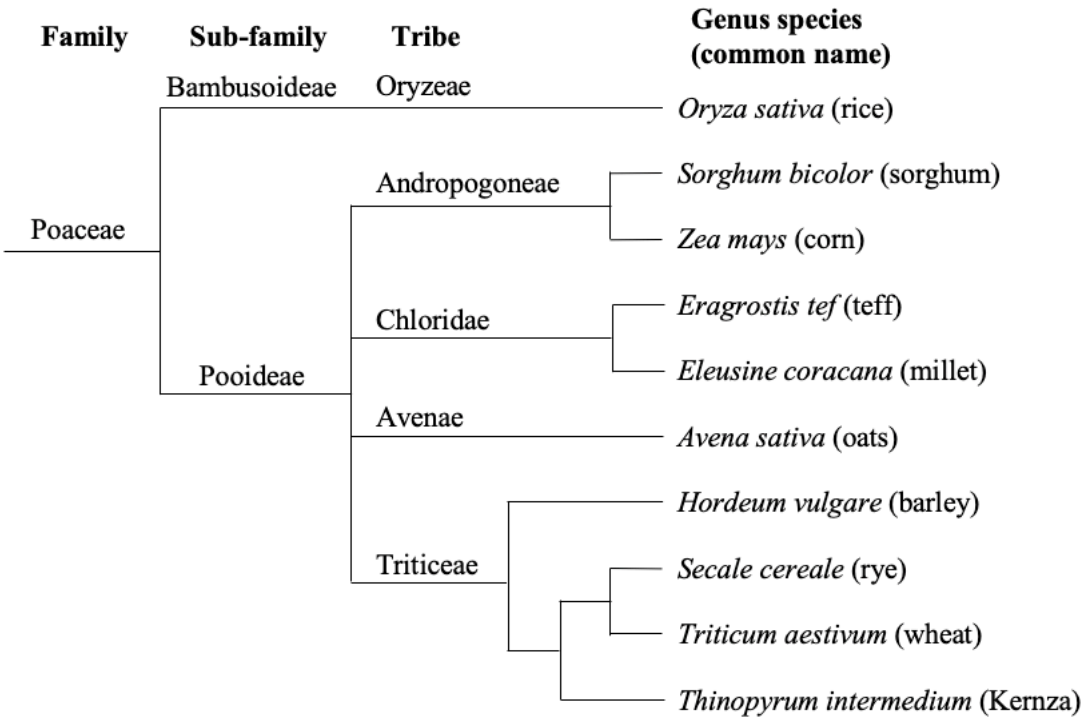


***Figure 1*. Phylogenetic tree of the grass family, Poaceae, with domesticated cereals and pseudo-cereals highlighted.** Within the Triticeae tribe, Kernza (*Thinopyrum intermedium*) is most closely related to wheat (*Triticum aestivum*) and rye (*Secale cereale*), and less so to barley (*Hordeum vulgare*). Figure adapted from [25,28].

Interest in the domestication of this grain as a food crop has primarily focused on its perennial nature and the associated environmental benefits. Most cereal crops grown around the world, including wheat and barley, are annuals that have a life cycle of only one season. Kernza, however, has the ability to grow over multiple years with grain production each year, while it also develops a deeply-penetrating root system. While wheat roots generally reach only 50 – 300 cm depending on soil conditions [29], Kernza roots have been shown to grow to depths greater than 3 meters, and at a much greater density than those of wheat [30] (***Figure 2***). The top meter of soil in Kernza fields can contain 15 times the amount of organic carbon sequestered compared to untilled annual wheat fields, and fundamentally acts as a carbon sink [30]. This densely-packed root system is ideal for more efficient water usage [31] and access to deeper and more stable water stores, both advantageous in climates with low or intermittent rainfall or

unpredictable weather patterns [32]. Kernza fields have also been shown to reduce nitrogen leaching when compared to wheat fields [33].

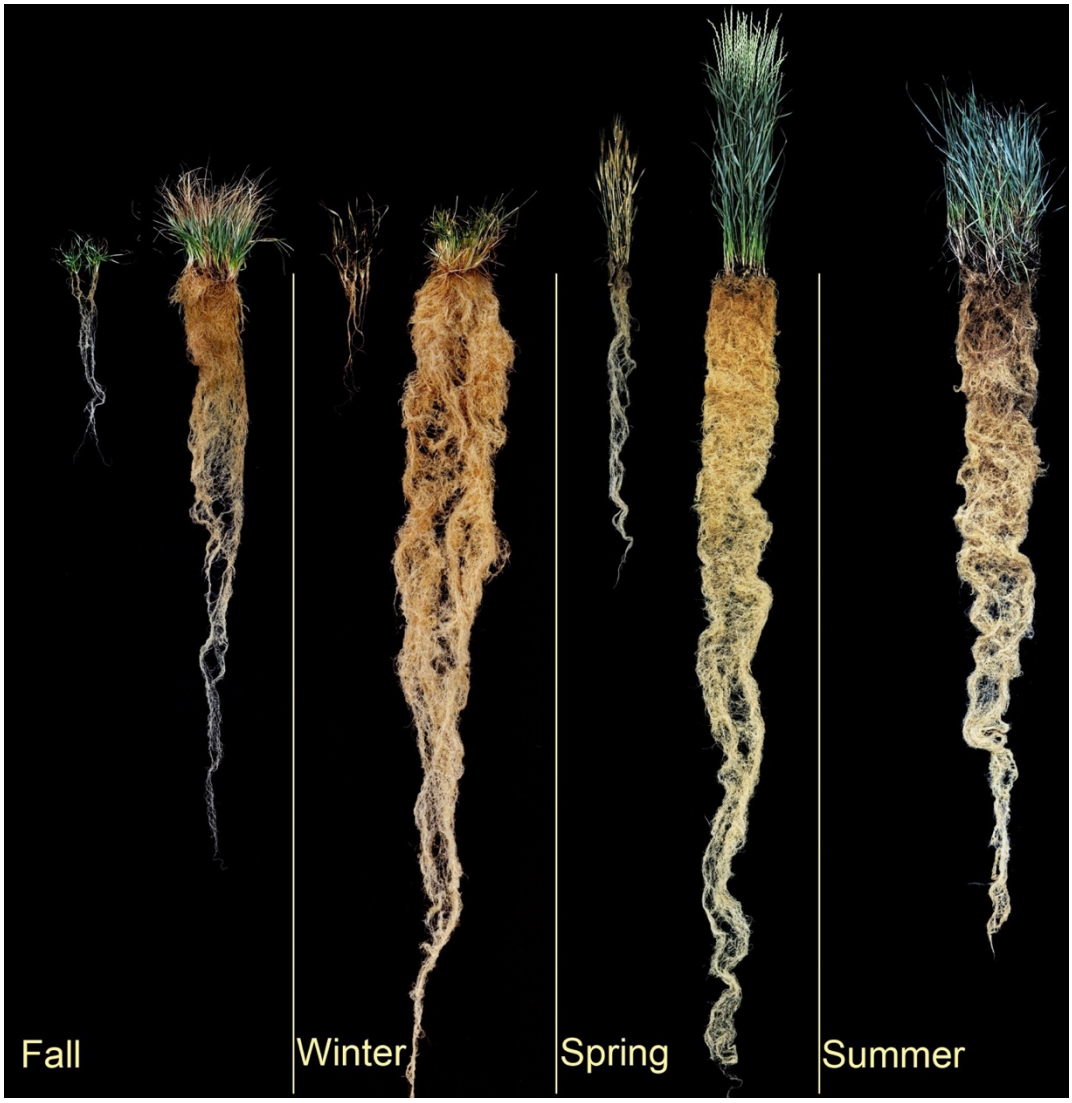


***Figure 2*. Comparison of above- and below-ground biomass of wheat (left) and Kernza (right) during each season.** During summer, wheat plants are frequently removed, and the soil is greatly reduced in organic material compared to perennial Kernza fields. Figure from Cascadian Farms [34].

As this species is still in the early stages of domestication, grain yields per hectare are currently much lower than both barley and wheat, averaging 696 kg/ha (621 lb/ac), relative to typical commercial wheat or barley operations in the US, at 3,475 kg/ha (3,100 lb/ac) and 4,181 kg/ha (3,730 lb/ac), respectively [35]. Unfortunately, current genetic lines of Kernza are unable

to maintain yields year-over-year, and the 'MN-Clearwater' variety produces similar yields the first two years of harvest, but exhibits a sharp reduction of 77 % in the third year. This reduced yield is postulated to be due to an increase in above-ground vegetative density in later years, and recent research indicates that defoliation for hay and straw production resulted in increased grain yield [36]. Thus current breeding and agronomic programs are focused on improving yields per plant, per year, and per hectare [24].

Nutritionally, refined Kernza flour is relatively similar to all-purpose white (wheat) flour and barley flour. All are similarly low in lipids (<2 % w/w) and rich in carbohydrates (>70 % w/w) (***Table 1***). However, whole grain Kernza has a carbohydrate content closer to 67 % and a starch content of 45 – 50 % (w/w), lower than the 65 % starch found in whole grain hard red wheat [37] or the 58 – 65 % found in barley varieties used for malting [38]. Kernza also has a protein content of over 17 % (w/w), significantly higher than the 9 – 12 % found in wheat or barley. Additionally, Kernza contains glutenin and gliadin proteins similar to wheat, and thus are not "gluten-free" [39].

***Table 1*. Nutritional composition of Kernza, wheat and barley flours of various types.** All values are based on a 100 g, dry basis sample. Cells with dashed lines were not reported. Kernza refined flour and all-purpose white flour and white wheat whole grain data adapted from [40]. Barley and malted barley data are adapted from [41] and [42], respectively.

| | | Kernza whole grain* | Kernza refined flour* | White wheat whole grain | All-purpose white flour (wheat) | Barley whole grain | Malted barley whole grain |
|---|---|---|---|---|---|---|---|
| *Moisture* | *%* | 8.6 | 8.1 | 13.7 | 11.9 | 12.11 | 8.21 |
| *Ash* | *%* | 2.4 | 0.6 | --- | 0.5 | 1.28 | 1.37 |
| *Calories* | *kcal* | 368 | 368 | 318 | 340 | 345 | 361 |
| *Protein* | *g* | 19.2 | 17.5 | 9.24 | 10.3 | 10.5 | 10.3 |
| *Carbohydrates* | *g* | 67.3 | 73.2 | 73.7 | 76.3 | 74.5 | 78.3 |
| *Dietary fiber* | *g* | 18 | 4.3 | 10.3 | 2.7 | 10.1 | 7.1 |
| *Soluble fiber* | *g* | 3.6 | 1 | --- | 0.9 | --- | --- |
| *Sugar* | *g* | 1.7 | --- | 1.1 | 0.3 | 0.8 | 0.8 |
| *Total fat* | *g* | 2.9 | 1.2 | 2.3 | 1 | 1.6 | 1.8 |
| *Saturated fat* | *g* | 0.5 | 0.3 | 0.4 | 0.2 | 0.33 | 0.38 |
| *Monounsaturated fat* | *g* | 0.5 | 0.1 | 0.3 | 0.1 | 0.21 | 0.25 |
| *Polyunsaturated fat* | *g* | 1.9 | 0.7 | 1.6 | 0.4 | 0.77 | 0.95 |
| *Trans Fat* | *g* | 0 | 0 | 0 | 0 | --- | --- |
| *Cholesterol* | *mg* | 0 | 0 | 0.1 | 0 | --- | 0 |
| *Calcium, Ca* | *mg* | 120 | 50 | 25 | 15 | 32 | 37 |
| *Iron, Pb* | *mg* | 5.5 | 3.7 | 2.6 | 1.17 | 2.68 | 4.71 |
| *Potassium, K* | *mg* | 480 | 140 | --- | 107 | 309 | 224 |
| *Sodium, Na* | *mg* | 0 | 0 | 13 | 2 | 4 | 11 |

* Data represents analysis of one sample of Clearwater Variety, MVTL, New Ulm, MN

Current Kernza penetration into the food industry is minimal but growing. It has been featured on restaurant menus as a flour in baked goods and as whole cooked grains [23], and General Mills has used it to produce cereal under their label Cascadian Farm Organic [43]. In the beverage world, Ventura Spirits (Ventura, CA, USA) has also produced a now-discontinued Kernza whiskey [44], and the food brand Patagonia Provisions (Sausalito, CA, USA) has been producing Kernza beers in collaboration with Hopworks Urban Brewery (Portland, OR, USA) since 2016. These beers, named “Long Root Pale Ale” and “Long Root Wit” used raw Kernza as a 15 % (w/w) adjunct in a grist consisting primarily of barley malt [45]. A small number of other

breweries in the US Midwest have also used Kernza in brewing beer at varying quantities [46]. Considering its brewing potential, the Agricultural Utilization Research Institute released a report in early 2021 [47] that compared brewer-specific aspects of malted and unmalted Kernza to malted barley and wheat, and provided guidance for brewers on its potential use. Both malted and raw Kernza were both enthusiastically endorsed as mash additions, with the caveats that worts produced with Kernza will likely have lower fermentable sugar content than those with barley malt, and that the small grain size may not allow for homogenous milling. Qualitative organoleptic aspects of Kernza beers were also included, but without quantitative significance.

## 1.2 Malting

For the vast majority of the beer brewing industry, malt is the primary ingredient and amounts to roughly 50 % of the cost of raw ingredients [48], and over 90 % of the mass of ingredients (excluding water) for all-malt beers [49]. The quality of malt produced is therefore of very high importance to brewers, and the production of this ingredient is maintained by maltsters, who comprise an entirely separate industry. During malting, raw grains undergo a controlled germination process in which three major categories of changes occur within the grain as it is transformed into malt: (1) structural molecules are degraded, (2) enzymes are activated and synthesized *de novo*, and (3) the substrates necessary for beer brewing are both produced and made accessible [17].

The barley grain is comprised of several biochemically distinct structures that serve separate functions (***Figure 3***). The hull (husk) encases the grain, and is primarily composed of insoluble fibers –mainly cellulose– and some polyphenols, and primarily acts as a protective covering that also aids in reducing moisture uptake [50]. The majority of barley varieties have a hull that is firmly attached to the grain, even after threshing, though the hull of wheat and some

"hulless" barley varieties can be easily removed by threshing [51]. The endosperm is the largest portion of the kernel, representing over 75 % of the total weight and serves as the nutrient substrate reserve for the germinating grain in the form of starch granules embedded in a protein-rich matrix [52]. The cell walls in the endosperm are primarily composed of non-starch polysaccharides; predominantly β-glucan and arabinoxylans (each 3 – 9 % of total dry grain weight), but also cellulose (1.4 – 5 %) and lignin (0.9 – 2 %) [52]. The embryo (germ) is located at the proximal end of the seed, representing only 2.5 % of the total weight, is the portion from which the seedling grows, and is rich in protein (34 %), lipids (15 %), and sugars (30 %) [53]. The scutellum, a thin layer of tissue between the embryo and the endosperm, is composed of hemicellulose and protein, and secretes hormones such as gibberellins during germination that stimulate enzyme release and synthesis in the aleurone layer [54]. The aleurone layer surrounds the endosperm and contains high levels of enzymes that are activated during malting to degrade structural components of the endosperm [55].

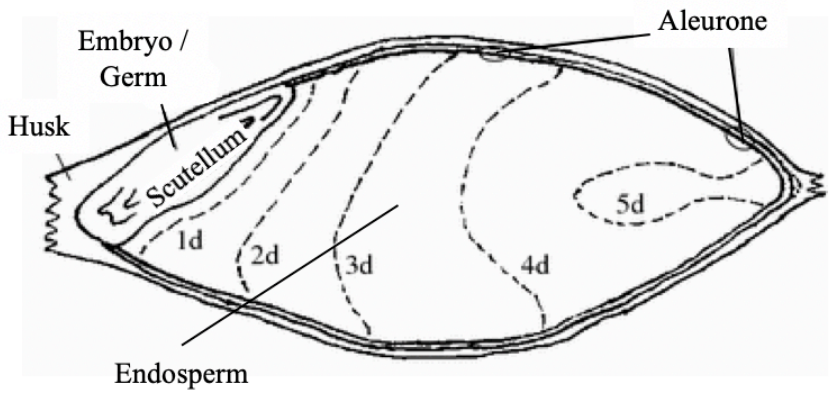


***Figure 3*. Physical parts of the barley grain in cross-section, showing modification during malting.** Dotted lines indicate the progression of modification of the endosperm from proximal to distal ends (left to right) over 5 days (1d to 5d) of germination. Figure adapted from [56].

After grains are cleaned and sorted, the transformation begins with steeping, where grains are fully immersed in tanks of water where they hydrate and swell until they reach 42 – 48 %

moisture (w/w) [17] (***Figure 4***). While immersion steeping uses a single continuous immersion of the grain in water, most malting operations today utilize multi-steeping methods where the water is drained periodically for 'air rests' and the grain is exposed to air [57]. These air rests allow for the grain to begin to sprout, called chitting [17], which increases the rate of moisture uptake during the following steep, and can reduce the total process time by a third [58]. Additionally, the inability of some barley grains to not germinate after steeping, called water sensitivity, can be overcome by employing an air rest when the grain has reached 35 – 37 % moisture and reimmersing until the final desired hydration has been reached [59]. The rate at which hydration occurs is dependent on a number of factors, including initial grain moisture content, steep water temperature, and in some cases, varietal genotype [60]. As portions of the grain become hydrated, they are rendered metabolically active and prepare the grain to begin germination in the next stage, including the initial solubilization of some portion of the β- glucan by the enzyme β-D-glucan solubilase present in raw grains [61].

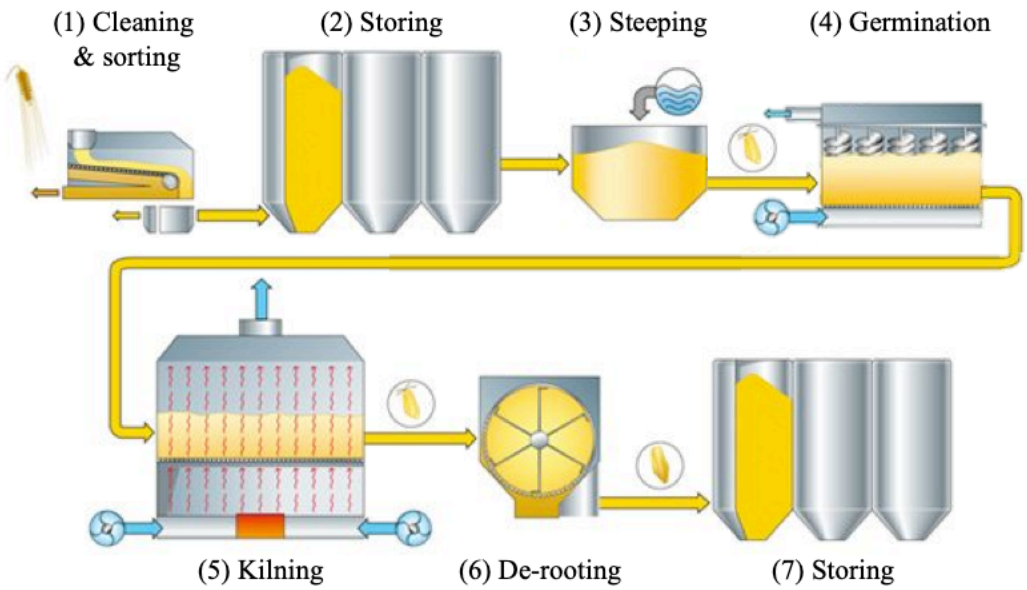


***Figure 4*. Industrial malting process flow.** Raw barley is (1) cleaned and sorted to remove foreign materials and undersized grains, (2) stored dry until ready for use, (3) steeped in water for 24 – 48 hours until grains are sufficiently hydrated, (4) germinated for 4 – 7 days with temperature, humidity and airflow maintained to control the rate of modification, (4) kilned for 24 hours at higher temperatures to dry the malt, (6) de-rooted to remove undesirable rootlets, and (7) stored dry until ready for packaging, shipping and use at the brewery. Figure adapted from [62].

After steeping, the grain is drained and distributed into troughs, drums or in some cases simply piles on the floor to begin germination. There, temperature and humidity are maintained with constant air flow to supply oxygen to the respiring grains, reduce the buildup of $CO_2$, and ensure homogeneity within the physical mass of the grain bed [63]. This stage generally lasts a 4 – 7 days, and maltsters strive for minimal moisture losses.

The germination phase is the most biochemically active, with hydration initiating the synthesis and mobilization of a multitude of endogenous enzymes [64]. Hydrolytic enzymes stored in the scutellum and aleurone layers are mobilized to the endosperm, where they begin to break down a variety of structural molecules into forms or concentrations more compatible with the brewing process. For example, the non-starch polysaccharide β-D-glucan is hydrolyzed primarily by the enzyme endo-(1→3),(1→4)-β-glucanase (EC 3.2.1.73) from 3 – 6 % w/w in raw

barley to 0.5 – 2 % in finished malt [65]. If malted improperly or incompletely, high concentrations of insoluble, undegraded β-glucan remain in the malt, resulting in extremely viscous wort [66]. Similarly, insoluble structural proteins in the endosperm, such as hordein in barley, are degraded by a host of endoproteases, whose overall activity increases approximately 20-fold during malting [67]. As these proteins are hydrolyzed, free amino acids and di- and tri-peptides are released [68]. These are then released into the wort during mashing, which have long been recognized for their importance in wort for yeast health and fermentation quality [69]. Additionally, this protein hydrolysis also exposes the starch granules for later hydration and enzymatic hydrolysis in the mash at the brewery [70].

As a result of the hydrolysis of structural proteins, some previously-bound enzymes important for beer brewing are liberated and made available for later use in brewing. β-amylases (EC 3.2.1.2), for example, are found intact in the starchy endosperm of raw grain in multiple forms [71]. Prior to malting, these are bound to proteins on their active site, and are not active until they are released from this bound form by proteases [72]. Sufficient β-amylase activity in the mash is paramount to produce properly fermentable wort, as it specifically cleaves the penultimate α-1,4 glycosidic bond from starch molecules to produce maltose [72], the primary fermentation substrate for brewing yeasts [73].

Concurrent with this structural degradation, many enzymes are also being synthesized *de novo*, particularly those that are important for starch hydrolysis in the mash [74]. These include limit dextrinase, α-amylase, and α-glucosidase, which hydrolyze starch molecules at specific linkages to produce mono- to oligosaccharides of various lengths. Limit dextrinase (EC 3.2.1.10) is a debranching enzyme found in small quantities in raw grain, but is also synthesized during malting [75]. It specifically hydrolyzes α-(1→6)-glucose linkages in branched amylopectin that

produces linear amylose starch, which is more easily degraded by α- and β-amylases [75]. Alpha-amylases (EC 3.2.1.1) exhibit the most dynamic change in abundance during malting, as these are virtually absent in ungerminated grains, and the majority are synthesized in the aleurone layer and later migrate to the endosperm [74]. In the mash, this enzyme is utilized to hydrolyze both amylose and amylopectin α-(1→4)-glucose linkages indiscriminately, resulting in glucose chains of various lengths and branching, but importantly also allows for β-amylases to cleave maltose off the newly-exposed ends [75].

"Modification" is the term used by maltsters to refer to the overall degradation of non-starch polysaccharides and structural protein molecules in the endosperm that proceeds along the length of the grain (***Figure 3***). It is closely assessed by the maltster to evaluate the quality of the malt produced. The reduction and degradation of insoluble β-glucan and proteins into smaller and more soluble forms is measured to determine whether the grain has sufficiently modified. Separately, starch-hydrolyzing enzymatic activity is also assessed to determine if sufficient enzymes have been synthesized and released.

Following germination, the malt is high in moisture and extremely biochemically active, so it is moved to a kiln for drying. Here, the elevated temperatures in the kiln drive moisture off from roughly 40 % (dry weight) to 4.0 – 4.5 %, which restricts further modification and enzyme activity by reducing water activity [76]. Additionally, the reduced water activity inhibits microbial activity and reduces quality losses during storage [77]. If the primary goal of kilning is only to drive off moisture, temperatures are chosen that reduce enzyme degradation. Limit dextrinase is known to show significant and irreversible reduced activity at temperatures exceeding 70 °C [78]. β-amylases show similar reductions in activity if kilning temperatures exceed 72 °C for extended times and show reduced activity by roughly 30 – 50 % in malts kilned

at temperatures ranging from 60 – 80 °C [79,80]. To preserve enzyme activity, kiln temperatures may be employed as low as 40 °C [81]. Many malts, however, are kilned at higher temperatures to also produce flavorsome Maillard reaction products. Pale malts are produced with the intent to balance enzymatic activity with flavor, and are frequently kilned with temperatures increasing from 60 °C to a maximum of 85 °C over the course of 24 hours [63]. For producing dark roasted and chocolate malts, final kiln temperatures can rise as high 180 – 220 °C, though these malts are almost exclusively used as minor additions to the mash, primarily for flavor and color [63].

From a malting perspective, Kernza has several physiological characteristics that encourage its potential as an alternative to barley. Many tropical grains, such as rice, corn, and sorghum, contain starches that will not gelatinize until they reach higher temperatures, 68 – 78 °C, 62 – 72 °C, and 68 – 78 °C, respectively [27]. Therefore, a cooking step is required prior their addition to the mash, which has the side effect of inactivating most of their necessary endogenous enzymes [63]. Some breeding lines of *T. intermedium*, however, have been show to contain starch that exhibits peak gelatinization at 60.2 – 62.4 °C [82]. This gelatinization temperature range is similar to those found in wheat and barley, at 58 – 64 °C and 51 – 60 °C, respectively [27], and indicate that Kernza mash may be used to contribute both starches and enzymes without a prior cooking step. Kernza also contains more carbohydrate compared to barley and wheat [83,84], and contains amylose and amylopectin of similar quantity, size, and branching structure as oats, barley and rice [82], which are already widely used in the brewing industry.

Kernza exhibits some potential impediments for successful malting or as a principal component of the mash. For example, the current breeding lines of Kernza are not completely dehulled, nor is the hull firmly attached, and thus mechanical methods are required to remove the

hull from the grain for homogeneity. Unfortunately, the mechanical dehulling methods currently used by industry [40] may be too damaging to the inconsistently-sized grains, resulting in numerous broken grains and damaged germs that negatively impact germination quality [85]. If properly dehulled, Kernza may require malting techniques similar to wheat and rye, which do not have an attached hull, and require careful management of the tightly-packed grain beds during germination to mitigate heat and carbon dioxide buildup due to lack of airflow [17]. Additionally, without the structural reinforcement provided by the hulls in the mash at the brewery, the wort does not percolate through the grain bed as efficiently, and filtering the mash in a lauter tun may even be halted completely as the grain bed compacts. For this reason, wheat beers traditionally contain at least 40 % malted barley for, among other things, the filter bed formed by the barley hulls [86]. An addition of rice hulls to the mash is also frequently used to reduce the risk of filtration issues, as is the employment of a mash filter [63]. For a 100 % Kernza beer, the hulls removed prior to malting might be added back to the mash and function much like rice hulls.

## 1.3 Brewing

Though the processing of malt is complex, it is ultimately only an intermediate product in the overall production of beer. After malt has been shipped to the brewery, it is stored under moderate humidity and temperature conditions to maintain quality, as its biochemical composition is the primary quality driver of wort, the substrate for fermentation by yeasts into beer.

At the brewery, the malt is milled and immediately added to the mash tun, where warm water is added to form a slurry, called the mash. The mill size is chosen to be fine enough to ensure rapid hydration of the particles, but not so small that the barley husk materials are

rendered ineffective for filtration later [59]. The finely ground malt, or 'grist,' may also contain other sources of starch, or 'adjuncts,' in addition to the malt [63].

Complete hydration of the grist is generally achieved within minutes for well-mixed mashes with typical liquor-to-grist ratios of 3:1 to 7:1, or 3 – 7 liters of water ('liquor') to 1 kg of grist [63]. Concurrent with the hydration of starches, solubilized components previously produced during malting are dissolved into solution, including the free amino acids, peptides, soluble proteins, and short chain saccharides discussed previously [17].

The endogenous malt enzymes are also released in the mash slurry, where they catalyze an extremely complex and interrelated cascade of biochemical changes. The starch-degrading enzymes –β-amylase, α-amylase, limit dextrinase, and α-glucosidase– hydrolyze amylose and amylopectin as previously described, releasing glucose, maltose, maltotriose, and linear and branched amylose and amylopectin oligosaccharides into solution [87]. The resultant mixture of degraded starch products are collectively referred to as 'extract,' and is the substrate that is later fermented by yeasts into ethanol [88].

The activity of this suite of enzymes, and thus the final composition of the extracted wort and final beer, is highly dependent on the conditions of the mash. Activity optimums for β-amylase, for example, are between 45 and 50 °C, whereas α-amylase is most active between 55 and 60 °C [87]. Furthermore, β-amylase is inactivated starting at 52.5 °C, and is completely inactive after only 180 seconds at 68 °C [87]. To optimize maltose production, a balance must be struck with temperatures chosen where both β-amylase and α-amylase are active simultaneously. As α-amylase indiscriminately cleaves the α-(1→4) bonds along the amylose and amylopectin chains, the amylose ends are exposed for β-amylase to act on and produce maltose, which is critical for the fermentability of the wort.

Malt proteases are also present in great abundance and are highly active in the mash. Here, they continue to degrade structural prolamins and other proteins and produce additional solubilized proteins and FAN [89], which make up approximately 5 % of the wort solids in an all-malt mash [90]. Carboxypeptidases, which are present in high quantities in malt, are active at 35 – 50 °C and are highly correlated with FAN production in the mash [91]. The vast majority of FAN (88 %), however, is produced earlier during malting, with only the remaining 12 % created in the mash [90].

The few β-glucanases remaining in the malt after kilning continue to degrade high molecular weight (HMW) β-glucans into s chains in the mash. The presence of these enzymes in the mash is critical for brewers using less-modified malts or a portion of completely unmalted grains, as those would otherwise contribute highly-viscous HMW β-glucans and greatly reduce or even halt the extraction of wort from the grain bed. These enzymes are more active at lower temperatures, near 45 °C, and are rapidly inactivated at higher temperatures [92]. Malt β-glucanases have a half-life of only 10 minutes at 50 °C and less than 2 minutes at 60 °C [92]. While the majority of soluble HMW β-glucan will have been completely hydrolyzed during germination in well-modified malts [93], additional HMW β-glucans may be further extracted into the wort by β-glucan solubilase, which is most active at temperatures higher than β-glucanase can withstand [94].

All told, mash regimes are chosen based on the composition of the grist, the malt quality, and the targeted final wort. An infusion mash is the simplest process and targets the highest fermentable sugar recovery by holding the mash temperature constant at 65 °C for one hour, and variations on the infusion mash are widely used by ale brewers using well-modified malt [95]. At this temperature, β-glucanase and proteases are largely inactive, but β-amylase and especially α-

amylase are highly active, resulting in a highly fermentable wort [96]. If large quantities of undermodified malts or raw grain adjuncts are used in the grist, however, FAN may be lower and β-glucan concentrations may be higher, resulting in downstream processing and quality issues [95]. Many other brewers attempt to address the non-overlapping enzyme temperature requirements by utilizing multi-temperature 'step mashes,' where the mash temperature is held for periods of time at progressively higher temperatures. Step mashes may include a 'β-glucan rest' at 35 – 45 °C to degrade β-glucan, a 'protein rest' at 50 – 55 °C to target additional proteolysis, and a 'sugar rest' at 63 – 70 °C with a goal of starch hydrolysis, all over the course of 1 – 2 hours [63].

From the mash tun, the grist and wort slurry may be separated through several methods. Traditionally, it is pumped to a lauter tun, where the wort is recycled from the bottom through the top and percolates back through the grain bed in a filtration process called vorlaufing. Afterward, the filtered wort is drained off the bottom into the boil kettle ('lautering') and additional fresh water is added to the top of the grain bed ('sparging') to filter through and extract residual soluble materials [63]. This process relies on husk materials from the malt to form channels in the grain bed through which the wort percolates as it is extracted and maintains a consistent flow rate by reducing the tendency of the grain bed to compress on itself[97]. Worts with high soluble protein or β-glucan concentrations tend to be thicker and more viscous, and thus are more prone to slow or completely halt mash separation [98]. Modern innovation has decreased time, energy, and water costs by incorporating centrifuges and mash filters, which separate the solids from the wort based on density and particle size. These also greatly reduce the risk of a stuck mash where the wort extraction has halted before it is complete [99,100].

Once the wort has been completely extracted from the spent grain solids, the liquid is boiled and hops are added at precise times to extract and convert specific compounds, but the contributions from malt to the beer are essentially complete. Further downstream, the boiled wort is cooled, filtered, and transferred to a fermentation vessel. Here, *Saccharomyces* spp. yeasts are added to consume the sugars, FAN and other enzymatic byproducts of the mash, and ethanol, $CO_2$ and other fermentation byproducts are produced in the final beer [49].

## 1.4 Proteomics

The term 'proteome' refers to the entirety of proteins in a given system at a specific time and condition, usually an organism, cell, or tissue [101]. In food and agriculture sciences, proteomics has been used in a multitude of ways, such as identifying specific proteins, aiding in functional analysis of those protein systems, or even ingredient authentication [102–104].

Historically, protein research on cereal grains has either focused on the proportion of specific protein groups or on quantifying total protein content using combustion or acid hydrolysis methods. The major protein groups in cereal grains were first defined based on their solubilities: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and alkali-soluble glutelins [105]. This perspective has allowed, for example, early research into the fate of barley's alcohol-soluble hordein (prolamin) proteins during germination and malting [106,107], which if large quantities remain intact are known to have outsized negative consequences on beer quality [108] and on consumer health for those experiencing celiac disease and non-celiac gluten sensitivity [109]. Since then, gel-based techniques such as sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) that separate proteins by size have been developed, where purified protein mixtures are loaded into gels and differentially migrate to an applied current based on their molecular weight [110]. Though much successful research has

been performed using these methods, they are hindered by an inability to load large amounts of proteins at a time, poor separation of very hydrophobic proteins, co-migration of multiple proteins in the same band and an failure to identify proteins if purified standards are not readily available [111].

More recently, mass spectrometry- (MS-) based techniques have been developed that provide for much greater breadth and specificity. Intact proteins are difficult to measure analytically due to their size, thus for MS-based proteomics, proteins are first purified from all other potential contaminants and subjected to enzymatic hydrolysis to reduce their size to easily ionizable peptides prior to injection into the analytical instrumentation [112] (***Figure 5***). The most widely-used enzyme for this is trypsin, which is known to cleave peptide chains at only the C-terminal side of lysine and arginine residues, unless a proline is present at the carboxyl side [113]. Because these trypsin cleavages are so predictable and generally relatively close together, the amino acid sequence of the resultant peptide chain can be estimated with surprising accuracy from the mass-to-charge (*m/z*) ratio spectral peaks after the peptides have been ionized in the mass spectrometer and recorded by the detector [114]. Protein identification and quantification estimates are then made based on the presence and abundance of fragmented peptides in the sample that are computationally compared to *in silico* fragmented peptides from a protein database [114]. A major limitation of this methodology is that proteins can only identified if they are present in the protein sequence database(s). Though some methods do not utilize on-line separation of the samples prior to injection in the mass spectrometer, “bottoms-up” proteomics uses high-performance liquid chromatography (HPLC) columns to separate the complex mixtures to elute a sample into the mass spectrometer using a gradient of solvents to separate peptides based on polarity and other biochemical aspects, allowing for thousands of MS scans

over the entire sample and higher resolution spectral analyses [112]. Additionally, label-free quantification (LFQ) is a method used to compare the relative abundances of proteins between samples, as it has been observed that ion peak intensity of each peptide is generally proportional to the concentration of the same peptide in a sample when using high resolution mass spectrometers [115].

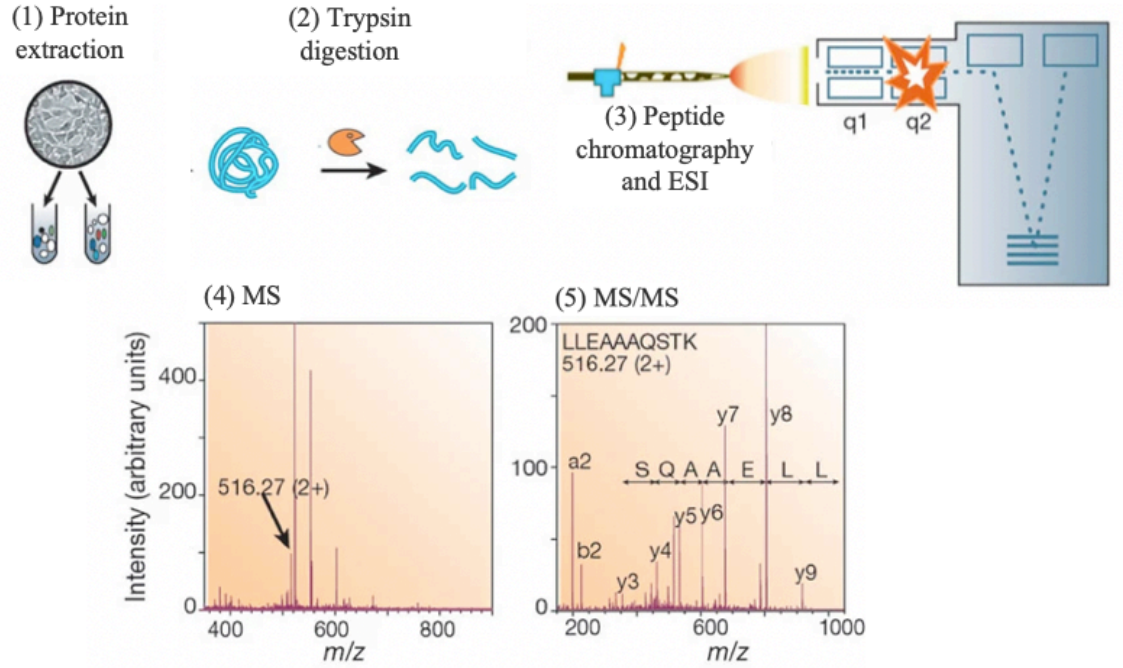


***Figure 5*. Generic proteomics workflow utilizing HPLC MS/MS.** Samples are (1) treated to extract and purify proteins from contaminants, (2) digested into peptides using the enzyme trypsin, (3) separated by HPLC and nebulized via electrospray ionization (ESI), (4) injected into a mass spectrometer with the resultant m/z spectrum, and (5) a tandem MS/MS spectrum generated by fragmentation of peptides within a specified m/z range. Figure adapted from [112].

In tandem with the development of these analytical technologies, research into cereal grain proteins has been undergoing an organizational shift. Instead of grouping proteins based on their solubilities, more research has reorganized these proteins relative to their functionality for the organism. For the proteins found in grains, these can be grouped into storage, metabolic, protective, and structural types [116].

Storage proteins are generally characterized by providing germinating seeds with nitrogen, sulfur, and carbon, and the group is predominantly comprised of prolamins and globulins. The prolamin superfamily of proteins is almost ubiquitous in cereal grains, and can be found in barley (hordein), wheat (glutenins and gliadins), rye (secalin), and oats (avenins), among many others. In barley, hordeins are the dominant protein in the endosperm, and comprise up to 80 % of the total protein in fully mature seeds [117,118]. Globulins make up the majority of the remainder of the storage proteins, and are produced during seed filling in the aleurone layer and are found in the embryo, endosperm, and aleurone layers of mature seeds [119,120].

Protective proteins are named for their role in protecting the seeds from biotic stressors such as pests or pathogens. Serine protease inhibitor proteins, also known as serpins, function to inhibit chymotrypsin-like enzymes used by insects and other pathogens. Other trypsin inhibitors have been found in in barley seeds and serve similar defensive functions [121]. Non-specific lipid transfer proteins (ns-LTPs) also protect the seed from foreign enzymes, but also reduce evaporative water loss by assembling into hydrophobic polymers [122]. Though protective proteins have not been shown to date to be linked with malting quality, serpins (*e.g.* Protein Z) [123] and lipid transfer proteins (*e.g.* LTP-1) [124] from malt have been shown to have positive impacts on foam quality when present at sufficient concentrations in beer.

Metabolic and structural proteins are a diverse and less-well-defined group. In barley and malt, the major focus of this group is on many of the enzymes previously discussed, including amylases, proteases, and lipoxygenases [17]. This group may also include proteins overlapping in other groups, such as ns-LTPs, for their eponymous role in shuttling phospholipids and other fatty acids [122], which are also grouped with protective proteins.

A number of malt and beer MS-based proteomics studies have been conducted in recent years (***Table 2***), but the most comprehensive barley malt proteome investigation was undertaken in 2018 by Mahalingam [125], in which barley was malted at lab scale and sampled as the grain was steeped, germinated and kilned. In this study, a total of 1,418 proteins were identified amongst all samples, with many present though all stages, though only a small number of proteins were identified as unique to any one of the sampling stages. Quantitation of the proteins at each stage showed that levels of α-amylase, for example, were more abundant later in germination, which is consistent with previous research on increased α-amylase activity throughout germination. Additionally, hierarchical clustering was used to identify groupings of proteins exhibiting similar changes in abundance during the malting stages. A recent study by Kerr *et. al.* in 2019 [126] used LC-MS/MS proteomics analysis on barley seeds to distinguish between malting- and feed-type varieties. They showed that malting barley varieties, even prior to malting, showed greater amounts of specific proteins related to both starch biosynthesis and hydrolysis, namely β-amylase, sucrose synthase 1, and sucrose synthase 2, than the amounts found in feed barley varieties. In 2018, Schulz *et al.* [127] analyzed samples of wort and beer brewed at benchtop-scale, and showed that the beer proteome was significantly different when less- or more-modified barley malt was used. More recently in 2021, Kerr *et al.* [128] showed that the abundances of some barley proteins are dependent on both the temperature of the mash and the amount of time held at a given temperature.

***Table 2*. Recent mass spectrometry-based proteomics research on barley and wheat in seed, malt, wort, and beer systems.**

| Grain | Material | References |
|---|---|---|
| barley | seed | Østergaard *et al.* 2002 [129] |
| barley | seed, malt | Jin *et al.* 2014 [130] |
| barley | malt | Flodrová *et al.* 2019 [131] |
| barley | malt | Jin *et al.* 2014 [132] |
| barley | malt | Mahalingam 2019 [133] |
| barley | malt rootlet | Iimure *et al.* 2012 [134] |
| barley | wort | Schulz *et al.* 2018 [127] |
| barley | wort, beer | Perrocheau *et al.* 2005 [135] |
| barley | wort, beer | Kerr *et al.* 2019 [136] |
| barley | beer | Berner *et al.* 2013 [137] |
| barley | beer | Fasoli *et al.* 2010 [138] |
| barley | beer | Picariello *et al.* 2012 [139] |
| barley | beer, malt, seed | Colgrave *et al.* 2012 [28] |
| barley, others | beer | Spada *et al.* 2020 [140] |
| barley, wheat, einkorn wheat | beer | Picariello 2015 [141] |
| barley, wheat | beer | Dong *et al.* 2015 [142] |
| wheat | germinating seed | Li *et al.* 2019 [143] |
| wheat | roots | Østergaard *et al.* 2002 [129] |

To date, very little research has been performed on the Kernza grain proteome. Work thus far has been limited to SDS-PAGE gel separation techniques to identify proteins mainly based on molecular weight, and focusing on broad categories of proteins, such as albumins and globulins. Rahardjo *et. al.* [144] compared several *T. intermedium* breeding lines with those from wheat, to examine the relative abundance of the proteins responsible for the gluten networks, which determine bread dough texture. The *T. intermedium* lines contained sufficient α-, β-, and γ-gliadins and some low molecular weight glutenins, but were deficient in high molecular weight glutenin subunit proteins [144]. Though the Kernza genome has been sequenced [24], it has not yet been converted into an proteomic database, a significant obstacle that hinders MS-based proteomic research on this species.

# 2. Materials and Methods

## 2.1 Malting

### 2.1.1 Raw Grain Selection

Samples of raw, sorted, dehulled 'M5' variety Kernza were obtained from Sprowt Labs (Burnsville, MN, USA) and identified as Lot #3. This grain was harvested from the Lincoln Pipestone Wellhead Protection Project in Lake Benton, MN, USA, where 54 acres of Kernza were planted in 2018 on a wellhead location to establish if Kernza is able reduce nitrates in groundwater more effectively than annual row crops [145]. This crop was not certified organic but was grown without the addition of synthetic pesticides or fertilizers. Roughly 24,000 lbs of grain were harvested in Fall 2019 and dehulled using an impact dehuller with a tough rubber impact surface. Grain moisture and deoxynivalenol (DON) content (***Table 3***), a mycotoxin and indicator of *Fusarium* spp. mold contamination, were measured prior to cleaning. A target of DON concentrations of <1.0 ppm has been established for grain and malt destined for beer brewing [146]. A 10 lb sample of this lot was shipped in paper bags in Fall 2020 to UC Davis and held at room temperature until use.

Samples of raw 'Copeland' barley were obtained from Admiral Maltings (Alameda, CA, USA) in November of 2020 and identified as 'Lot TL6'. This barley was grown and harvested at Cascade Farms in Tulelake, CA, USA in 2019 using organic practices and without supplemental irrigation after planting. Grain moisture and protein content were analyzed by the Hartwick College Center for Craft Food & Beverage after reception at Admiral Maltings. Rapid visco analysis (RVA) was also performed to assess the potential for loss of germinative capacity during storage [147], and the reported RVA of ≥120 for this lot indicates a low risk of rapid germination loss in storage.

***Table 3*. Reported quality parameters for raw Kernza and barley lots used for malting**, as provided by the grain suppliers: Admiral Maltings and Perennial Pantry, respectively.

| | | Kernza 'M5' Lot #3 | Barley 'Copeland' Lot TL6 |
|---|---|---|---|
| *Moisture (wet basis)* | *%* | 13.0 | 10.5 |
| *Protein (dry basis)* | *%* | - | 10.6 |
| *RVA* | | - | 180 |
| *DON* | *ppm* | 0.3 | - |

### 2.1.2 Micromalting

Kernza kernels were sifted and sorted to remove broken kernels, foreign seeds, husks, and other plant material using USA Standard Testing Sieves (Fisher Scientific, Waltham, MA, USA) and retaining the portion between No. 10 (1.4 mm) and No. 14 (1.00 mm) sieves. The barley had been sorted prior to reception and did not require additional cleaning. Both grains were each portioned into four individual 400 g units in plastic bags and shipped to the Hartwick College Center for Craft Food & Beverage (NY, USA) for malting on an automated dual tank 2SG Steep Germinator Curio Malting unit (Milton Keynes, UK) and kilned in a two-unit 2K Curio Malting MMK kiln. Kernza was divide into four treatments (***Table 4***): long (A), mid (B), and short (D) steep times with air rests, all with a low kiln temperature of 55 °C for 22 hours, and a mid steep time with air rests (C) with a high temperature step-wise kilning ranging from 55 °C to 85 °C for 22 hours. All Kernza germination times were kept constant at 15 °C for 96 hours. Barley was divided into two treatments: a low kiln temperature of 22 hours at 55 °C (A), and a step-wise kiln temperature ranging from 55 °C to 85 °C, over a total of 22 hours (B, C, D). Steep times and temperatures for barley were identical for all treatments at 8 hours submerged ("wet"),

14 hours exposed to air ("dry"), 6 hours wet, and 12 hours dry, all at 14 °C. Germination times and temperatures were held constant for all barley treatments at 15 °C for 96 hours.

***Table 4.*** **Steep, germination and kilning treatments used in this study** in paired Curio Malting steep/germination and kiln units at Hartwick College with temperatures ( °C) and times in hours (h). Shorter and longer steep times were tested to mitigate the low and slow germination of Kernza during bench trials. All germination times were kept constant. Low kiln temperatures are used to dry the grain and retain high enzyme activity, and the higher kiln temperatures used here are typical for pale malts and can add flavor complexity from Maillard reactions.

| | Kernza | | | | Barley | |
|---|---|---|---|---|---|---|
| | Long steep t, low kiln T (A) | Mid steep t, low kiln T (B) | Mid steep t, high kiln T (C) | Short steep t, low kiln T (D) | Low kiln T (A) | High kiln T (B, C, D) |
| *Steep* | 6 h wet (14 h air) 8 h wet (12 h air) @ 14 °C | 6 h wet (14 h air) 5 h wet (12 h air) @ 14 °C | 6 h wet (14 h air) 5 h wet (12 h air) @ 14 °C | 6 h wet (14 h air) 2 h wet (12 h air) @ 14 °C | 8 h wet (14 h air) 6 h wet (12 h air) @ 14 °C | 8 h wet (14 h air) 6 h wet (12 h air) @ 14 °C |
| *Germ* | 96 h @ 15 °C | 96 h @ 15 °C | 96 h @ 15 °C | 96 h @ 15 °C | 96 h @ 15 °C | 96 h @ 15 °C |
| *Kiln* | 22 h @ 55 °C | 22 h @ 55 °C | 6 h @ 55 °C, 6 h @ 65 °C, 6 h @ 72 °C, 4 h @ 85 °C | 22 h @ 55 °C | 22 h @ 55 °C | 6 h @ 55 °C, 6 h @ 65 °C, 6 h @ 72 °C, 4 h @ 85 °C |

Raw grains (sample 1) were held in a -20 °C freezer until use at UC Davis. During the malting process, samples of roughly 10 grams of both grains from each treatment were removed at specified times (***Figure 6***): directly after steeping / start of germination (sample 2), and 24, 48, 72 and 96 hours after the start of germination (samples 3 – 6, respectively). These malt samples were flash-frozen with liquid nitrogen and held at -20 °C for a minimum of 24 hours until all samples had been collected. Flash-freezing was followed by freeze-drying under vacuum in a Labconco Freeze Dryer model 75034 (Kansas City, MO, USA), until the frost disappeared from the surface of the container, or roughly 48 hours, then portioned into Wirl-Pak plastic bags (Madison, WI, USA). The finished malt after kilning (sample 7) was transferred to Ziploc (Racine, WI, USA) plastic bags, which were shipped to UC Davis and stored at -20 °C in a freezer until use.

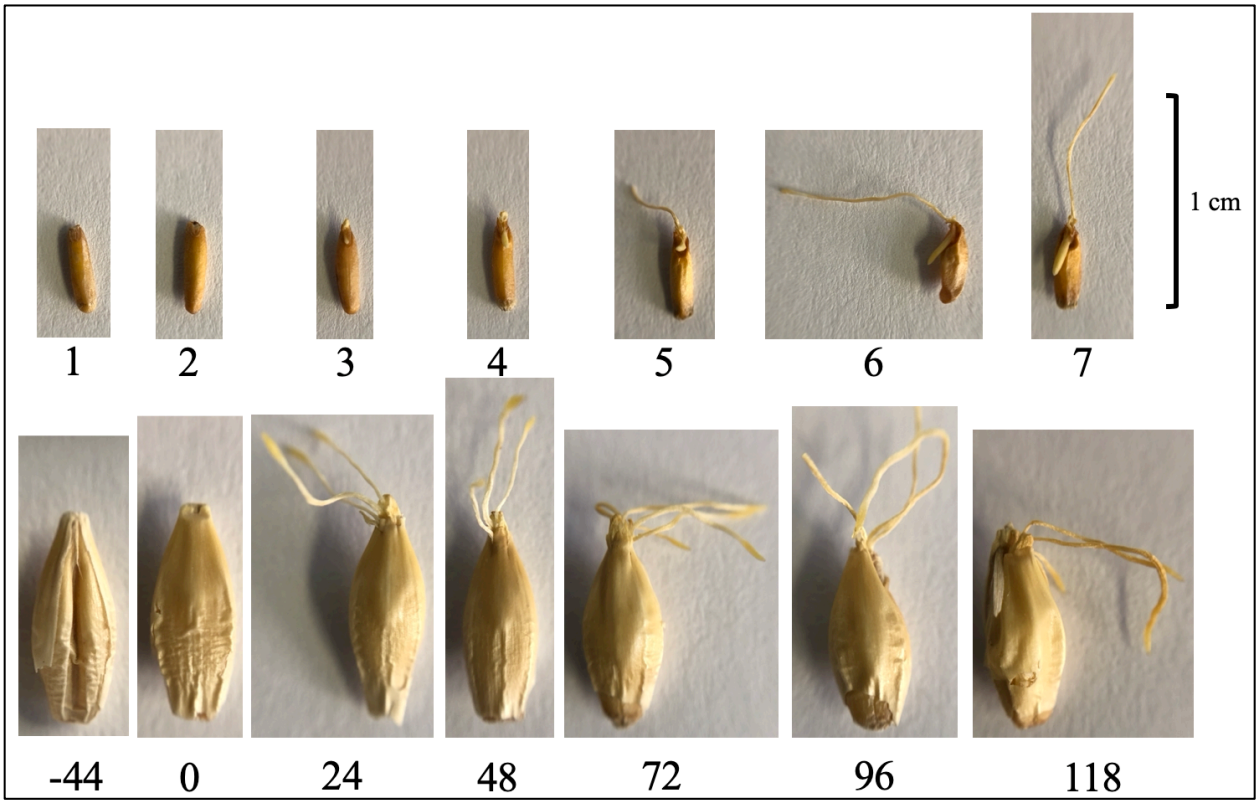


***Figure 6*. Kernza (top) and barley (bottom) grain samples from each successive malting stage.** Samples shown to scale, and arranged in time series with sample numbers listed in the center from raw (1), post-steep (2), every 24 hours of germination after steeping (3 – 6), and after kilning (7). Time from start of germination, in hours, for each sample point is listed at the bottom.

### 2.1.3 Traditional Grain & Malt Analysis

#### 2.1.3.1 Germinative Energy, Germinative Capacity, & Water Sensitivity

Prior to malting, the germinative capacity, germinative energy, and water sensitivity for both grains was assessed following ASBC method Barley-3: Germination [148]. Here, kernels from each species were transferred separately to two 90 mm polystyrene petri dishes (Falcon Plastics, Los Angeles, CA, USA), each containing 100 kernels and either 4 mL or 8 mL of deionized water and two No. 1 filter papers (Whatman, Germany), in duplicate. The dishes were closed and held at room temperature for 72 hours, with the chitted kernels removed every 24 hours. After the 72 hours, the remaining ungerminated kernels were counted, and 2 mL of a

solution of 0.75 % (w/v) hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) was added to the petri dish, and held at room temperature for an additional 48 hours before counting again.

#### 2.1.3.2 Starch Gelatinization

Starch gelatinization temperatures in the raw grains and highest-extract malt samples (below) was measured via differential scanning calorimetry (DSC) using a TA Instruments DSC250 (New Castle, DE, USA), in duplicate, as described previously [149]. To the supplied aluminum pan, 1.5 mg of flour was weighed and 2.5 μL deionized water was added (*i.e.* 3:5 m:v). The pan was hermetically sealed with an aluminum lid and allowed to equilibrate to room temperature. Isothermal scans were performed while equilibrating the sample at 20 °C for 3 min, then while heating the sample from 40 °C to 100 °C at a rate of 5 °C per minute, with an empty pan used as a reference. Onset ($T_o$), and peak ($T_p$) starch gelatinization temperatures were calculated using the supplied TA software based on the endothermic transition peaks.

#### 2.1.3.3 Moisture Content

Moisture content for the four selected malts and the original, unmalted raw grain samples was measured in duplicate following ASBC method Malt-3: Moisture [150] using 5.0 g of finely-ground malt and desiccating in a Shel Lab SMO1 forced-air oven (Cornelius, Oregon, USA) at 102 °C for 3 hours, with the change in mass attributed to evaporative water loss. Moisture was reported as a percentage of the total weight of the malt or grain including water, or "wet basis".

#### 2.1.3.4 Protein Content

Total protein was measured on the finely ground grain and malts using the Dumas combustion method following ASBC method Malt-8: Protein [151] using a Leco FP-528

elemental analyzer (St. Joseph, MI, USA). A nitrogen-to-protein conversion factor of 6.25 was used for the barley samples [152] and the wheat conversion factor of 5.70 [153] was used to for Kernza, as they are more closely related. Both total nitrogen and total protein are reported as a percentage by weight in dry matter.

#### 2.1.3.5 Friability

Friability of the two barley malt samples was calculated according to ASBC method Malt-12: Friability [154] using a Pfeuffer friabilimeter (Kitzingen, Germany). The friabilimeter presses whole kernels with a roller into a screen drum and retains only the unmodified portions of the grain larger than the 1 mm pore size of the screen. These retained portions are weighed and divided from the total mass of malt to estimate the proportion of the grain that remained unmodified after malting. A portion of the Kernza grains were too small for this method and allowed for unground kernels to pass through the drum, resulting in a potential over-estimation of modification. Thus, assessment of modification for the Kernza malts did not use this method.

#### 2.1.3.6 Total β-glucan

Total β-glucan in the raw and malted grains was quantified using a methodology in development by Held, S. and Fox, G. (unpublished) using the supplied reagents from the Megazyme Mixed Linkage β-glucan Assay (K-BGLU) Kit (Wicklow, Ireland) and prepared following EBC Methods 4.16.1 and 3.10.1 as follows. Kernels were milled to a fine flour with a benchtop MIAG burr mill (Braunschweig, Germany), and 1.0 g of the malt flour samples and 0.5 g of the non-malted flour samples were weighed into 50 mL polypropylene centrifuge tubes, in duplicate. The flour was combined with 5 mL of 50 % (v/v) ethanol (Koptec, King of Prussia, PA, USA), vortexed, and boiled for 5 minutes in a boiling water bath. An additional 5 mL of 50 % ethanol was added to each tube and tubes were vortexed and centrifuged at 1,000 x *g* for 10

minutes. The supernatant was discarded, 10 mL of 50 % ethanol was added, and the tubes were vortexed and centrifuged again at 1,000 x *g* for 10 minutes, with the supernatant discarded afterward. Next, 5 mL of a 20 mM, pH 6.5 sodium phosphate buffer (Acros Organics, Fairlawn, NJ, USA) was mixed with the remaining pellet before boiling for 5 minutes on a hot plate with intermittent mixing. The tubes were removed and allowed to equilibrate to 40 ºC in a separate water bath (Blue M, Blue Island, IL, USA) for at least 5 minutes, after which 0.2 mL of 10 U lichenase (Megazyme, Wicklow Ireland) was added, and the samples were digested for one hour at 40 ºC with regular mixing. At the end of incubation, the final volume of the tubes was diluted to 15 mL using DI water. Finally, the tubes were centrifuged at 1,000 x *g* for 10 minutes and aliquots of the supernatants were analyzed by the Thermo Scientific Gallery Plus Beermaster discrete analyzer (Waltham, MA, USA), with technical duplicates, using a custom program designed to mimic the benchtop method and using only reagents supplied with the Megazyme K-BGLU kit. There, 8 µL of 0.2 U β-glucosidase in a 50 mM pH 4.0 sodium acetate buffer was added to 8 µL of the sample and allowed to incubate for 17 minutes at 37 °C, followed by an addition of 240 µL of the Glucose Determination Reagent (GOPOD Reagent) and a second incubation for 22 minutes at 37 °C. The final absorbance was measured at 520 nm and compared to a true sample blank containing 8 µL of the final diluted sample, 8 µL sodium acetate buffer (50 mM, pH 4.0), and 240 µL GOPOD reagent. The absorbance at this wavelength was then compared to the absorbance of the supplied 0.1 mg/mL D-glucose standard, the product of the β-glucan hydrolysis. The amount of β-glucan in the original sample was thus estimated from the change in absorbance following the digestion of β-glucans to D-glucose by lichenase and β-glucosidase. Total β-glucan is reported as percent by weight of dry matter using moisture values previously analyzed.

### 2.1.3.7 Extract

Following malting, all treatments for both Kernza and barley, in addition to the raw grains, were assessed for malt quality following the method adapted from ASBC Malt-4: Extract [155]. Kernels were ground to a fine flour on a MIAG benchtop burr mill (Dresden, Germany), portioned by 25 g into mash tins, and soaked in an automated, temperature-controlled mash bath (IEC, Melbourne, Australia) with 100 mL of deionized water at 65 °C for 60 minutes. Following this, deionized water at 65 °C was added to bring the final mass of malt and water to 250 g (*i.e.* 1:8 G:L), and the wort and malt slurry was passed through a 2555 ½ Whatman fluted filter (Germany), with the first 50 mL poured back over the grain bed and allowed to continue filtering until either 200 mL was collected or 2 hours had elapsed, whichever came first. Density of the extracted wort was measured in brix using a Reichert Brix/RI-Check digital refractometer (Depew, NY, USA). Brix values were converted to degrees Plato (°P) and extract values were calculated and reported as fine grind, dry basis according to the equations described in ASBC Malt-4: Extract [155].

From the Kernza treatments, the worts from the raw grain, malts from the modified steep treatment resulting in the highest extract value, and the high temperature kiln treatment were retained for additional analysis. The worts from the raw barley and both the high and low temperature kiln treatments were selected for retained analysis.

### 2.1.3.8 FAN, Wort β-glucan, pH, Color, & Clarity

On the selected retained worts (above), free alpha-amino nitrogen (FAN), color, and wort β-glucan were measured on a Thermo Fisher Gallery Plus Beermaster discrete analyzer (Waltham, MA, USA) using supplied reagents and standard programs with sample dilutions as needed. Two technical replicates were analyzed for all samples. Here, FAN is quantified based

on the reaction of alpha-amino nitrogen on primary amines with o-phthaldialdehyde (OPA) and measuring the intensity of the absorbance at 340 nm. β-glucan is quantified based on the reaction of the diluted wort with a calcofluor reagent that results in the formation of a photometric calcofluor-β-glucan complex chromaphore. The absorbance of the resultant solution is measured at 405 nm and a side wavelength of 600 nm to quantify the concentration of β-glucan in solution. FAN and β-glucan are reported as concentrations in mg/L of the wort. Wort pH was measured using a Mettler Toledo SevenCompact pH/conductivity meter with an InLab Ultra Micro pH electrode (Columbus, OH, USA). Color is reported in SRM based on absorbance at 430 nm. Clarity of the worts was assessed visually and reported qualitatively.

#### 2.1.3.9 Soluble Nitrogen & Soluble Protein

Soluble nitrogen was measured on the wort produced above via the Dumas combustion method following EBC-Analytica 4.9.3 - Soluble Nitrogen of Malt: Dumas Combustion Method [156] on a Leco FP-528 elemental analyzer (St. Joseph, MI, USA) at the UC Davis Analytical Laboratory. From this, the amount of soluble nitrogen in the raw grain or malt was calculated as percent by weight of dry matter. Nitrogen-to-protein conversion factors of 6.25 for barley and 5.70 for Kernza were used again as above, and soluble nitrogen and soluble protein are both reported.

#### 2.1.3.10 Alpha-Amylase Activity & Diastatic Power

Starch-hydrolyzing enzymatic activity for all six selected grain and malt samples defined above were also assessed, following a modified protocol from the ASBC method Malt-7: Alpha-Amylase [157] and Malt-6: Diastatic Power [158]. Enzymes were first extracted from 12.5 g of finely-milled flour using 250 mL of a 0.5 % sodium chloride solution, and held at 20 °C for 2.5 hours with intermittent mixing. Following the infusion, the mixture was filtered using a fluted

paper filter, returning the first 50 mL and collecting for a maximum of 30 minutes, then diluted to a ratio of 1:5 with fresh 0.5 % sodium chloride solution. Alpha-amylase activity was measured following using a Thermo Fisher Gallery Plus Beermaster discrete analyzer (Waltham, MA, USA) using supplied reagents. Diastatic power (DP) was assessed following Kiviluoma *et. al.* [159], again using the Thermo Fisher Gallery Plus Beermaster discrete analyzer (Waltham, MA, USA) and a 1 % (w/w) special soluble starch solution (ASBC, St. Paul, MN, USA) as a standardized substrate.

### 2.1.4 Amino Acid Analysis

Amino acid compositions of raw Kernza and barley were determined using an L-8800a (Hitachi High-Tech America; Santa Clara, CA, USA) amino acid analyzer at the Molecular Structure Facility at UC Davis, utilizing ion-exchange chromatography to separate amino acids followed by a post-column ninhydrin reaction detection system. Raw grains were milled to a fine flour and roughly 5 mg was hydrolyzed with 200 µL of 4.5 N NaOH at 110 ºC for 24 hours and neutralized with 200 µL of 4.2 N HCl. To this, 600 µL NorLeu diluent was added, and 50 µL was injected into the instrument. Individual amino acid concentrations are reported as both a percentage of all amino acids (mg / 100 mg protein), as well as percentage of total mass on a dry basis (mg / 100 mg flour).

### 2.1.5 Proteome Analysis

#### 2.1.5.1 Sample Preparation

To extract and prepare proteins for proteomic instrumental analysis, raw grain and malt from all stages of the modified steep treatment resulting in the highest extract value, as well as the high temperature kiln treatment for Kernza, in addition to raw grain and all stages of both the high and low temperature kiln treatments for barley, as outlined above, were removed from the

freezer for milling. Five kernels from each stage, in triplicate, were frozen with liquid nitrogen in a stone mortar and pestle and ground by hand, with additional liquid nitrogen added as needed, to achieve a fine flour. Each replicate was then transferred to a 1.5 mL polypropylene microcentrifuge tube (Eppendorf Hamburg, Germany), and returned to the -20 °C freezer for storage until later use.

Upon thawing on the benchtop, proteins from the flour were extracted, denatured, alkylated, digested and purified essentially as described in [126], with some modifications. To precipitate the proteins, 10 mg of flour was resuspended in a 1.5 mL polypropylene microcentrifuge tube (Eppendorf, Hamburg, Germany) containing 600 µL of a solution containing 6 M guanidine hydrochloride (Spectrum Chemical, New Brunswick, NJ, USA), 50 mM tris HCl buffer pH 8 (Sigma-Aldrich, St. Louis, MO, USA), and 10 mM 1,4-dithiothreitol [DTT] (Sigma-Aldrich, St. Louis, MO, USA) prepared fresh. The slurry was incubated at 30 °C for 30 minutes with shaking at 1,000 rpm with a Multi-Therm Heat-Shaker incubator (Benchmark Scientific, Edison, NJ, USA). To that, 2-iodoacetamide (Spectrum Chemical, New Brunswick, NJ, USA) was added to a final concentration of 20 mM and incubated at 30 °C for 1 hour with shaking at 1,000 rpm to alkylate the exposed cystine residues. To quench the excess acrylamide, DTT was added to a final concentration of 10 mM, vortexed and then centrifuged at room temperature at 18,000 rcf for 10 minutes to clarify. The protein concentration of the supernatant was measured using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) in a 96-well plate on a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). Based on the protein concentration, an aliquot of the supernatant calculated to contain roughly 100 µg of protein was transferred into a new 1.5 mL polypropylene microcentrifuge tube containing 1 mL of a 1:1 (v/v) methanol/acetone solution

(Fisher Chemical, Waltham, MA, USA) and vortexed. This solution was incubated in a freezer at -20 °C for 16 hours. After incubation, the tubes were centrifuged at room temp at 18,000 rcf for 10 minutes, the supernatant was discarded, and the tubes allowed to air-dry in a fume hood. To the dry protein pellet, 100 µL of a solution containing 2 µg proteomics-grade trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 100 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) in Type 3 water was added for a trypsin-to-protein ratio of 1:50 (w/w). This solution was mixed gently and incubated at 37 °C for 16 hours with shaking at 1,500 rpm. Following the digestion, the pH of each sample was adjusted with an addition of trifluoracetic acid (TFA) (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 1 % TFA to halt the digestion.

The resultant peptides of the final solution were desalted using BioPureSPN MIDI™ SPE $C_{18}$ columns (The Nest Group, Ipswich, MA, USA), wetted with 200 µL of 100 % LCMS-grade acetonitrile (Spectrum Chemical, New Brunswick, NJ, USA), and equilibrated with 200 µL of 0.1 % TFA in Type 3 water. Next, 100 µL of the final peptide solution were passed through the column, followed by a wash with 100 µL of 0.1 % TFA in Type 3 water to remove salts, and eluted into a new 1.5 mL polypropylene microcentrifuge tube (Eppendorf, Hamburg, Germany) using three rounds of 100 µL of 50 % acetonitrile and 0.1 % TFA in Type 3 water. For each wetting, equilibration, washing and elution stage, microcentrifuge tubes were centrifuged at 1,000 rcf for 2 minutes or until the column was dry, whichever came first. Peptide recovery was measured in triplicate on a QuBit3 Fluorometer (Life Technologies, Carlsbad, CA, USA) using the supplied reagents. Following this, peptides were dried in a SpeedVac vacuum concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature and resuspended in a solution of 0.1 % TFA in Type 3 water to a final peptide concentration of 1.0 µg/µL and stored in a freezer at -20 °C until transfer to the UC Davis Proteomics Core for instrument analysis.

### 2.1.5.2 HPLC MS/MS Instrumental Analysis

At the Proteomics Core, peptides were directly loaded onto an IonOpticks (Parkville, Victoria, Australia) 25 cm x 75 µm, 1.6 µm $C_{18}$ Aurora column with a Captive Spray emitter. Peptides were separated using a Bruker nanoElute UHPLC (Billerica, Massachusetts, USA) at 400 nl/minute using gradients containing variable ratios of Solvent A (0.1% formic acid in water) to Solvent B (0.1 % formic acid in acetonitrile). Gradient conditions were 0 to 60 minutes (2 % to 14 % Solvent B), 60 to 90 minutes (14 % to 24 % B), 90 to 100 minutes (24 % to 34 % B), 100 to 110 minutes (34 % to 95 % B), and 110 to 120 minutes (95 % B). Eluting peptides were then further separated using trapped ion mobility spectrometry (TIMS) on a Bruker timsTOF Pro mass spectrometer (Billerica, Massachusetts, USA). Mass spectrometry data was acquired using the DDA PASEF method [160]. The acquisition scheme used was 100 ms accumulation, 100 ms PASEF ramp (at 100 % duty cycle) with up to 10 PASEF MS/MS scans per topN acquisition cycle. The capillary voltage was set at 1700 V, and capillary gas temp to 200 °C. The target value was set at 20,000 a.u. with the intensity threshold set at 500 a.u., and a m/z range surveyed between 100 to 1700. Precursor ions for PASEF-MS/MS were selected in real time from a TIMS-MS survey scan using a non-linear PASEF scheduling algorithm. The polygon filter (200 to 1700 m/z) was designed to cover ions within a specific m/z and ion mobility plane to select multiply charged peptide features rather than singly charged background ions. The quadrupole isolation width was set to 2 Th for m/z <700 and 3 Th for m/z 800.

### 2.1.5.3 Data Processing

Mass spectrometry raw files were processed with MSFragger v3.3 (www.nesvilab.org). Because the proteome of *T. intermedium* has not yet been databased, the Kernza sample spectra were searched against the complete Swiss-Prot and TrEMBL UniProt databases

(www.UniProt.org) of *Hordeum vulgare* (barley; taxon ID: 4513; 211,370 entries), *Triticum aestivum* (wheat; taxon ID: 4565; 143,870 entries), and *Secale cereal* (rye; taxon ID: 4550; 820 entries) and the cRAP database of common laboratory contaminants (www.thegpm.org/crap; 115 entries), all downloaded July 29, 2020. Barley sample spectra were searched separately against only the complete Swiss-Prot and TrEMBL UniProt database for *H. vulgare* and the cRAP database. Decoy sequences were generated and appended to the original database for MSFragger. A maximum of two missing cleavages were allowed, the required minimum peptide sequence length was 7 amino acids, and the peptide mass was limited to a maximum of 4,600 Da. Carbamidomethylation of cysteine residues was set as a fixed modification, and methionine oxidation and acetylation of protein N termini as variable modifications. The initial maximum mass tolerances were 70 ppm for precursor ions and 35 ppm for fragment ions. A reversed sequence library was generated and used to control the false discovery rate (FDR) at less than 1 % for peptide spectrum matches and protein group identifications. Decoy database hits, proteins identified as potential contaminants, and proteins identified exclusively by one site modification were excluded from further analysis. Label-free protein quantification was performed with the IonQuant algorithm [161]. All other MsFragger parameters were kept at their default values. The complete data set reported in this study comprises 48 raw files. Samples were grouped by mass spectrometric acquisition methods.

All Kernza and barley protein razor intensity values were exported into Microsoft® Excel 2019, Version 16.16.27 (201012), and the data was filtered to retain only proteins present in at least two of the three replicates for each sample stage; thus if the protein was present in zero or only one of the replicates, it was considered not present at that sample stage. For those proteins deemed present, the average of the available razor intensity values was calculated from the

replicates. From here, the average relative razor intensity for each protein at each stage was calculated by dividing the average razor intensity at that sample stage by the maximum average razor intensity across all stages, resulting in a unitless value for each stage, ranging from 0 to 1. A value of 1 indicated the razor intensity of the protein at that stage is at the maximum value for all stages, and therefore the highest abundance relative to all stages. Additionally, all proteins were annotated from the UniProtKB database using each unique protein identifier entry code. Proteins were annotated with protein name, organism, reviewed status, protein length (in Daltons), keywords, gene ontology (GO) and GO IDs, function, and pathway.

## 2.2 Mashing

### 2.2.1 Malt Selection

To prepare malt for mashing, 25 grams of the barley and Kernza malt from the final kilned stage of treatment A, as defined above, was portioned from the freezer in triplicate. Each replicate was separately frozen with liquid nitrogen in a stone mortar and pestle and ground by hand, with additional liquid nitrogen added as needed to achieve a fine flour. This flour was then transferred to a 50 mL polypropylene centrifuge tube (Falcon Plastics, Los Angeles, CA, USA) and returned to the -20 °C freezer for storage until later use.

### 2.2.2 Micromashing

Two mash profiles were chosen (***Figure 7***): an infusion mash of a constant 65 °C for 60 minutes and a step mash starting 45 °C and held for 20 minutes, increasing to 55 °C and held for another 20 minutes, and then increasing to 65 °C and held for a final 20 minutes.

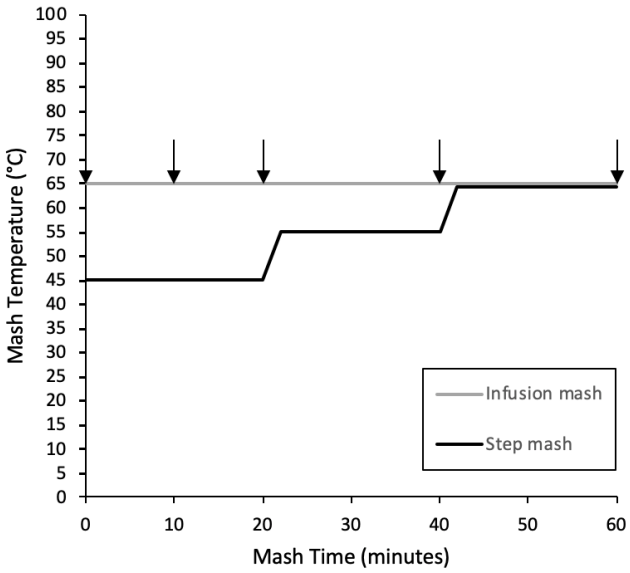


***Figure 7.*** **Mash profiles used for micromashing.** Infusion mash was held at a constant 65 °C for 60 minutes, while the step mash consisted of three 20-minute holds at 45 °C, 55 °C, and 65 °C. Sample times for both mash profiles are marked with arrows at 0, 10, 20, 40 and 60 minutes.

To mash very small volumes of the samples, the mashes were handled essentially as described previously [128], with some modifications. First, 200 mg of malt flour was portioned into 1.5 mL polypropylene microcentrifuge tubes (Eppendorf, Hamburg, Germany). For the separate "infusion" and "protein rest" mash schedules, 5 time point samples were prepared, in triplicate. Type 3 water was warmed on a hot plate to 2 °C above the starting mash temperature as 'strike water.' To each of the tubes, 1 mL of strike water was added and vortexed to create a slurry. Tubes were then immediately inserted into a Multi-Therm Heat-Shaker incubator (Benchmark Scientific, Edison, NJ, USA), preheated to the appropriate starting temperature and shaking set to 1,500 rpm.

At 10, 20, 40 and 60 minutes after the start of the mash, the triplicate samples for that timepoint were removed, centrifuged at 16,100 rcf, and the supernatant decanted into a new 1.5 mL microcentrifuge tube with the solids discarded. The supernatant was immediately inserted into a Labnet AccuBlock Digital Dry Bath D1200 static heat block (Woodbridge, NJ, USA) set

to 102 °C and held for 2 minutes to inactivate thermosensitive enzymes. Samples were then cooled quickly in an ice slurry and held until all samples had been collected. The 0-minute sample was prepared by adding 1 mL of strike water at the given temperature, vortexing, and immediately centrifuging and following the steps described above.

### 2.2.3 Traditional Wort Analysis: FAN, Protein, pH, Density, & Extract

Free amino nitrogen (FAN) and protein were on each sample with two technical replicates on the Thermo Fisher Gallery Plus Beermaster discrete analyzer (Waltham, MA, USA) using supplied reagents and standard programs with sample dilutions as needed. FAN was measured using similar to the worts produced previously for malt assessments (above). Wort protein is quantified here by measuring the absorbance of protein-cupric ion complexes at 540 nm with a side wavelength at 700 nm and uses a bovine serum albumin (BSA) standard solution for calibration. FAN and protein are reported as concentrations in the final wort produced in mg/L. Wort pH was measured with a Mettler Toledo SevenCompact pH/conductivity meter with an InLab Ultra Micro pH electrode (Columbus, OH, USA). Wort density was measured in brix using a Reichert Brix / RI-Check digital refractometer (Depew, NY, USA) and converted to degrees Plato (°P). Extract values were calculated and reported as fine grind, dry basis according to the equations described in ASBC Malt-4: Extract [155] but with the liquor to grist ratio of 1:5 used here.

### 2.2.4 Proteome Analysis

#### 2.2.4.1 Wort Sample Preparation

To extract and prepare proteins for proteomic instrumental analysis, proteins from the wort were extracted, denatured, alkylated, digested and purified essentially as described

previously [127], with some modifications. To precipitate the proteins, the roughly 1 mL of collected wort collected in the 1.5 mL microcentrifuge tubes was treated with an addition of 100 µL of 4 mg/mL sodium deoxycholate (Spectrum Chemical, New Brunswick, NJ, USA) in 100 % w/v trichloroacetic acid (Spectrum Chemical, New Brunswick, NJ, USA), vortexed, and incubated in a freezer at 0 °C for 30 minutes. The sample was then centrifuged at 16,100 rcf for 11 minutes and the supernatant decanted to waste. The protein pellet was then resuspended in the same tube with 1 mL of ice-cold acetone by vortexing and incubated again at 0 °C for 15 minutes. The sample was then again centrifuged at 16,100 rcf for 11 minutes with the supernatant decanted to waste and allowed to air-dry in a fume hood with tube cap open. To denature the proteins, the pellet was resuspended in the same tube with 100 µL of a solution comprised of 8 M USP-grade urea (Research Products International, Prospect, IL, USA), 50 mM ammonium bicarbonate (Spectrum Chemical, New Brunswick, NJ, USA) and 5 mM 1,4-dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) in Type 3 water. This was then incubated at 56 °C for 30 minutes with shaking at 500 rpm with a Multi-Therm Heat-Shaker incubator (Benchmark Scientific, Edison, NJ, USA). To this, 2-iodoacetamide (IAA) (TCI America, Portland, OR, USA) was added to a final concentration of 25 mM to alkylate the cystine residues, and was incubated at room temperature in the dark for 30 minutes. Excess IAA was quenched with the addition of DTT to a final additional DTT concentration of 5 mM.

The total protein concentration of each sample was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with the addition of a Reducing Agent Compatibility Kit due to the urea and DTT in solution, and measured using a 96-well plate on a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). Based on the protein concentration for each sample, a volume of sample

containing approximately 50 µg protein was transferred to a new 1.5 mL polypropylene microcentrifuge tube. To this, 2 µg of proteomics-grade trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added for a trypsin-to-protein ratio of 1:25, and an additional calculated volume of 50 mM ammonium bicarbonate was added to dilute the urea in each sample to below 2 M. This solution was mixed gently and incubated at 37 °C for 16 hours, with shaking at 1,500 rpm.

Following the digestion, the pH of each sample was adjusted with an addition of trifluoracetic acid (TFA) (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 1 % (v/v) TFA to stop the digestion. The resultant peptides were desalted using BioPureSPN MIDI™ SPE $C_{18}$ columns (The Nest Group, Ipswich, MA, USA) as described previously, lyophilized in a SpeedVac vacuum concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature and re-suspended in a solution of 0.1 % (v/v) TFA in Type 3 water to a final peptide concentration of 1.0 µg/µL and stored in a freezer at -20 °C until transfer to the UC Davis Proteomics Core for instrument analysis.

#### 2.2.4.2 HPLC MS/MS Instrumental Analysis & Data Processing

Peptides from each sample injection were analyzed and the resultant data processed at the UC Davis Proteomics Core as described above. The complete data set reported in this study comprises 60 raw files. The data reported here are razor intensity values for each protein, as well as the relative abundance values, calculated as described above.

## 3. Results and Discussion

### 3.1 Barley & Kernza Malting

### 3.1.1 Germination

Prior to the start of any malting run, the viability and germination potential of the raw grains must be assessed to ensure the likelihood of proper malt modification. The barley samples used in this study generated both germinative energy and capacity (*i.e.* viability) values greater than 99 %, indicating that this lot was likely to generate consistent malt data (Table 5). The Kernza samples, however, only had germinative energy values of 77 % and 80 %, indicating that a significant portion of the grains in this lot were unlikely to germinate during these malting runs. Furthermore, the germinative capacity of 78 % indicates that these seeds are likely not dormant, but dead or irreversibly damaged, and may never germinate. Similar low germination values have been observed in dehulled Kernza previously, and may be the result of damage to the embryo during the dehulling process [85]. Current continued improvement breeding at the Land Institute is focused on decreasing seed shatter and improving free-threshing [24], which may result in better germination post-dehulling. Conversely, maltsters may consider using hulled Kernza to decrease the risk of damaged kernels. However, as the hulls are only partially adhered to the kernels, if hulled Kernza is used, an excessive proportion of detached hulls may be found in the grain beds while steeping and malting. The water sensitivity assay for the Kernza resulted in a negative value, -3 %, indicating that more seeds germinated in the greater volume of water, implying that a longer steep time might be advantageous for higher germination rates. Barley water sensitivity is typically assessed to validate that barley will still germinate after steeping for extended periods in water, and results in a positive value. In this case, the barley used here was not sensitive to excessive steeping in the 8 mL treatment.

***Table 5*. Results of germination tests for raw Kernza and barley.** All barley grains germinated under all conditions, indicating this lot will produce a reliable barley malt reference. Germinative energy and capacity for the Kernza were considerably lower, indicating a portion of the kernels were unable to sprout and may not produce a homogenously-modified malt. The negative water sensitivity value indicates the Kernza germinated better in the treatment with excessive water and increased steep times might be advantageous.

| | | Kernza | Barley |
|---|---|---|---|
| *Germinative energy, 4 mL* | % | 77 | 100 |
| *Germinative energy, 8 mL* | % | 80 | 100 |
| *Germinative capacity* | % | 78 | 100 |
| *Water sensitivity* | % | -3 | 0 |

### 3.1.2 Traditional Grain and Malt Analysis

#### 3.1.2.1 Starch Gelatinization

In the mash, starch granules are exposed to warm water where the starches hydrate and swell, making them accessible to the endogenous thermosensitive malt enzymes [63]. The starch gelatinization onset and peak temperatures for malted Kernza measured here were similar to barley (***Table 6***), at 58.3 °C and 64 °C, respectively; within the range previously reported on other breeding lines of *T. intermedium* [82]. The starches found in the endosperms of barley and wheat have gelatinization temperatures ranging from 51 - 60 °C and 58 - 64 °C, respectively, with oats and rye within this range as well [27]. Endogenous amylases are also active at these temperatures [162], and thus historical mashing techniques have targeted this range for brewing with malts and raw grains of these cereals. Corn starches gelatinize between 62 – 72 °C, and those found in sorghum and rice at 68 – 78 °C. Thus, if corn, sorghum, or rice are to be used in the mash, a separate cooking step is required prior to their addition to hydrate the starches, which consequently renders many of their endogenous enzymes inactive. Therefore, the gelatinization temperatures for the starches in Kernza are ideal for mash additions without a cereal cooking

step, unlike corn, sorghum or rice. Malted Kernza displayed onset and peak temperatures roughly 0.5 °C greater than raw Kernza, implying similar efficacy in the mash. The increase in gelatinization temperatures from raw to malt was exhibited by barley as well, where peak gelatinization increased by 1.2 °C after malting. Previous research has shown similar increases in peak starch gelatinization temperatures from raw to malted barley [163], but without reasoning attributed. The temperature change is potentially an effect of the modification of some starches in the grain during malting, where starches partially hydrate during the steep and are somewhat degraded either enzymatically during germination or thermally during kilning [164].

***Table 6*. Results of traditional analyses on raw and malted Kernza and barley and the worts they produced.** Reported values are the averages of biological and technical replicates and are reported with their associated standard deviations. Superscripts indicate statistical similarity to other values in the same row by two-tailed *t*-tests ($p < 0.05$). Dashes indicate values that were not measured.

| | | Kernza | | | Barley | | |
|---|---|---|---|---|---|---|---|
| | | Raw | Malt | Malt | Raw | Malt | Malt |
| | | Unmalted | Lower temp kiln | Higher temp kiln | Unmalted | Lower temp kiln | Higher temp kiln |
| *Moisture (%)* | *%* | 10.11 ± 0.11 | 5.43 ± 0.10[a] | 3.82 ± 0.21 | 9.68 ± 0.06 | 5.56 ± 0.00[a] | 4.06 ± 0.03 |
| *Starch gelatinization onset (To)* | *°C* | 57.7 ± 0.8[b] | 58.3 ± 0.1[b] | - | 61.2 ± 0.1[a] | - | 60.7 ± 0.6[a] |
| *Starch gelatinization peak (Tp)* | *°C* | 63.6 ± 0.6[a] | 64.0 ± 0.1[a] | - | 65.3 ± 0.5 | - | 66.5 ± 0.1 |
| *Starch gelatinization enthalpy* | *J/g* | 1.15 ± 0.15[a] | 1.08 ± 0.26[a] | - | 0.78 ± 0.20[ab] | - | 0.78 ± 0.04[b] |
| *Friability* | *%* | - | - | - | - | 85.9 | 82.7 |
| α-a*mylase (dry basis)* | *DU* | 2.1 ± 0.6[a] | 18.2 ± 0.8 | 13.4 ± 0.4 | 1.3 ± 0.7[a] | 84.3 ± 1.7 | 68.0 ± 2.7 |
| *Diastatic power (dry basis)* | *°L* | 102 ± 2.5[b] | 112 ± 2.5[a] | 90 ± 2.5 | 34 ± 2.5 | 154 ± 2.5 | 108 ± 2.5[ab] |
| *Total nitrogen (dry basis)* | *%* | 2.88 | 3.00 | 2.96 | 1.77 | 1.86 | 1.87 |
| *Total protein (dry basis)* | *%* | 16.42 | 17.12 | 16.88 | 11.07 | 11.65 | 11.66 |
| *Soluble nitrogen (dry basis)* | *%* | 0.60 ± 0.00 | 1.38 ± 0.00 | 1.22 ± 0.00 | 0.40 ± 0.00 | 0.86 ± 0.00 | 0.84 ± 0.00 |
| *Soluble protein (dry basis)* | *%* | 3.42 ± 0.00 | 7.87 ± 0.00 | 6.93 ± 0.00 | 2.49 ± 0.00 | 5.36 ± 0.00 | 5.28 ± 0.00 |
| *Soluble/total nitrogen ratio, DB (S/T)* | *%* | 20.9 | 46.0 | 41.1 | 22.5 | 46.0 | 45.3 |
| *Extract, FGDB* | *%* | 63.2 ± 0.7[a] | 72.4 ± 1.5[a] | 67.1 ± 5.0[a] | 32.5 ± 2.7 | 84.4 ± 0.0 | 82.4 ± 0.7 |
| *Total β-glucan (dry basis)* | *%* | 0.85 ± 0.08[a] | 0.49 ± 0.00[c] | 0.61 ± 0.07[bcd] | 1.82 ±0.05 | 0.77 ± 0.04[ab] | 0.58 ± 0.00[d] |
| *Wort β-glucan* | *mg/L* | 354 ± 21 | 33 ± 2 | 49 ± 2 | 660 ± 25 | 125 ± 5 | 98 ± 1 |
| *Color* | *°SRM* | 1.5 ± 0.0[a] | 3.4 ± 0.9 | 3.8 ± 0.6 | 1.4 ± 0.0[a] | 1.1 ± 0.0 | 1.6 ± 0.0 |
| *FAN* | *mg/L* | 46.8 ± 0.2 | 172.9 ± 12.9[a] | 139.4 ± 12.3 | 62.7 ± 1.6 | 206.9 ± 1.9 | 189.3 ± 4.2[a] |
| *pH* | | 6.40 ± 0.1 | 5.95 ± 0.00[b] | 6.09 ± 0.04[a] | 6.08 ± 0.00[ab] | 6.08 ± 0.01[a] | 5.98 ± 0.01 |
| *Clarity* | | hazy | hazy | hazy | very hazy | clear | clear |

### 3.1.2.2 Friability

After the barley malt has been produced, the maltster typically performs a friability test to determine the extent to which the grain was modified. Well-modified malts typically exhibit friability of >80 % [165], and the barley malt references here were deemed well-modified with friability values of 85.9 % and 82.7 % for the lower and higher temperature kilned samples, respectively (***Table 6***). The individual Kernza malt kernels, however, were too small for this instrument, as some whole and only partially milled kernels were able to pass through the drum sieve, which would bias the degree of modification estimated for these malts using this method. Modification for the Kernza, therefore, was estimated using both total-to-soluble protein ratios in the malts and FAN concentrations in the produced worts, below.

### 3.1.2.3 Alpha-Amylase Activity & Diastatic Power

Starch-degrading enzyme activity, the primary utility of malt in the brewery, was measured in two ways: α-amylase activity and diastatic power (DP). Alpha-amylase activity showed a marked increase by malting the Kernza, from 2.1 dextrinizing units (DU) in the raw grain to 18.2 DU in the lower temperature kilned malt and 13.4 DU in the higher temperature kilned malt (***Table 6***). The increase in α-amylase activity during malting was substantially lower than the increase exhibited by the barley, where α-amylase was measured at 1.3 DU in the raw kernels and 84.3 DU in the lower-temperature kilned malt. The higher-kilned barley malt displayed lower activity, 68.0 DU, which is likely attributable to the decreased thermostability of α-amylase enzymes at the higher kiln temperatures [166]. The DP of the barley malt also increased significantly from raw, at 34 °L, to the lower-temperature kilned malt, at 154 °L, but did not increase as much in the higher-temperature kilned barley malt, at 108 °L. Although DP is a measure of the collective activity of a number of starch-degrading enzymes without specific

attribution to any one enzyme, it has been shown to be more correlated with β-amylase than α-amylase or limit dextrinase activity [167]. Low α-amylase activity values are generally correlated with slower mash conversion, but DP is generally considered the best overall measure of the conversion that is likely to occur in the mash [87,162]. As β-amylase is the most thermosensitive at these at higher temperatures [168], it follows that increased kiln temperatures results in lower DP values [166]. The malted Kernza did display a similar correlation between lower kiln temperatures and higher DP activity, with the lower kilned malt measuring at 112 °L and the higher kilned malt at 90 °L. Interestingly, DP of the raw Kernza did not increase as significantly when malted as it did with barley. The DP of the unmalted, raw Kernza was measured at 102 °L, which only slightly increased after malting. The high starting DP value pre-malting, coupled with an apparent lack of meaningful change in DP in both malting treatments, indicates that the raw Kernza grains in this sample already had a high level of overall starch-degrading enzymatic activity, which was retained and relatively unaltered by malting. In barley, β-amylase is known to be present in the endosperm of mature seeds and is concentrated in the sub-aleurone layer in an inactivated form bound to insoluble proteins. β-amylase is released from this bound form during malting [17]. It may be that Kernza contains most of its β-amylase in unbound forms prior to germination, and further investigation into β-amylase activity and composition, as well as the activity of other starch-degrading enzymes, may elucidate this further.

#### 3.1.2.4 Total Nitrogen & Total Protein

The raw Kernza displayed a dry weight protein content of 16.42 % (2.88 % nitrogen), slightly lower than the 17.11 % (3.00 % nitrogen) of the lower temperature kilned malt and 16.86 % (2.96 % nitrogen) of the higher temperature kilned malt (***Table 6***). Total protein in wheat ranges from 6 – 27 % by weight, though most commercial varieties are between 8 – 16 % [27].

The dry weight protein content for the barley was substantially lower, at 11.07 % (1.17 % nitrogen) in the raw grain, 11.65 % protein (1.86 % nitrogen) in the lower temperature kilned malt, and 11.66 % protein (1.87 % nitrogen) in the higher temperature kilned malt. Malting barley of good quality typically exhibits protein contents ranging from 10.0 – 13.5 % [169], and malting barley outside of this range is often rejected and diverted to animal feed. For barley malt, higher protein concentrations are generally associated with slower water uptake during steeping [170], and lower relative amounts of starch, but higher enzymatic activity, specifically β-amylase [171]. Additionally, higher levels of protein are associated with increased formation of protein-polyphenol complexes during brewing that results in downstream quality issues including haze and filtration challenges [172]. Thus, the higher protein content of Kernza in both raw and malt forms may result in haze formation downstream during brewing, and appropriate mitigation techniques should be utilized if a clear final product is desired.

#### 3.1.2.5 Soluble Nitrogen, Soluble Protein, & Kolbach Index

The soluble protein content of the Kernza was calculated to be 3.42 % (0.60 % nitrogen) of the dry weight in the unmalted samples, which increased to 7.87 % (1.38 % nitrogen) and 6.93 % (1.22 % nitrogen) in the low and high temperature kilned malts, respectively (***Table 6***). The soluble protein in the unmalted barley reference was calculated to be 2.49 % (0.40 % nitrogen) of the dry weight, significantly lower than the 5.36 % (0.86 % nitrogen) and 5.28 % (0.84 % nitrogen) in the low and high temperature kilned malts. On their own, some solubilized proteins positively contribute to foam quality and mouthfeel of the finished beer [123,124], but others may reduce overall quality in the form of haze [173]. Additionally, low levels of soluble protein are correlated with beers that are thin and insipid [49].

More importantly, the ratio of soluble nitrogen to total nitrogen (S/T, or Kolbach index) in the dry malt is used as a method to assess the extent of modification the grain has undergone during malting, and thus the effectiveness of the malting process. The two barley malt references were calculated to have Kolbach indexes of 46.0 % and 45.3 % for low and high kilned samples (***Table 6***). Similarly, the two Kernza malt samples were calculated to have Kolbach indexes of 46.0 % and 41.1 % for the low and high temperature kilned treatments. Malts are generally considered to have undergone a sufficient level of modification if 40 – 47 % of the proteins have become solubilized [174], thus indicating that the Kernza malt samples here are sufficiently modified. Similarly, the barley malts produced ratios squarely within this range, and can thus be used as an appropriate reference to represent a typical well-modified malt.

#### 3.1.2.6 Extract

The extract value for the barley references used here increased from 32.5 % unmalted to 84.4 % in the lower temperature kilned malt and 82.4 % in the higher temperature kilned malt (***Table 6***), a relative increase of over 150 % for both. The Kernza samples, however, did not exhibit a similar increase. Raw Kernza grain extract was 63.6 %, compared to 72.4 % in the lower temperature kilned malt and 67.1 % in the higher temperature kilned malt, a relative increase of less than 15 % in both. Kernza's lack of extract increase by malting may be due to the aforementioned high diastatic power present in raw Kernza, which did not markedly increase by malting it. Extract values are a representation of the combined effect of both the amount of available starch and the available activity of the endogenous starch-degrading enzymes, represented by the proportion of the dry weight of the malt that is likely to be converted to fermentable saccharides in the mash. Though starch is not synthesized during malting, it is made accessible to the starch-degrading enzymes during the malting process by enzymatic degradation

of proteins and non-starch polysaccharides that encase the starch molecules in the endosperm of unmalted kernels. The extract values for both Kernza malts found here are below a standard accepted range of 78 – 84 % for well-modified barley malt [170], and as such, it may either have (1) lower levels of starch than the barley references, (2) starch that is not available to the endogenous enzymes to degrade into soluble derivatives during the laboratory mash, or (3) starch-degrading enzymatic activity that is too low to degrade all the starches available in the malt. Additional inquiry into the composition of the carbohydrates in the raw and malted Kernza, as well as the amounts of other structural non-starch polysaccharides in the endosperm such as arabinoxylan, may elucidate this deficiency. Furthermore, the aforementioned propensity for damage to the embryo during the dehulling of Kernza may have decreased the overall finished malt quality.

### 3.1.2.7 Total β-glucan & Wort β-glucan

Total β-glucan content was found to 0.85 % dry weight in the raw Kernza grain, which decreased to 0.49 % in the lower kilned malt and 0.61 % in the higher kilned malt. The β-glucan content of the barley here decreased from 1.82 % in the raw grain to 0.77 % in the lower temperature kilned malt and 0.58 % in the higher temperature kilned malt (***Table 6***). β-glucan is the major non-starch polysaccharide found in the endosperm of raw barley, accounting for 3 – 6 % dry weight, but in much lower concentrations in well-modified barley malt (0.5 – 2 % w/w) [65]. In raw wheat, β-glucan is found in much lower quantities; generally 0.5 % w/w [175], to which the Kernza appears to be more similar. In under-modified malts, the large remaining quantities of high molecular weight β-glucans are extracted into the wort, where they increase the wort viscosity and result in mash filtration issues and decreased beer quality downstream [176]. Under-modified malts, therefore, require mashing techniques such as a ‘β-glucan rest,’

where the mash is held at 35 – 45 °C, and endogenous β-glucanase enzymes are active and able to degrade the polysaccharide into less troublesome lower molecular weight chains [63]. The smaller quantities of β-glucan found here in the Kernza malt indicate a β-glucan rest is not recommended.

When malts are assessed for their quality by producers, however, β-glucan content is more frequently measured and reported as the amount extracted into wort under standardized mashing conditions, as this is a more reliable predictor of how the malt will function in a brewery [61]. Here, the reference barley exhibited wort β-glucan of 660 mg/L with unmalted grain that decreased to 125 mg/L in the low kiln temperature and 98 mg/L in the high kiln temperature malts (***Table 6***). This decrease follows the trend exhibited in the barley total β-glucan assay, above. The wort β-glucan in the Kernza malt wort tracked similarly to the total β-glucan assay as well, with unmalted grain resulting in 354 mg/L wort β-glucan, decreasing to only 33 mg/L and 49 mg/L in the low and high temperature kilned malts, respectively. The European Brewing Convention (EBC) recommends a β-glucan limit of 250 mg/L in wort, with the Institute of Brewing & Distilling (IBD) recommending a more conservative 200 mg/L [165], and the American Malting Barley Association (AMBA) advocating for the lowest limit of 100 mg/L [174], though wort β-glucan values produced with barley malt are frequently reported in excess of 200 mg/L [177]. Thus, as before, these results imply that wort produced with Kernza should not necessitate a β-glucan rest, but do show that β-glucan content was reduced by malting.

#### 3.1.2.8 FAN

The Kernza exhibited an increase in wort FAN by malting, from 46.8 mg/L with the raw grain to 173 mg/L and 139 mg/L with the low and high temperature kilned malt samples,

respectively (***Table 6***), or a relative increase of over 175 % for both. The reference barley increased wort FAN by 200 % by malting, from 62.7 mg/L using raw grain to 206.9 mg/L and 189.3 mg/L using the low and high temperature kilned malt samples, respectively. Free amino nitrogen (FAN) is an another measure of the degree of protein hydrolysis that has occurred during malting, and to a lesser degree during mashing [90]. In barley malt, 88 % of total FAN present in wort is estimated to have been produced during malting, while only 12 % is produced during mashing [90]. FAN values have been recognized for their importance for some time, as nitrogen in this form is essential for yeast health, especially during the growth phase [178]. FAN below 130 mg/L in wort prior to pitching yeast [179] may result in decreased fermentation efficiency and negatively impacts beer quality and stability [180]. FAN is not, in actuality, only a measure of free amino acids, as it also includes di- and tri-peptides with exposed alpha-amino nitrogen [181], and does not include proline due to the inability of the o-phthaldialdehyde reagent to react with the alpha-amino nitrogen bound in a ring structure [182]. Though proline is by far the most abundant free amino acid found in barley worts [183], it is not assimilated by brewing yeasts under normal fermentation conditions due to its structure, and thus its absence from the FAN measurement is appropriate for brewing applications [69]. The amount of FAN in these Kernza worts is sufficient for yeast health and fermentation in an all-Kernza malt grist. Interestingly, though, increased FAN is generally associated with higher levels of malt protein [184], which was not seen here when the Kernza and barley malts are compared and the higher protein content of the Kernza taken into account. This suggests that the Kernza malts may require additional modification to reach higher FAN levels, or that the amino acid profiles of these free amino acids may be more heavily weighted with proline than barley malt worts, and as

this amino acid is not accounted for in the FAN measurement. Further investigation into the amino acid profiles of these worts may elucidate this further.

#### 3.1.2.9 Color & Haze

Additional notes on the wort quality include a slight increased color value of both Kernza malts and the observation of slight haze on both the malted Kernza sample worts (***Table 6***). The color values for both Kernza malts were below 4 SRM, acceptable for very light-colored worts. The observed haze indicates that turbidity-mitigation strategies should be considered when brewing with Kernza malts if clear beers are desired.

Taken together, these data strongly indicate that both samples of Kernza malt and the barley malt references malted well, and exhibit a high degree of modification under both kilning conditions.

### 3.1.3 Amino Acid Analysis

Kernza and barley showed strong similarities regarding most of the amino acid relationships measured here (***Table 7***). Proline (Pro) made up more than 12 % of total protein by weight for both grains, and Glx, a combination of glutamic acid and glutamine, was above 20 % for both. However, Kernza Glx values are markedly higher, at 27 %, compared to 20 % in barley. The third-most represented amino acid for both grains was glycine (Gly), at 6.9 % for Kernza and 7.4 % for barley. Though the relative amounts of glutamic acid and glutamine are not clear based on this assay, they are the most prevalent amino acids in prolamins, along with glycine and proline [116]. As prolamins are the most prevalent proteins in many other cereal grains [27], it might be inferred that Kernza is rich in storage proteins similar to the hordeins found in barley or glutenins found in wheat. The barley values reported here are consistent with previously-reported amino acid composition data [52]. Barley is known to be limiting in lysine and threonine [27],

and as such, Kernza can be considered limiting in these amino acids as well, as these two amino acids are lower in the Kernza sample compared to the barley sample. Additionally, when the amino acids are viewed relative to the total dry weight of the grain (***Figure 8***), Kernza is more abundant in all amino acids due to its higher overall protein content. The difference between the two species in their Glx and proline content is pronounced here as well.

***Table 7*. Amino acid compositions of raw Kernza and barley grain as a molar percentage of each amino acid to total protein**, as determined by ion-exchange chromatography. Glx denotes a combined value of glutamic acid and glutamine. Asx denotes a combined value for aspartic acid and asparagine.

| | Kernza "M5" | Barley "Copeland" |
|---|---|---|
| *Alanine* | 5.14 | 6.64 |
| *Arginine* | 3.24 | 3.8 |
| *Asx* | 5.17 | 6.73 |
| *Cysteine* | 2.94 | 2.69 |
| *Glx* | 27.09 | 20.42 |
| *Glycine* | 6.89 | 7.36 |
| *Histidine* | 1.98 | 1.97 |
| *Isoleucine* | 3.67 | 3.79 |
| *Leucine* | 6.72 | 7.11 |
| *Lysine* | 2.52 | 3.51 |
| *Methionine* | 1.63 | 1.74 |
| *Phenylalanine* | 3.81 | 4.2 |
| *Proline* | 12.13 | 12.23 |
| *Serine* | 5.77 | 5.25 |
| *Threonine* | 3.53 | 3.78 |
| *Tryptophan* | 0.79 | 1.03 |
| *Tyrosine* | 1.99 | 1.93 |
| *Valine* | 4.99 | 5.82 |

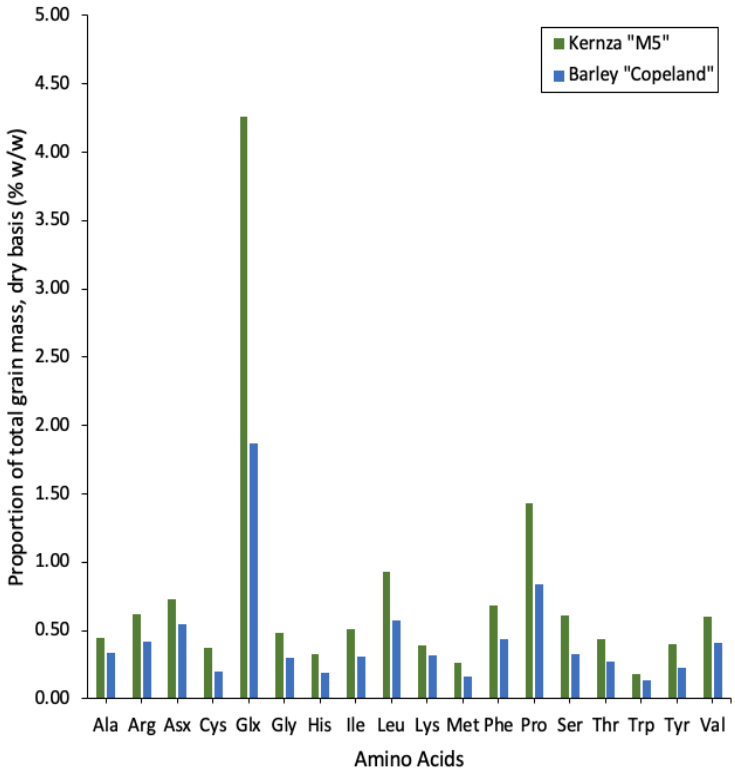


***Figure 8*. Amino acid compositions of raw Kernza and barley grain as a proportion of each amino acid to the total grain mass on a dry basis**, as determined by ion-exchange chromatography. Glx denotes a combined value of glutamic acid and glutamine. Asx denotes a combined value for aspartic acid and asparagine.

### 3.1.4 Malt Proteome Analysis

In total, over 2,000 separate proteins were identified over all the Kernza malting stages (***Table 8***). Kernza malting process samples identified 2,135 proteins, with 1,471 (69 %) of those containing identifiable names from the database, though other annotations were available through the UniProt database inferred through homology to other known proteins. Of all these named and unnamed proteins, 1,496, or roughly two thirds, were from the wheat (*T. aestivum*) database, 616 proteins were from barley (*H. vulgare*), and 23 were from rye (*S. cereale*). The low number of *S. cereale* proteins is likely due to the much smaller database of proteins relative to the those available for *T. aestivum* and *H. vulgare*. The unnamed proteins were proportionally similarly distributed between *T. aestivum* and *H. vulgare*. Of all the barley malting process

samples, 2,805 proteins were identified, and 1,942 (69 %) were identified by name. This coverage is consistent with previous proteomic barley malt studies [185], indicating that the extraction, tryptic digestion, and instrumental methods were successful.

***Table 8*. Count of proteins identified in Kernza and barley from all malting stages**. Data includes both all proteins and only those with identified names from the UniProt databases. Nearly 3,000 barley proteins were identified in all the barley malt samples. In the Kernza malting samples, over 2,000 proteins were identified, with the majority from wheat, roughly one quarter from barley, and a small number from rye. For all groups, over two-thirds of the proteins were able to be annotated with defined names.

| | Kernza | | Barley | |
|---|---|---|---|---|
| | Total | Named | Total | Named |
| *T. aestivum* (wheat) | 1,496 | 1,006 | - | - |
| *H. vulgare* (barley) | 616 | 442 | 2,805 | 1,924 |
| *S. cereale* (rye) | 23 | 23 | - | - |
| Total | 2,135 | 1,471 | 2,805 | 1,924 |

Of all the named Kernza proteins, there was significant overlap with *T. aestivum* and *H. vulgare*, with 669 of the total 1,471 (46 %) appearing in both species, and 11 proteins shared between all three species, including *S. cereale* (***Figure 9***). This is expected, as a large portion of the Kernza genome has been shown to be shared with *T. aestivum*, and Kernza is also more distantly related to *H. vulgare* [25] (***Figure 1***). Only 7 proteins were shared between *T. aestivum* and *S. cereale*, and no identified proteins were shared between only *H. vulgare* and *S. cereale*.

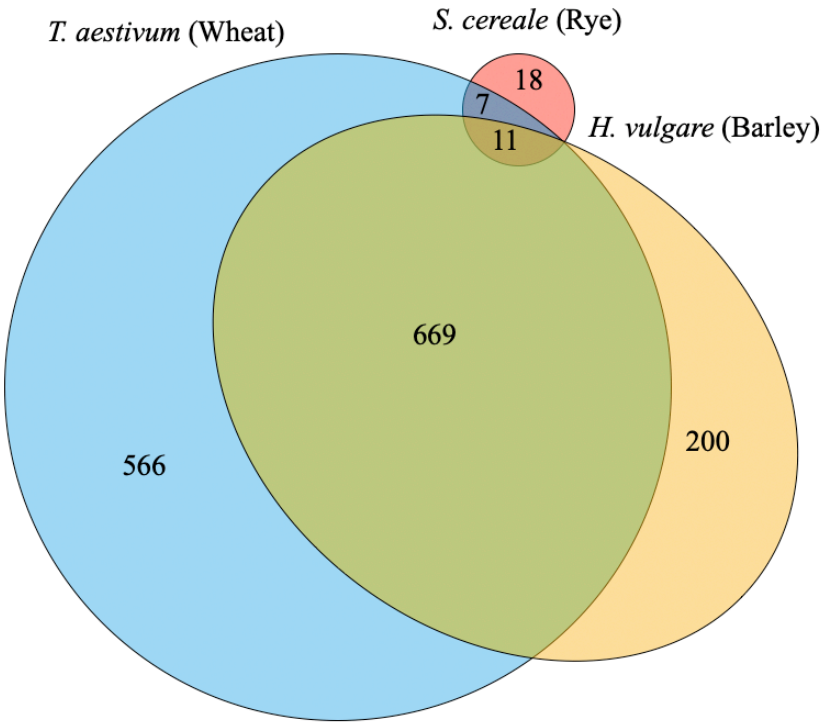


***Figure 9*. The distribution of proteins from with defined names identified in Kernza over all malting stages.** The majority of named proteins were from the *T. astivum* database, with over half overlapping with *H. vulgare*, following the taxonomy outlined in ***Figure 1***. Only a small number were identified from *S. cereale* database, likely due to the low proteome coverage for that species in the database available to search. Venn diagram generated in eulerAPE v3.

As the Kernza was malted, the number of proteins present increased from 1,217 in the raw grain to 1,651 in the final green (unkilned) malt after 96 hours of germination. This equates to a change from 57 % to 77 % of total protein representation when compared to all proteins identified over all malting stages, indicating an increase in protein diversity over the malting process (***Figure 10***). The barley exhibited a more extreme increase, with 1,363 proteins identified in the raw grain and 2,453 proteins in the green malt, or from 49 % to 87 % of all proteins identified over all malting stages.

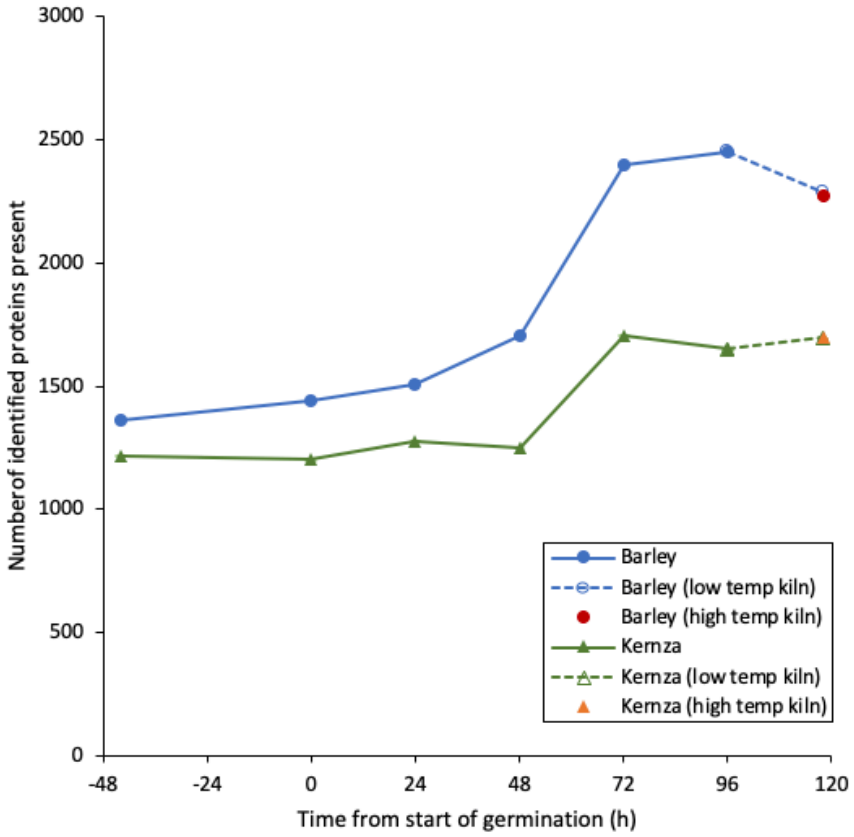


***Figure 10*. Change in the total number of identified proteins in Kernza and barley during malting and kilning.** Sampling points include raw (-44 h), post-steep (0 h), germination (+24, 48, 72, 96 h) and after 22 hours of kilning (+118 h).

The trend in protein diversity exhibited a similar inflection point during germination for both grains. Kernza samples at 72 hours into germination displayed an increase of 456 proteins (36 %) from the previous 24 hours, whereas the barley at 72 hours had increased by 689 proteins, or 40 %, over the same period. Prior stages, however, were not as consistent between the two species. From raw grain through 48 hours into germination, Kernza only exhibited a 3 % increase in the number of proteins identified, whereas barley exhibited a steady increase of 25 % over the same period. From 72 hours until the end of germination, the count of proteins for both grains was relatively unchanged, with neither Kernza nor barley differing by more than 4 %. The much slower increase over the first days of germination in the Kernza indicate that a longer germination period may have been advantageous to achieve a more fully-modified malt. Wheat

malts are known to frequently require similar germination time extensions relative to barley malts [186], and this may be true for Kernza as well. The application of exogenous gibberellic acid is frequently used in the malt industry to increase the rate of modification and enzyme synthesis in barley and wheat malts [187], and its use may be appropriate here as well.

In both kiln treatments of the Kernza malt, the total number of proteins identified were essentially the same, with 1,697 and 1,698 proteins identified in the low and high kilned samples, a slight increase from the 1,651 proteins at the last stage of germination. Interestingly, of the proteins found in both of the kilned Kernza samples, 80 were not present in the green malt, indicating that additional protein synthesis may have continued as the temperature of the kiln was slowly raised. Therefore, the malt at the 96 hour germination point may not have been fully modified and might have benefitted from additional germination time. The kilned barley malt references displayed significantly different effects from kilning, with many fewer proteins present after kilning than were found in the final germination stage. In the low- and high-temperature kilned samples, 2,272 and 2,288 proteins were identified, respectively, which represented a 7 % decrease from the green malt.

Several non-enzymatic proteins known for their importance to beer were identified in finished Kernza malt. Protein Z is a serpin-type protein found in high concentrations in the endosperm of barley, and due to its low surface hydrophobicity, it is one of the most common proteins identified in beer foam [123]. The three isoforms of Protein Z that have been identified in barley are Protein Zx (BSZx), protein Z4 (BSZ4), and protein Z7 (BSZ7), and though BSZ7 is the most prevalent (80 %), all are foam-positive and haze-forming when found in beer [188]. Though barley Protein Z was not identified in any of the Kernza malt samples, similar proteins in the serpin superfamily were identified, including Serpin-N3.2, Serpin-Z1A, and Serpin-Z1C

from wheat and other serpin-domain containing proteins from both wheat and barley. All the wheat-related serpin proteins generally maintained their abundances over the malting process, though many of the barley-related serpins decreased effectively to zero after the 3rd day of germination. No research to date has been conducted on serpin proteins from wheat in beer. A number of non-specific lipid transfer proteins (ns-LTPs) were also found in the raw Kernza grain, and some of these maintained their abundance during the malting process. Ns-LTPs are another class of foam-positive proteins found in beer derived from barley malt that have been long known for their foam-positive attributes [124]. These results indicate that haze formation and foam-positive attributes are likely attributes of beer brewed with Kernza malt consistent with the increased haze noted on the Kernza worts reported in ***Table 6***.

Storage proteins are the most abundant proteins in quiescent, raw grains prior to germination, accounting for over 75 % of all proteins in cereals [117,118]. Overall, the storage proteins in Kernza were significantly reduced in average abundance during malting (***Figure 11a***). In the Kernza samples, 39 individual seed storage proteins were identified, which included avenin, gliadin and glutenin from wheat, and gliadin and ferritin proteins from rye, all of which are prolamin-type proteins similar to the hordein found in barley. The abundance and diversity of these proteins corroborates previous studies that have identified prolamin proteins in Kernza [144] and thus Kernza products should not be labeled 'gluten free.' The overall group of storage proteins in Kernza was drastically reduced in abundance starting at 48 hours into germination and continued through to 96 hours, similar to the rate and degree of change occurring in the barley malt. The reduction in the abundance of proteins in this group is indicative of the protein hydrolysis occurring throughout germination, and the resulting hydrolysis products are known drivers of beer quality, including haze, foam quality, and FAN. Due to the higher starting total

protein content of the Kernza relative to the barley used in this study, the decrease in storage protein abundance likely resulted in higher concentrations of FAN and soluble protein found in Kernza wort when compared the barley wort reported in ***Table 6***.

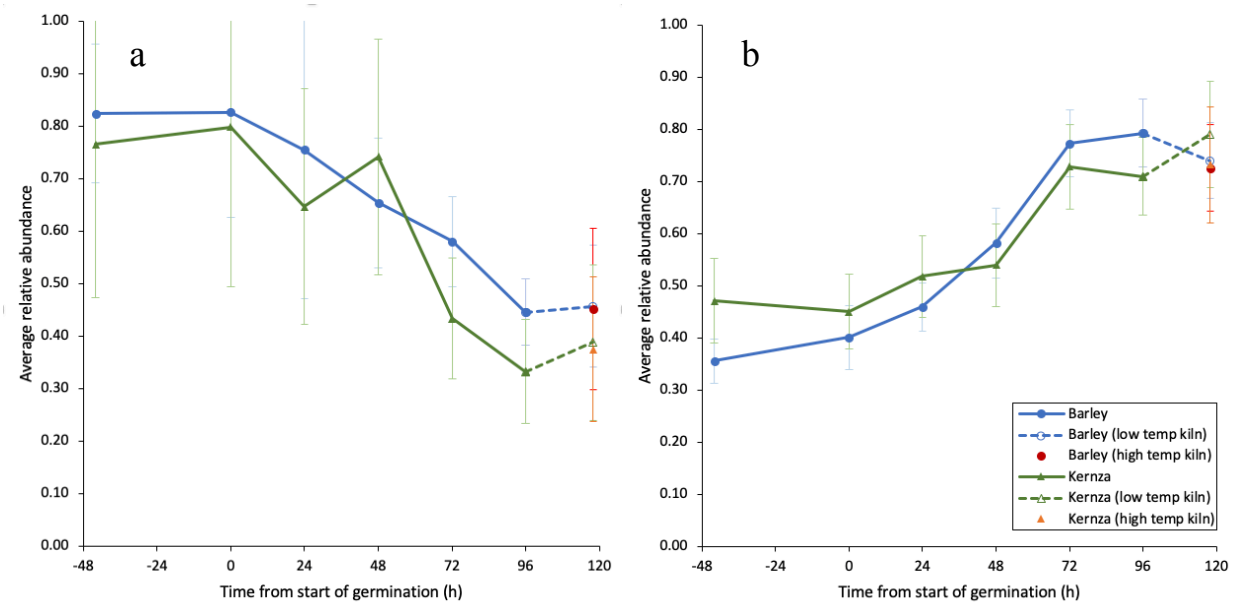


***Figure 11.*** **Changes in the average relative abundance of (a) all seed storage proteins and (b) all protease-associated proteins in Kernza and barley during malting and kilning.** Sampling points include raw (-44 h), post-steep (0 h), germination (+24, 48, 72, 96 h) and after 22 hours of kilning (+118 h). Seed storage proteins for both species exhibited a similar rate of decline when taken in aggregate, likely due to endogenous enzymatic protein hydrolysis characteristic of malt modification, of which the proteins associated increased were increasing in abundance concurrently. Error bars represent standard deviations.

Protease-associated proteins exhibited an opposite trend for both Kernza and barley malts (***Figure 11b***). For both Kernza and barley, over 100 individual proteins within this group were identified over all the sample points, including multiple carboxypeptidases and aminopeptidases. This protein group increased steadily for both species over the malting period, and the maximum average relative abundance was reached at 72 hours into germination. This level appeared to hold steady over the final 24 hours for both, yet decreased slightly after the barley samples were kilned. Interestingly, the Kernza proteins appeared to have increased slightly after kilning at low

temperatures, further implying that additional biosynthesis and modification were occurring in these grains at this point, and a longer germination time may have been warranted.

The abundance of carboxypeptidases enzymes for both species increased drastically during germination (***Figure 12***), reaching peak relative abundance at 96 hours, although there was no leveling-off as seen in the proteases overall. Carboxypeptidases in the barley malt are known for their ability to degrade endosperm cell wall proteins to release free amino acids [189], and have been singled out for their activity related to the release of β-glucan from proteins in the endosperm walls into soluble and active forms [190]. Additionally, malt carboxypeptidases have been shown to be one of the most important classes of enzymes active in the mash to produced additional FAN [191]. These results indicate for both the barley and Kernza used here, the increase in abundance that these enzymes exhibited might have continued with additional germination time. Additionally, Kernza did not exhibit as substantial an increase in carboxypeptidases from raw to green malt as did the barley, which may imply that either (1) the starting amounts of these enzymes are higher in Kernza or (2) the synthesis of these enzymes during germination is not as dramatic as with barley. Additional research into the activity and presence of these enzymes should be investigated in Kernza with attention to the concurrent enzymatic activity.

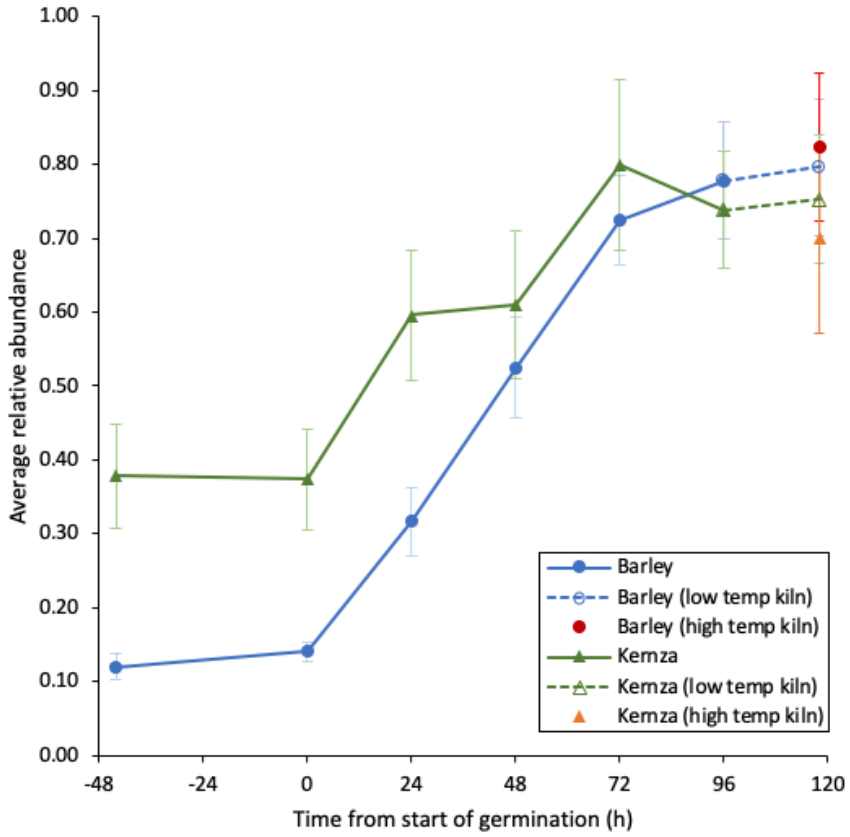


***Figure 12*. Change in average relative abundance of all carboxypeptidase enzymes for Kernza and barley during malting and kilning.** During malting and kilning, these enzymes increased in abundance to a much higher degree than protease enzymes as a whole (Figure 11***b***). Sampling points include raw (-44 h), post-steep (0 h), germination (+24, 48, 72, 96 h) and after 22 hours of kilning (+118 h). Error bars represent standard deviations.

Carbohydrate metabolism-associated proteins followed a similar pattern in both grains, increasing markedly over the malting timeline. This group encompassed 45 proteins found in the barley samples and 54 in Kernza, including the key malting enzymes α-amylase, β-amylase and endoglucanase, each of which were found in both grains. Overall, roughly 50 % of all of the proteins in each of the grains' groups were present in the raw grains for both Kernza and barley (***Figure 13***), and increased steadily during germination to 85 % in the green Kernza malt and 95 % in the green barley malt, with only slight changes to both malts after kilning. The increase in carbohydrate-metabolism proteins is consistent with previous research showing that a number of

these starch-degrading enzymes are synthesized *de novo* during malting, although some are present in raw grains [192], further exemplified by the diastatic power measured prior to malting reported in ***Table 6***.

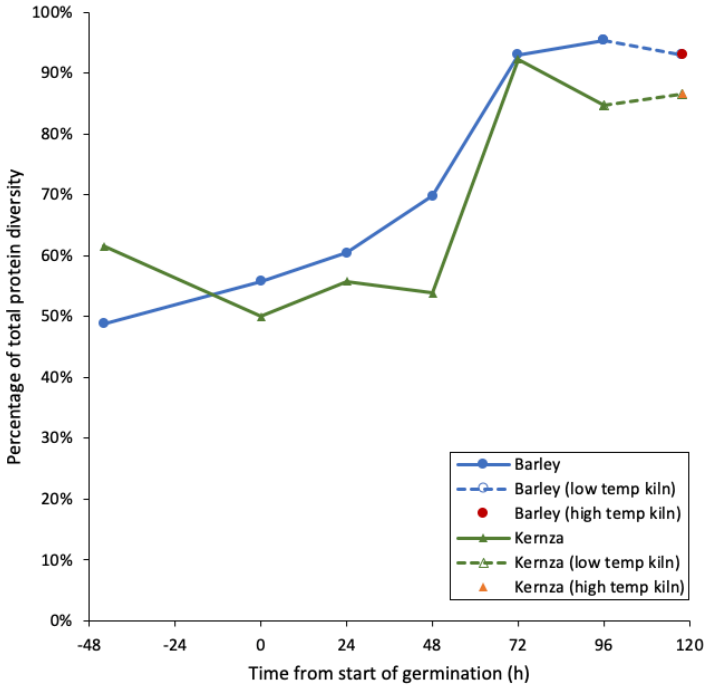


***Figure 13*. Change in overall representation of carbohydrate metabolism-associated proteins for Kernza and barley during malting and kilning.** Only roughly half of all the proteins in this group were present in the raw samples, yet this increased to 95 % in the barley and 85 % in the Kernza after 96 hours of germination. Sampling points include raw (-44 h), post-steep (0 h), germination (+24, 48, 72, 96 h) and after 22 hours of kilning (+118 h).

β-amylases, for example, were present in the raw grain of both species, with seven different β-amylases identified from all three grain species in the Kernza, and only one in barley. The highest relative abundances were either in the raw state, after steep or at the first day of germination (***Figure 14***), and for most β-amylases, the change in abundance was anti-correlated with the changes in the greater carbohydrate metabolism-group. Many of the β-amylases decreased to 66 % or less from their highest abundance by the end of the kilning stages. These data confirm that β-amylases are present in both raw barley and Kernza but, unfortunately, does

not distinguish between the bound, inactive form and the active form that has been released by endopeptidases during malting.

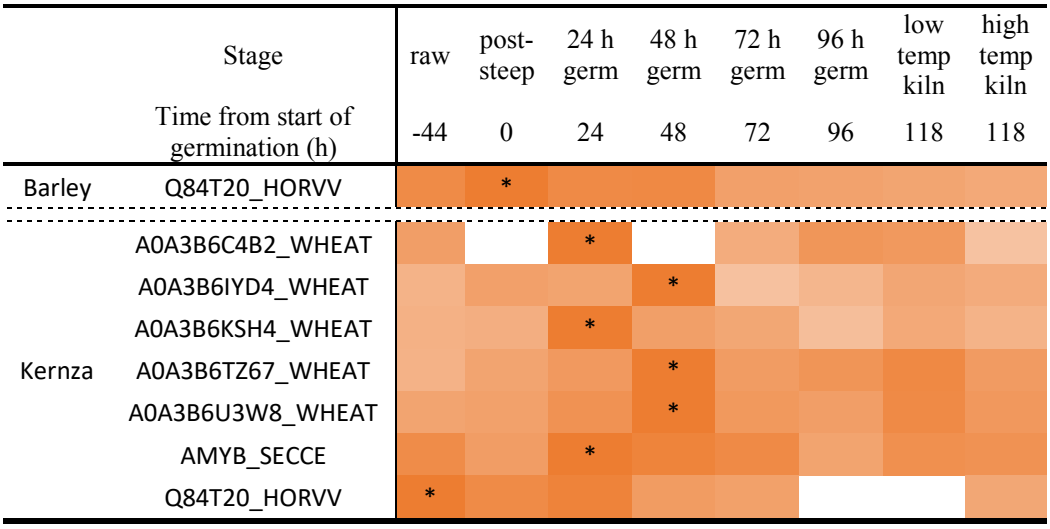


***Figure 14*. Heatmap showing change in relative abundance for individual β-amylase enzymes for barley and Kernza malts.** Individual proteins are identified by UniProt accession codes and abundances are shown at each sample point**.** White color indicates the protein is not present with a color gradient progressing to dark orange indicating higher relative abundance and * denoting the stage with the highest relative abundance.

The overall relative abundance of proteins associated with carbohydrate metabolism increased over germination, concurrent with the increase in the diversity of all proteins, and this change was similar in both barley and Kernza (***Figure 15***). Multiple forms of α-amylase present in both grains increased substantially in abundance over time (***Figure 16***). Here, α-amylase was effectively nonexistent in Kernza until 48 hours into germination, all were present at 72 hours, and the highest relative amounts were exhibited at the end of germination. There was little decrease in the abundance of most Kernza α-amylases due to either kilning treatment. The barley malt α-amylases however, were present at 24 hours into germination, indicating that these grains were synthesizing the enzyme earlier. While the total relative amount of most barley α-amylases peaked at the end of germination after the low temperature kiln, similar to Kernza, the α-amylase activity measurements in ***Table 6*** indicate that the total amount of α-amylase in the barley malt is

either greater or more active than in the Kernza malt. Additional quantitative methods are needed to estimate the relative abundances of α-amylase enzymes between barley and Kernza to elucidate this disparity in α-amylase activity. Additionally, the protein A0A3B6PSD9_WHEAT was present in the raw grains of Kernza, which may be an indication that this species exhibits a form of late-maturity α-amylase expression similar to wheat, in which cool temperatures during grain development can promote the synthesis of this enzyme and subsequently degrade the available starch prior to harvest [193]. Though this issue is of greater concern for baking applications, it may be a sign of pre-germination and thus impact malting quality later.

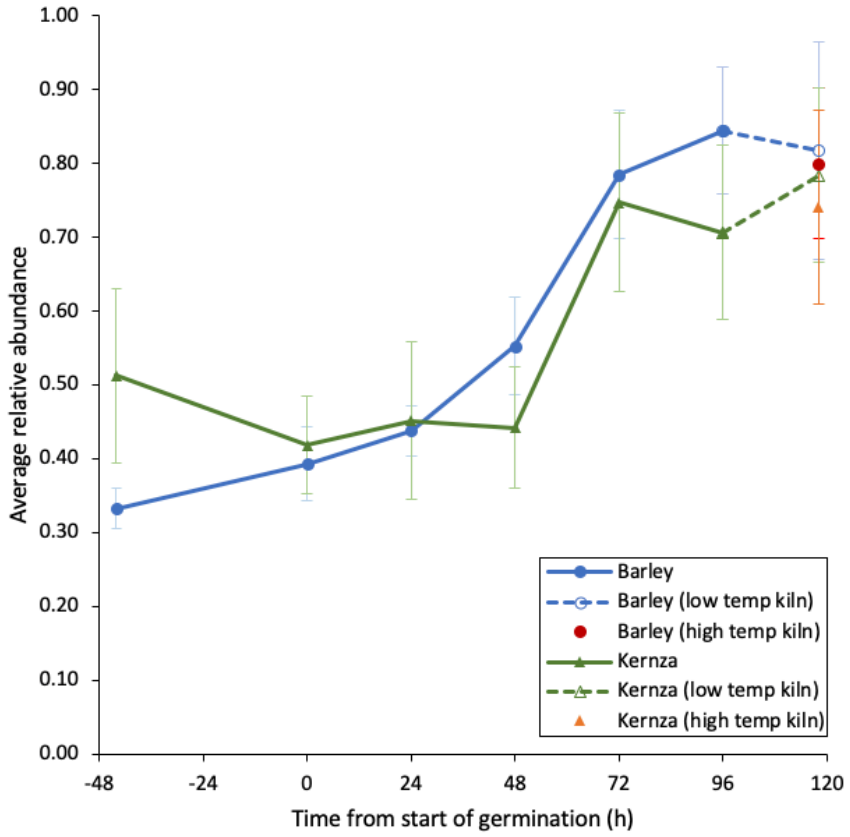


***Figure 15*. Change in relative amount of all carbohydrate metabolism-associated proteins for Kernza and barley during malting and kilning.** Sampling points include raw (-44 h), post-steep (0 h), germination (+24, 48, 72, 96 h) and after 22 hours of kilning (+118 h). Both grains exhibited sharp increases in the abundance of this protein group over the course of malting, with the most dramatic increase at 48 hours for both. Error bars represent standard deviations.

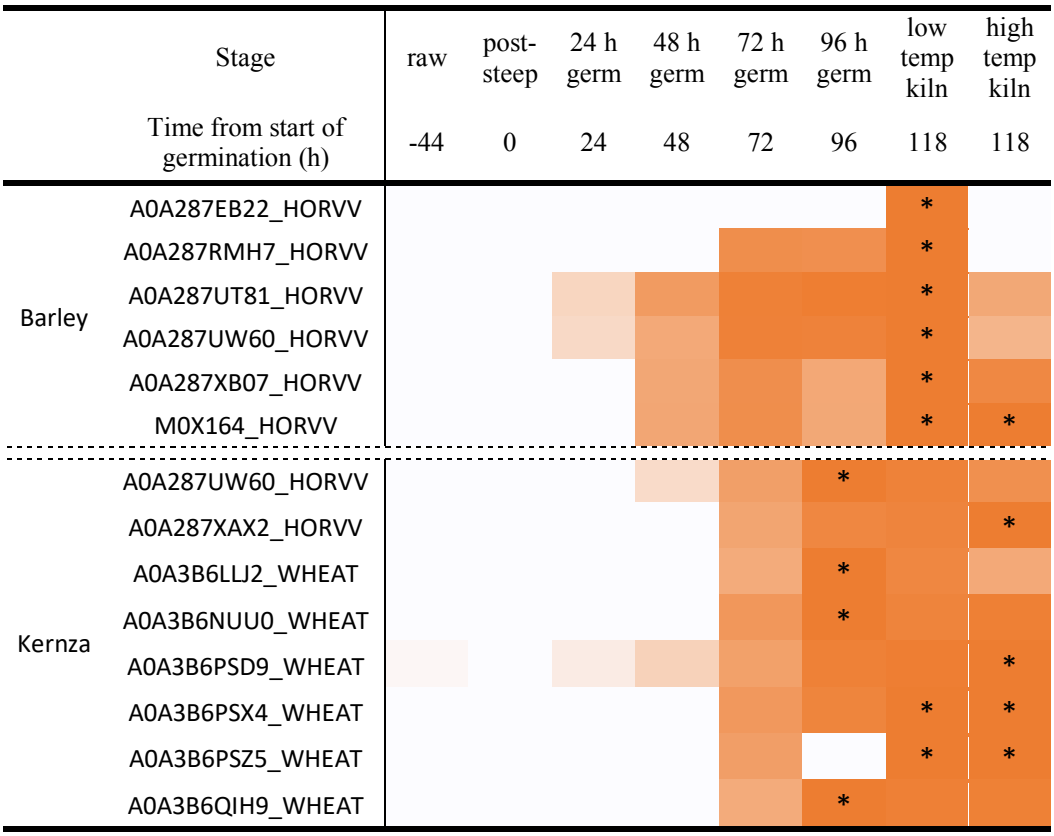


***Figure 16*. Heatmap showing change in relative abundance for individual α-amylase enzymes for barley and Kernza malts.** Individual proteins are identified by UniProt accession codes and abundances are shown at each sample point**.** White color indicates the protein is not present with a color gradient progressing to dark orange indicating higher relative abundance and * denoting the stage with the highest relative abundance.

The carbohydrate metabolism group also included a large proportion of chitinases (EC 3.2.1.14), which function to defend germinating grains against fungal pathogens [194]. Though these enzymes are not functional in the brewery per se, their presence and activity are crucial for the protection of germinating grains at the malt house. For example, the high humidity and moderate temperatures used during malting can promote the growth of *Fusarium* spp. and other molds that in turn can produce mycotoxins and hydrophobic compounds [195]. Beer quality may be impacted if malts with high concentrations of these compounds are used, as they may result in excessive foam production and a phenomenon known as 'bottle gushing' [195]. Of the chitinases

identified here, 6 of a total 11 were present in the raw barley and 7 out of the total 8 were present in the raw Kernza.

Additionally, the kilning temperature treatments appeared to have diverse effects on the abundance of different carbohydrate metabolism-associated enzymes for both species (***Figure 17***). Most of these enzymes identified in the final barley germination stage displayed reduced abundance after both kilning treatments, except for ribokinase (EC 2.7.1.15) and xylose kinase (EC 2.7.1.17). In addition, the barley chitinases (EC 3.2.1.14) and endoglucanase (EC 3.2.1.4) appeared to increase slightly after the high kiln treatment. Conversely, the Kernza enzymes in this group generally appeared to have increased after kilning, though less so in the higher temperature kiln treatment. Potentially these grains were continuing to synthesize these enzymes even as moisture was being lost in the kiln. Enzyme synthesis and activity during kilning has not been widely investigated, and further research into this area should be considered.

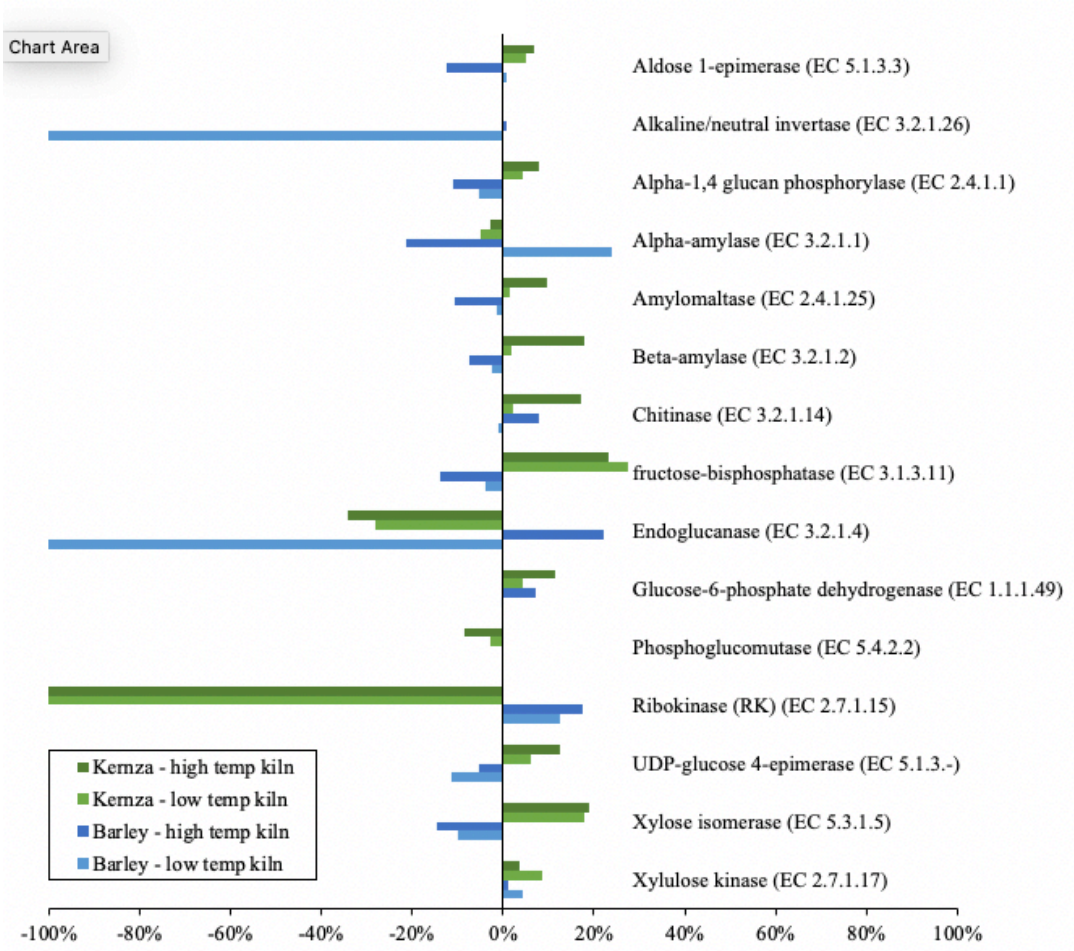


***Figure 17*. Difference in the relative abundance of carbohydrate metabolism-associated enzymes after low and high temperature kilning treatments for both barley and Kernza.** A change of -100 % indicates that the protein was not present after kilning. Most barley enzymes decreased in both kiln treatments, while the majority of the Kernza enzymes increased, though less so in the high temperature kiln.

## 3.2 Barley & Kernza Mashing

### 3.2.1 Traditional Wort Analysis

#### 3.2.1.1 Wort Density & Extract

In this study, the extract values for the infusion mashes, as calculated using a grist to liquor ratio of 1:5, were similar for both barley and Kernza, at roughly 67 % (***Table 9***). The lower extraction when compared to the same malts when using a grist-to-liquor ratio of 1:8 in the malt analysis previously (***Table 6***) can be attributed to both the decreased contact time with the

solids and wort resulting from the centrifuge and decanting separation methods used here, in addition to the decreased amylolytic activity due to the increased mash thickness [196]. However, the step mash profile produced a substantial increase in the barley extract, to 79.5 %, where the Kernza only increased slightly to 70.3 %. Infusion mashes at 65 °C are generally favored by ale brewers using highly modified malts, and result in a wort that is rich in extract and highly fermentable [197]. Step mashes, especially those that include a protein rest at 45 °C, are less frequently used by modern brewers, and were developed to better utilize undermodified malts that were more common in the past [49]. Though the Kernza malt produced here appears to have been less modified than the barley reference, a step mash was not necessary to substantially increasing the extract, implying that a step mash may not be essential for Kernza malts if they are more modified, but additional research is needed for confirmation.

***Table 9*. Results of traditional analyses on the final Kernza and barley worts for both infusion and step micromashes.** All values are based on a grist-to-liquor (G:L) ratio of 1:5, more concentrated than the 1:8 ratio used for data reported above in ***Table 6***. Superscripts indicate statistical similarity based on a two-tailed *t*-test to other values in the same row ($p < 0.05$).

| | | Kernza | | Barley | |
|---|---|---|---|---|---|
| | | Infusion mash | Step mash | Infusion mash | Step mash |
| *Wort gravity* | *°P* | 10.97 ± 0.21 | 12.53 ± 0.15[a] | 12.23 ± 0.51[a] | 13.63 ± 0.21 |
| *Extract (FGDB)* | *%* | 67.4 ± 2.7[a] | 70.3 ± 1.1 | 67.0 ± 1.1[a] | 79.5 ± 0.8 |
| *FAN* | *mg/L* | 268.0 ± 2.3 | 293.6 ± 4.7[a] | 235.1 ± 4.6 | 300.6 ± 17.3[a] |
| *Protein* | *mg/L* | 13.80 ± 0.28 | 16.58 ± 0.37 | 7.06 ± 0.10 | 7.58 ± 0.06 |
| *pH* | | 5.89 ± 0.01 | 5.87 ± 0.01[a] | 5.83 ± 0.03[b] | 5.86 ± 0.01[ab] |

### 3.2.1.2 FAN & Wort Protein

FAN was significantly higher in the infusion mash of the Kernza malt when compared to the barley infusion mash (***Table 9***). Because the Kernza grain is so much higher in protein than the barley, it follows that more FAN would be produced during malting, where the majority of FAN is created [90]. However, when the step mash was used, both malts produced similarly high FAN concentrations of ~300 mg/L. Additionally, the soluble protein concentrations of the worts were much higher for both Kernza mash treatments, at nearly double the concentration of each of the barley treatments. The wort protein concentrations were also significantly higher in the step mash treatments for both grains, but the 20 % increase exhibited by the Kernza was much greater than the 7 % increase exhibited by the barley. This implies that even given the higher starting amount of protein in the Kernza malt, significant proteolytic activity is still available in the malt when mash conditions are conducive.

## 3.2.1 Mash Proteome Analysis

The total number of individual proteins identified in all of the Kernza and barley mash stages (***Table 10***) was much lower than in the malted samples reported previously in ***Table 8***. In the Kernza mash samples, 806 proteins were identified, with 578 (72 %) of those with identifiable names from the UniProt database, though additional annotations were available from their homology to other proteins. Of all the Kernza mash proteins, roughly two thirds were from the wheat (*T. aestivum*) database, similar to the malt samples, with the other third from barley (*H. vulgare*) and only 12 from rye (*S. cereale*). The low number of *S. cereale* proteins is again likely due to the low proteome coverage of the database for the species used here. The unnamed proteins were proportionally distributed between *T. aestivum* and *H. vulgare*. Across all the barley mash samples, 1,179 proteins were identified, of which 839 (71 %) were identified by

name. This method is only able to identify proteins present in the wort solution, not in the remaining solids, and thus the reduction to nearly half of the proteins compared to the malting stages is expected as many proteins are not extracted during this process.

***Table 10*. Count of proteins identified in Kernza and barley worts from all micromash stages**. Data includes both all proteins and only those with identified names from the UniProt databases. Over 1,100 barley proteins were identified across all barley malt samples. In the Kernza mash samples, over 800 proteins were identified, again with the majority from wheat, roughly one quarter from barley, and a small number from rye.

| | Kernza | | Barley | |
|---|---|---|---|---|
| | Total | Named | Total | Named |
| *T. aestivum* (wheat) | 574 | 409 | - | - |
| *H. vulgare* (barley) | 220 | 157 | 1,179 | 839 |
| *S. cereale* (rye) | 12 | 12 | - | - |
| Total | 806 | 578 | 1,179 | 839 |

Across all the named Kernza proteins in the mash samples, there was significant overlap with *T. aestivum* and *H. vulgare*, with 234 (29 %) of the total 806 proteins appearing in both species, and 6 proteins shared between all three species, including *S. cereale* (Figure 18). Only two proteins were shared between *T. aestivum and S. cereale*, and no identified proteins were shared between only *H. vulgare* and *S. cereale*. Only 10 proteins identified were found only in *S. cereale.*

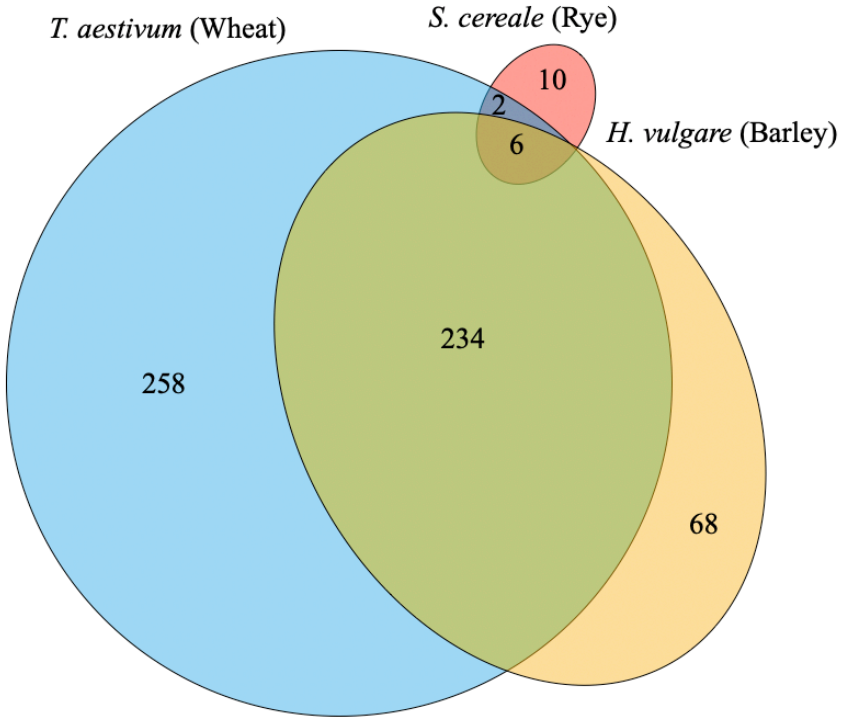


***Figure 18*. Distribution of proteins with defined names identified in Kernza worts over all micromashing stages.** The majority of named proteins were from the *T. astivum* database, with nearly half overlapping with *H. vulgare*. Only a small number were identified from much smaller *S. cereale* database. These relationships match the overall protein makeup in the malting samples above (Figure 9), where the majority of the proteins were identified from the *T. astivum* proteome. Venn diagram generated in eulerAPE v3.

For both species, the total number of wort proteins changed dramatically over the course of the two mash treatments (Figure 19). Both barley mashes began and ended with higher total numbers of proteins than the corresponding Kernza mash treatment. This may be due to the higher solubility of the proteins in the malted barley when compared to the Kernza, but may also be due to database searching impairments imposed by the use here of surrogate species to search the Kernza proteins. For each Kernza mash, only two thirds of the number of proteins in the barley mashes were identified, and this difference held for the duration of the infusion mash and at the beginning of the step mash, By the end of the step mash, however, the total number of proteins identified in the Kernza mash had increased to nearly 90 % of those found in the comparable barley mash. This was primarily due to the decrease in the total number of barley

proteins; which were roughly half of their initial number by the end of both mash treatments, whereas the Kernza proteins had only decreased by roughly 40 %.

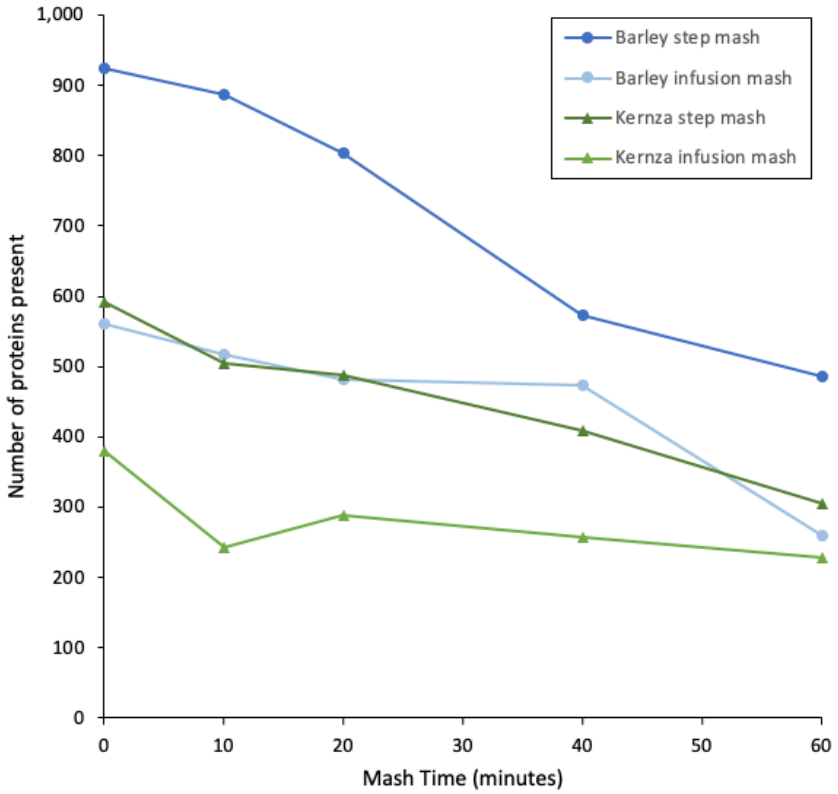


***Figure 19*. Change in total number of identified barley and Kernza wort proteins over the 60-minute step and infusion mashes**. The overall protein diversity decreased for all mashes, though most dramatically in the step mashes with both Kernza and barley malts. More proteins were identified in the step mashes at every sample point compared to the infusion mash with the same malt.

Conversely, several proteins showed increases in abundance over both mash treatments. In the infusion mash, 117 barley proteins increased in abundance. Though the majority of these were identified as 'uncharacterized' or 'predicted proteins' without annotations, they did include a number of serpin and non-specific lipid transfer proteins which are both associated with higher foam quality. In the barley step mash, 269 proteins had increased in abundance by the end of the 60 minutes, which represented nearly half of the proteins identified in the mash at this point. In the Kernza infusion mash, 73 proteins increased in abundance, including a number of prolamin

proteins, including serpins and ns-LTPs, indicating that the foam-positive qualities of beers made with Kernza are increased by malting and mashing in this method. This increase in abundance may be due to the extended time at a lower temperature during the step mash, allowing for sustained proteolytic enzyme activity in the mash to release these proteins into solution.

Overall, the average protein abundances generally followed the same trend as protein diversity, decreasing substantially over the 60 minutes for both grains and mash treatments (***Figure 20***). In the worts from both species, average relative protein abundances started and ended higher in the step mash than in the higher temperature infusion mash, though the difference was less pronounced at the end of the 60 minutes.

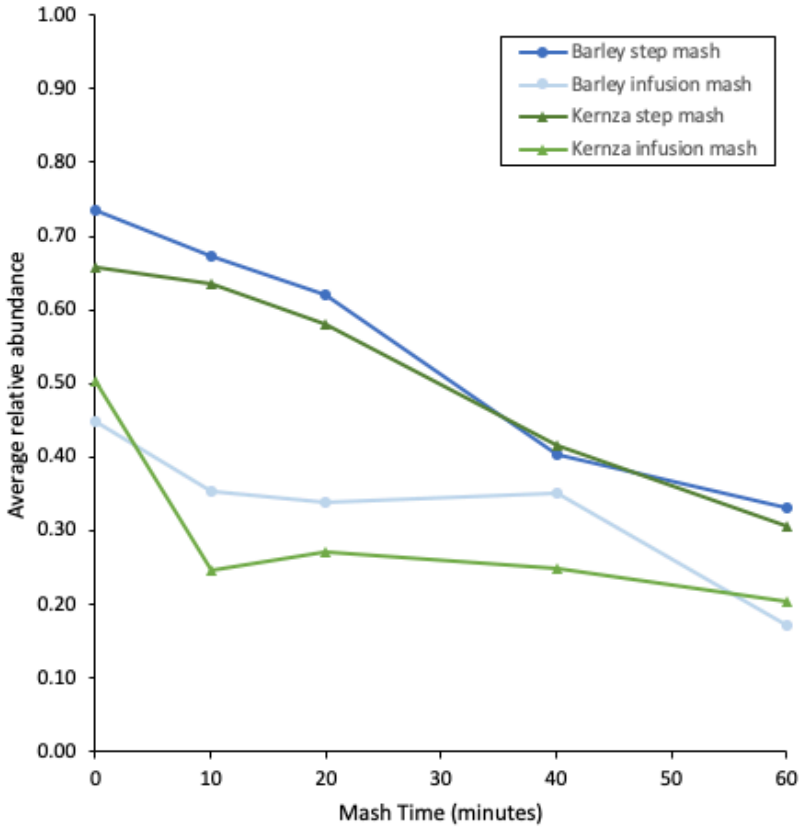


***Figure 20*. Change in average relative abundance of all barley and Kernza proteins over the 60-minute step and infusion mashes.** Barley and Kernza step mashes tracked similar

declines and amounts of overall abundance, which were consistently higher than either of the infusion mashes. For clarity, standard deviation error bars are not shown.

Abundance trends for proteins associated with carbohydrate metabolism were distinctly different between the two mash treatments (***Figure 21***). The infusion mash for both species produced a steep drop in average protein abundance after 10 minutes, which was relatively unchanged afterward, reflecting the thermostability of many of these enzymes. The barley step mash, however, started at a much higher average protein abundance, nearly 0.9, meaning that almost all the proteins were at their highest abundances. Eventually proteins gradually decreased to levels only slightly higher than the infusion mash. The Kernza step mash, interestingly, did not start at such a high abundance, but gradually increased to roughly 0.85 at 20 minutes, where it tracked the gradual decrease of the barley proteins almost exactly, down to roughly 0.3 at the end of the 60 minutes. This increase in the Kernza wort protein abundance may indicate that either proteolysis is solubilizing additional mostly-intact proteins at these low starting mash temperatures, or that more proteins are able to be released into the wort at these lower temperatures without precipitating out of solution. Though the concentration of soluble protein is known to increase during mashing at these temperatures [198], it is unknown to what extent they are degraded. Further investigation into this phenomenon is needed.

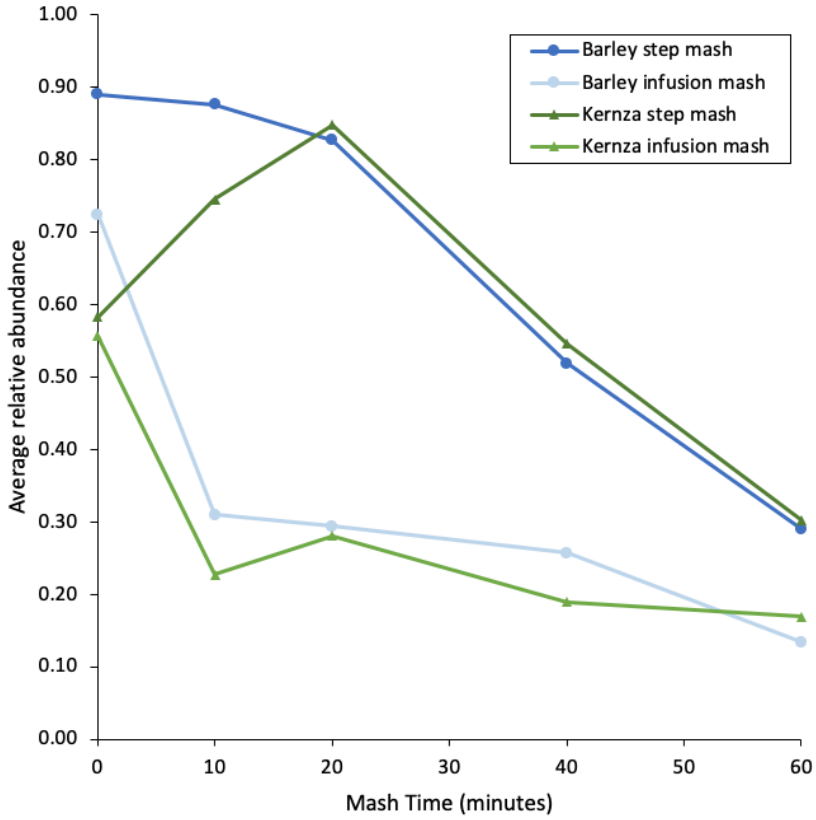


***Figure 21*. Change in average relative abundance of all barley and Kernza carbohydrate metabolism-associate proteins over the 60-minute step and infusion mashes.** Barley and Kernza infusion mashes tracked similar declines and amounts of overall abundance, which were consistently lower than either of the step mashes. Kernza step mash proteins increased in abundance until 20 minutes, after which they tracked the decline exhibited in the barley mash. For clarity, standard deviation error bars are not shown.

β-amylases in the infusion mashes of both species quickly reduced in abundance from time 0 to nearly non-existent in all following time points (***Figure 22***). This rapid reduction is consistent with the thermosensitivity of β-amylase, which is known to rapidly degrade at 65 °C. Thus, in the step mashes with lower initial temperatures, β-amylase abundances peaked at 20 minutes, and were nearly absent at the 60-minute sample point, after temperatures had increased to 65 °C.

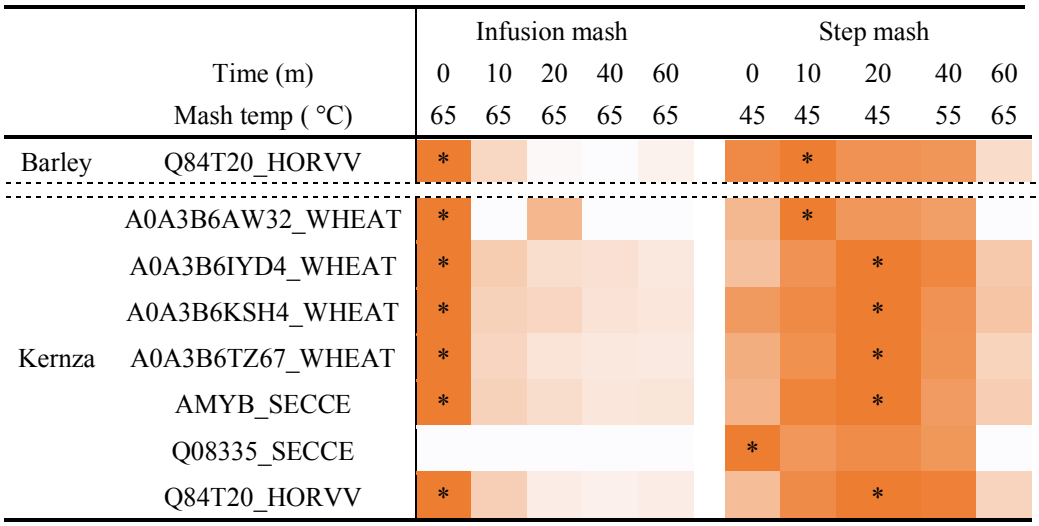


***Figure 22*. Heatmap showing change in relative abundance of individual β-amylase enzymes in barley and Kernza micromash worts.** Individual proteins are identified by UniProt accession codes and abundances are shown at each sample point**.** White color indicates the protein is not present; a color gradient progressing to dark orange indicates higher relative abundance and * denoting the stage with the highest relative abundance.

Additionally, the α-amylases from both the barley and Kernza were quickly reduced in abundance after only 10 minutes in the 65 °C infusion mash. For barley in the step mash, though, the highest α-amylase abundances were at the very end of the 60 minutes, implying that they were maintained in the mash for longer and thus able to degrade starch for longer. Alpha-amylases in the Kernza step mash peaked in abundance after 20 minutes, when the mash temperature was still only 45 °C, and quickly were reduced in abundance after the temperatures was increased in the following stages. Taken with the β-amylase abundance change, it stands to reason that the step mash profile produced a higher extraction wort for both Kernza and barley. However, because this type of assay can only be used to identify and quantify proteins, further inquiry to the overall amylolytic enzyme activity at these stages should be pursued to investigate the result of this phenomenon.

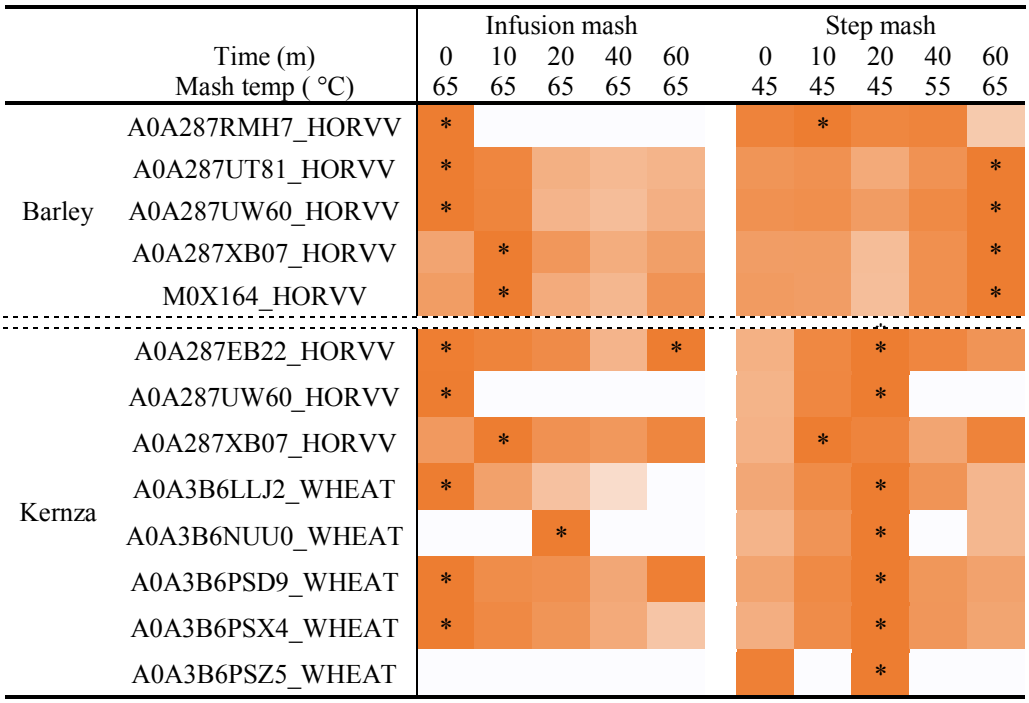


***Figure 23*. Heatmap showing change in relative abundance of individual α-amylase enzymes in barley and Kernza micromash worts.** Individual proteins are identified by UniProt accession codes and abundances are shown at each sample point**.** White color indicates the protein is not present with a color gradient progressing to dark orange indicating higher relative abundance and * denoting the stage with the highest relative abundance. Alpha-amylases followed a similar rapid reduction in abundance in both infusion mashes, while the α-amylases in the Kernza step mashes peaked in abundance at only 20 minutes.

Many of the peptidases, which here include numerous aminopeptidases and carboxypeptidases, followed a similar pattern to the carbohydrate metabolism-associated enzymes in the infusion mashes, and were only found in the first sample at 0 minutes, and nearly none afterward (***Figure 24***). The step mashes were less consistent, where these individual peptidases were differently abundant across the time series. However, there were fewer changes in the abundance of these proteins in the step mashes compared to the infusion mashes. The sustained presence of these free amino acid-producing enzymes was reflected in the higher measured FAN in the step mash worts compared to the infusion mash worts reported ***Table 9***.

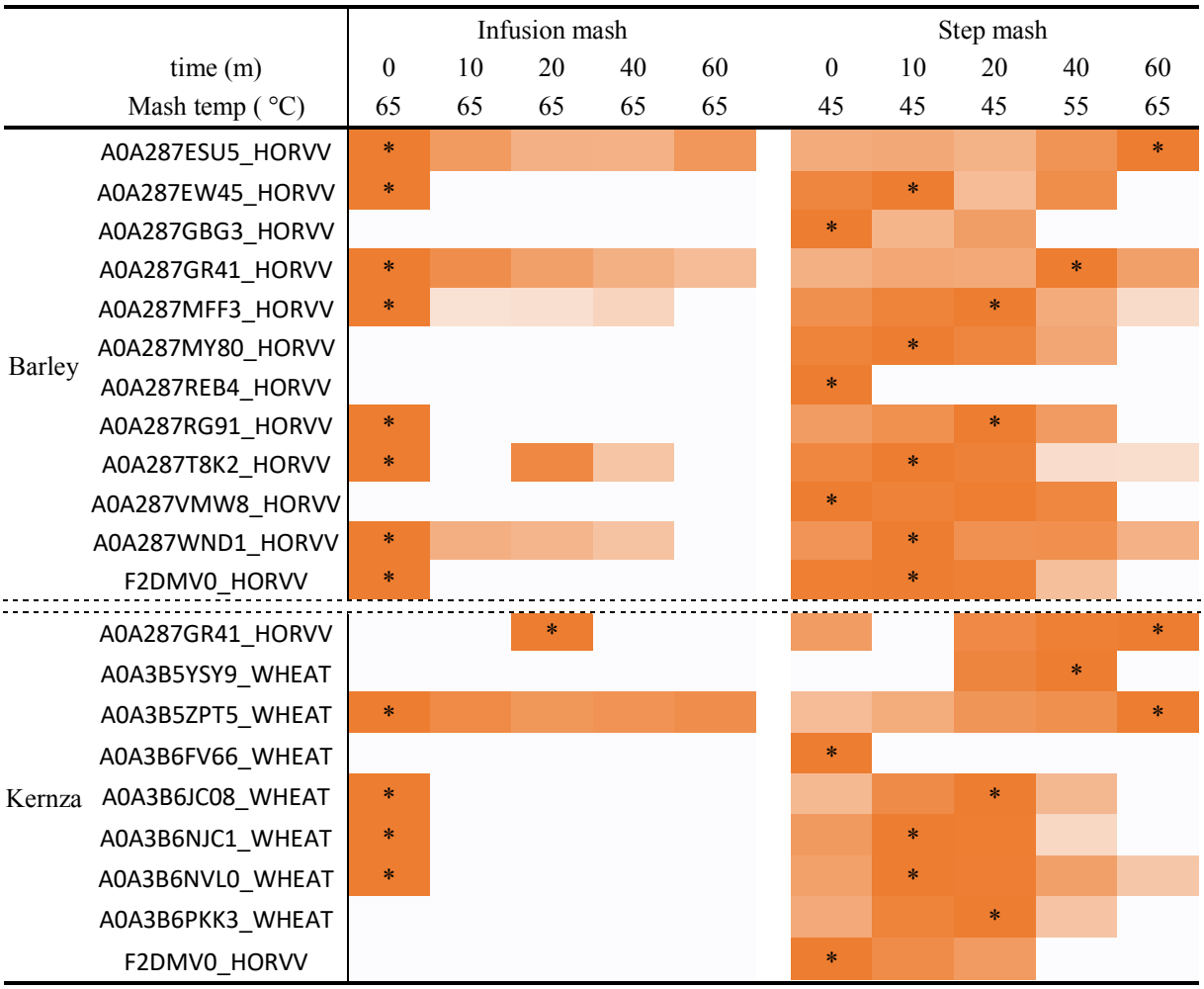


***Figure 24*. Heatmap showing change in relative abundance of individual peptidase enzymes in barley and Kernza micromash worts.** Individual proteins are identified by UniProt accession codes and abundances are shown at each sample point**.** White color indicates the protein is not present with a color gradient progressing to dark orange indicating higher relative abundance and * denoting the stage with the highest relative abundance.

## 4. Conclusions

The malt industry has been almost completely dominated by barley for centuries. Aspects intrinsic to this grain's biology have worked in its favor to drive this singular usage, including an endosperm rich in carbohydrates, evenness in its modification during malting and a relatively high starch-hydrolyzing enzyme activity when properly processed. However, these traits are not unique to barley, and other cereals have the potential to be used similarly. Investigations into

other species may provide an opportunity to address environmental issues associated with barley agriculture and the malt and beer industries as a whole.

The recent and ongoing domestication of Kernza provides an opportunity to disrupt this myopic industry. Here, the raw Kernza grains were malted under similar conditions to a barley reference. The resultant Kernza malt produced wort with high FAN and low wort β-glucan consistent with malt quality standards. However, though the extract exhibited by this malt were high, they were lower here than the barley malt reference, and slightly lower than typical malt standards. By malting Kernza, all aspects were shown to have substantially increased relative to the raw unmalted grain. Starch-hydrolyzing enzymatic activity, paramount to the conversion of starch to sugars in the mash for later fermentation by yeasts into alcohol, was increased in the Kernza by malting, and diastatic power in the final malt was of levels sufficient to completely convert an all-malt mash. Alpha-amylase activity increased seven-fold in the Kernza malt yet was only one fifth of the of barley malt. Notably, though, diastatic power was not substantially increased by malting Kernza, as it did with the barley reference, signifying that β-amylase activity is also high in unmalted, raw Kernza.

Assessment of the proteome of malted Kernza and barley produced additional insights into the potential for this grain as a malt. Overall protein diversity increased substantially during malting, indicating the pronounced biological activity and protein synthesis under malthouse-like germination conditions. Both carbohydrate metabolism- and protease-associated proteins increased in overall abundance, coinciding with a decrease in the abundance of structural storage proteins. Both are indicators of proper modification from a quiescent raw grain to a highly bioactive malt that contains internal components primed for activation in the mash.

Furthermore, when these malts were applied to micro-scale mash treatments, insights into the potential application of the grain were seen. By comparing a typical 60-minute infusion mash at 65 °C and a step mash with temperatures ranging from 45 °C to 65 °C over 60 minutes, Kernza produced values comparable with the barley malt reference. Here, it was clear that Kernza malt is adaptable to both applications widely used in breweries and is not in need of additional exogenous enzymes to produce worts of high fermentability. However, the step mash treatment did produced worts of higher extract and FAN, similar to the worts produced using step mash treatments with the barley reference.

Here, the proteome evaluation of the Kernza and barley malts showed striking similarity in the presence of several mash-active enzymes and foam-positive proteins. Moreover, similarities in the two grains were seen in changes in abundances of specific mash-active proteins, as well as the differential effects the two mash treatments had on them. While all mash treatments for both malts resulted in marked reductions in both overall protein diversity and individual protein abundances, the step mashes maintained both higher protein diversity and individual abundances throughout the 60-minute mashes. This was especially pronounced in the rapid decrease of starch-hydrolyzing enzymes abundances in the higher temperature infusion mash for both species, leading to the aforementioned lower resultant FAN and extract measured in these worts.

These results taken together indicate that Kernza is poised for recognition by the malt and beer industries. Though the Kernza malt did not perform as well as the industry standard barley in all regards, many of the measured values were only slightly inferior. When the environmental impact of these grains is considered, the relative value of this malt improves. Additionally, though germination was slower and less pronounced than the barley reference here, additions of

gibberellic acid may aid to hasten modification. Additionally, these results may aid the continued breeding programs of this relatively new grain to better support those applications.

## Supplemental Data

Raw MS files are available by request to the author.

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