Improved Methods for High-Throughput Extraction and Assay of Green Barley Malt Proteinase Activity Facilitating Examination of Proteinase Activity Across Large-Scale Barley Populations

Mark R. Schmitt and Allen D. Budde

ABSTRACT

We report efficient sample extraction and assay methods allowing quantitative determinations of proteinase activities from barley malt. The improved methods are used to assay >2,200 developmental lines of malting barley for two subsets of proteinase activity. The distributions of the resulting activities suggest differences in population structures between the two types of proteinases. Comparison of the activities of the green malt proteinases with standard malting quality measurements show highly significant correlations that differ between the proteinase subsets. The pH 4.5 hydrolysis of the artificial substrate Z-Phe-Arg-AMC correlates well with the traditional malting quality measurements, supporting the role of cysteine-class proteinases in mobilization of grain reserves during malting and mashing. Results from assays of gelatin hydrolysis at pH 6.0 suggest that these proteolytic activities may be involved in other aspects of seed C and N dynamics also linked to malting quality measurements. The differences between the pH 4.5 and 6.0 activities assayed here and their association with malting quality measurements suggest different physiological roles for the two proteinase activities in several aspects of seed germination. Either assay could be useful for population surveys, depending on the particular facet of seed metabolism under study.

When cereal grains germinate, they undergo a series of events necessary for mobilization of stored seed reserves, providing nutrients for the developing seedling before photosynthesis. Although the processes involved are complex, they have been extensively studied and can be grouped into four general steps: 1) grain hydration, 2) synthesis of enzymes to degrade storage materials (largely carbohydrates and proteins in barley), 3) digestion of the storage compounds into monomers or oligomers by the carbohydrate and protein hydrolases, and 4) growth of the roots and shoots. Malting and brewing take advantage of this process by carefully controlling environmental conditions to induce the grain to produce a particular mix of amino acids, carbohydrates, and other metabolites or hydrolysates that can be used to support yeast fermentation, and that contribute to other desired sensory attributes in the final product. In contrast, the process of protein mobilization in germinating seeds is poorly understood, in part due to the complexity of the system components. Potential proteolytic substrates are much more diverse; they include not only storage proteins but also metabolic and structural proteins. In addition, seed proteinases that may contribute to protein mobilization are substantially more numerous than the starch-degrading carboxypeptidases. Zhang and Jones (1995) have identified over 40 distinct endoproteinase activities on two-dimensional proteinase activity gels (zymograms) using readily digestible artificial substrates. These activities fall into four diverse classes based on the catalytic mechanism. Of the barley malt proteinases, the cysteine-class proteinases are often considered the most important to malting quality, based on diversity and abundance, and can be grouped into four general steps: 1) grain hydration (heating and drying) to partially germinate the grain, before the mobilized grain reserves are used for significant seedling growth.

Two classes of seed components that are of particular interest to malsters and brewers are storage carbohydrates and proteins. Mobilization of these two principal classes of stored reserves are conceptually similar processes in that polymers of a limited set of subunits (sugars or amino acids) are hydrolyzed by one or more enzymes (carbohydrases or proteinases) to monomers or oligomers. The basic components involved in starch degradation are reasonably well understood. Starch (composed of various combinations of straight-chain amylose and branched-chain amylopectin) is degradable in the combined action of four major enzymes (α-amylase, β-amylase, α-glucosidase, and limit dextrinase) to produce a limited subset of monomer-, di-, and oligosaccharides. Historically, barley breeding programs have been successful generally by selecting for desired levels of α-amylase and diastatic power as metrics for starch conversion to fermentable sugars. Nonetheless, the process of protein mobilization in germinating seeds is poorly understood, in part due to the complexity of the system components. Potential proteolytic substrates are much more diverse; they include not only storage proteins but also metabolic and structural proteins. In addition, seed proteinases that may contribute to protein mobilization are substantially more numerous than the starch-degrading carboxypeptidases. Zhang and Jones (1995) have identified over 40 distinct endoproteinase activities on two-dimensional proteinase activity gels (zymograms) using readily digestible artificial substrates. These activities fall into four diverse classes based on the catalytic mechanism. Of the barley malt proteinases, the cysteine-class proteinases are often considered the most important to malting quality, based on diversity and abundance, and can be grouped into four general steps: 1) grain hydration (heating and drying) to partially germinate the grain, before the mobilized grain reserves are used for significant seedling growth.

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1 USDA-ARS, Cereal Crops Research Unit, 502 Walnut Street, Madison, WI 53726. Mention of trade names or commercial products in this presentation is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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manipulations. While the steps needed to execute that assay are manageable for limited numbers of lines, the assay complexity and number of manipulations is prohibitive for larger studies such as those using association genetics methods to link genotype and phenotype data on a larger scale (>1,000 lines; www.barleycap.org). To quantify the proteolytic components of significantly larger numbers of lines in more comprehensive studies, more efficient methods for sample extraction and proteinase activity assay are needed.

In this report, we present such methods for extraction and analysis of proteinase activities from germinated barley seeds and demonstrate their efficacy by analyzing >2,000 lines for two classes of proteolytic activity. Applying these methods to larger, genetically defined populations will facilitate a better understanding of the genetic underpinnings of protein mobilization in barley and allow development of improved tools, allowing barley breeders to rapidly develop new malting cultivars.

**MATERIALS AND METHODS**

**Biological Materials**

Barley samples from the 2004 crop year submitted by U.S. public sector barley breeders to the USDA-ARS Cereal Crops Research Unit (CCRU) for malting and malt quality analysis were used in this study. Lines were selected for this study without regard to submitting institution, genetic background, production site, or malting characteristics. They were obtained in linear sequence from the samples submitted for routine malting quality analysis at CCRU. Lines tested constituted approximately 40% of the lines submitted to CCRU for malting quality assessments from crop year 2004, including both 2- and 6-row barleys from Midwest and Western breeding programs. As such, the lines tested do not constitute a structured population, but rather represent a sampling of the advanced lines currently under development by U.S. malting barley breeding programs.

**Malts and Malting Quality Evaluations**

Micromalts were prepared from 170 g (dwb) samples following standard protocols used at CCRU. Sample steeping was conducted in a custom-designed apparatus (Standard Industries, Fargo, ND) with alternating cycles of 4 hr of immersion at 16°C and 4 hr of air rest. Total steeping time was adjusted to provide a final out-of-steep grain moisture of 45%, using an empirical model based on kernel weight. The grain samples (170 g, dwb) were germinated in a second apparatus also from Standard Industries for 5 days at 17°C (rotating the cans containing the germinating barley for 3 min every 30 min) at humidity approaching 100%. At the end of the germination period, small aliquots of the resulting green malt were subsampled into 20-mL vials and frozen at −80°C. The remaining bulk of the green malt was kilned over 24 hr to a final temperature of 85°C (Peterson et al 2001). The resulting kilned malt was analyzed for malting quality attributes following American Society of Brewing Chemists standard methods (ASBC 2004).

The frozen green malt was cleaned of rootlets, nonadherent hull materials; and detached acropyles by gently rubbing in a kitchen strainer (1-2 mm mesh) using a prechilled (liquid N2) ceramic pestle, and sieving the malt through the strainer to remove fragmented rootlets and other loose material. Samples were kept frozen by storage on dry ice and frequent immersion in liquid N2 during processing. Cleaned, frozen green malt was returned to storage in a freezer at −80°C until extraction and analysis.

**Green Malt Extraction**

Cleaned green malt samples were extracted using a 96-well format homogenizer (Mini-BeadBeater-96, Bio-Spec Products, Bartlesville, OK; www.biops.com). For each line tested, 10 kernels (weighing approximately 0.5 g) were placed in a 2.0-mL screw-cap microfuge tube along with 1.2 mL of prechilled buffer (50 mM sodium acetate pH 5.6) and three prechilled 6.35-mm stainless steel balls (Bio-Spec Products). The tubes were placed in a prechilled aluminum extraction block for the Mini-BeadBeater-96 and homogenized for three cycles of 30 sec each. The block and sample tubes were returned to an ice bath for cooling between cycles. After homogenization, vials were transferred to a polypropylene microplate format rack (Bio-Spec) and centrifuged for 20 min at 5,000 rpm in a Beckman Coulter Avanti-J25 centrifuge using a JS-5.9 microplate carrier rotor. Supernatants were removed manually using a fine-tip polypropylene transfer pipet and aliquoted into polystyrene 96-well plates. The plates were subsequently scaled with adhesive-backed aluminum plate seals. The sealed microplow plates were returned to −80°C storage until assayed for proteinase activity. Immediately before assay, the plates were removed from storage and thawed rapidly on a thermomixer (Eppendorf North America, Westbury, NY; www.eppendorfna.com) regulated to 10°C. Thawed plates were mixed well using the thermomixer before sampling for assay.

For each extraction, the fresh weight of green malt in the 10 homogenized kernels was recorded and used to account for variations in the kernel weight of individual samples of malt.

**Microplate Proteinase Assays**

The proteinase activities of green malt extracts were assayed using fluorogenic substrates in 96-well microplates on a microplate fluorometer (SpectroMax Gemini XS, Molecular Devices, Sunnyvale, CA; www.moleculardevices.com). SoftMax Pro 4.3.1 LS software was used to continuously monitor fluorescence during the reaction time course. The software also provided automated rate calculations for fluorescence changes in individual treatment wells, and the corresponding correlation coefficients for the linear rate curve fits.

Two proteinase substrates were used in this study. For analyzing the activities of cysteine proteinases, the fluorogenic peptide Z-Phe-Arg-AMC (Bachem, Torrance, CA; www.bachem.com) was used (Davy et al 1998), with the reaction volumes modified for assay in 96-well microplates. Activity was assayed in a reaction mix (final concentrations 25 mM sodium acetate, pH 4.5, 10 μM Z-Phe-Arg-AMC) by following changes in the fluorescent signal (kinetic [time course]) mode set for excitation 320 nm, emission 420 nm, auto cutoff, 6 reads/data point, PMT medium). The assay pH (4.5) used by Davy et al (1998) and this study approximates the pH reported for the endosperm (Jones 2005). The proteolytic reactions were started by adding 25 μL of green malt extract to 175 μL of reaction premix, with increases in fluorescence measured in the microplate fluorimeter at 37°C. The initial, linear portion of the reaction time course was used to calculate the rate of substrate proteolysis. Assays using Z-Phe-Arg-AMC were done in triplicate.

Where appropriate, cysteine proteinase inhibitor E-64 (t-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane) from Sigma Chemical (St. Louis, MO; www.sigmaaldrich.com) was used at a final assay concentration of 10 μM to confirm the proportion of peptide hydrolysis that was due to cysteine-type proteinases.

A more digestible substrate (DQ-Gelatin, catalog #D-12054, Invitrogen/Molecular Probes, Carlsbad, CA; www.probes.invitrogen.com) was used to study the overall proteolytic activity in green malt at pH 6.0, matching that in Congress mashes at our facility (Jones and Budde 2003). The assays were buffered to pH 6.0 using 25 mM sodium acetate. The final concentration of the DQ-Gelatin substrate in the assay well was 33 μg/mL. Assays were conducted as above except for wavelengths used (excitation 485 nm, emission 530 nm, cutoff 515 nm) as appropriate for the different fluor. Reactions were started as above by adding 25 μL of a green malt extract to 175 μL of reaction premix equilibrated to 37°C, with rates of proteolysis also calculated as above. Assays using DQ-Gelatin as a substrate were conducted in quadruplicate. Note that pH 6.0 is reported to be optimal for activities of the two serine proteinases characterized to date (Terp et al 2000; Fontan-
that pH level is also near the lower limit of utility of the fluor in DQ-Gelatin due to its reduced fluorescent signal below pH 6.

For both substrates used, proteolysis rates and correlation coefficients for the rate calculations were automatically calculated by SoftMax Pro software and exported to an Excel worksheet where data was assembled and processed further. Correlations between proteinase activities and melting quality parameters and the corresponding significance levels were calculated (SAS Institute Inc., Cary, NC; www.sas.com).

RESULTS AND DISCUSSION

High-Throughput Fluorogenic Proteinase Assays

For a high-throughput proteinase assay to be useful in estimating the proteinase activities across relatively large barley populations, the assay should 1) be quantitative and reproducible; 2) require a minimal number of manipulations or sample transfers; 3) be adaptable to common formats (e.g., 96- or 384-well microplate) for ease of sample handling, storage, and analytical measurements; and 4) be either specific to a particular class (e.g., cysteine proteinases) or broadly adaptable to several classes of proteinase activities, depending on the information desired. The microplate-format assays using one of several fluorogenic substrates meet these requirements. The advantage of fluorogenic substrates for high-throughput assay systems is that the signal generated upon their hydrolysis can be detected in the reaction mixture without the need for further sample processing, allowing the proteolytic activity to be determined in real time. Due to the sensitivity of the fluorescent signal and the small reaction volumes necessary for signal detection, the assays are well suited to running in 96- or 384-well format fluorescent microplate readers. Use of this common format for sample transfers, storage, processing, and assay coupled with the efficiencies provided by use of 8- or 12-place automatic pipettors greatly increases sample throughput.

In addition to the specific fluorogenic peptide substrates used by Davy et al. (1998), a number of fluorogenic protein substrates for general surveys of proteolytic activity are also available commercially from several vendors including Pierce and Invitrogen/Molecular Probes. Derivatives of gelatin, casein, and albumin offer a variety of protein substrates, paralleling earlier colorimetric assay systems using resorufin-casein (Ahoivaskas and Naskali 1990) or azogelatin (Jones et al. 1998), but with the advantages offered by the fluorogenic assay systems. In this study, we used the gelatin derivative DQ-Gelatin due to the demonstrated susceptibility of gelatin to a variety of proteinases of different mechanistic classes (see Jones 2005 for review). In early experiments evaluating the various fluorogenic proteins as malt proteinase substrates, we found both greater signal intensity and reaction linearity with the gelatin derivatives compared with either casein or albumin derivatives (M. R. Schmitt, unpublished data). Several of the fluorogenic proteins (including DQ-Gelatin) are available with green or red fluorophores, either of which may be suitable, and only requiring different excitation/emission wavelengths or filter sets on the microplate fluorimeter.

Quantitation of Proteinase Activity by Microplate Fluorogenic Assays

For an assay to be useful for quantitating proteolytic activity, it should be reproducible, provide a linear time course sufficient to calculate a rate, and report activity that is proportional to the amount of proteolytic activity added to the assay system. Data presented in Fig. 1 shows that a fluorogenic assay with DQ-Gelatin as a substrate meets those requirements. Figure 1A shows a captured display from the reaction time courses for four sets of assays of a Harrington green malt extract in a three-fold dilution series (with four replicates for each condition), showing the excellent repeatability of the assay. During the initial portion of the time course used to calculate the linear rate fit, the data points for the replicate assays were essentially superimposed. Figure 1B shows a simplified display (only one replicate assay per dilution) demonstrating automatic calculation of reaction rate (Vmax/sec) and associated goodness-of-fit (R2) statistic for rate determination for the three-fold dilution series. Note the excellent r2 values for the rate calculations in the higher activity wells. Even for assays with 10- to 30-fold lower activities (wells F7 and F9 compared with well F3), the linear rate determinations are still good, with r2 values >0.9 for the calculated fit. When the mean rates (± standard deviations) for the four replicates shown in Fig. 1A are plotted as a function of their relative dilution (Fig. 1C), it is clear that the assay responds to the amount of added proteinase activity in a linear fashion, at least over a 30-fold range in activity. Comparable

![Fig. 1. Typical reaction time course for fluorogenic proteinase assays. Results from a DQ-Gelatin/pH 6.0 assay. A. Assay repeatability showing four replicate curves of a three-fold dilution series. B. Automatic fits to initial rates for the four dilution curves. Automatic rate and goodness-of-fit calculations (Vmax/sec and R2, respectively) from the data provided by SoftMax Pro. C. Linear regression of calculated rates for the serial dilutions. Values plotted are mean rate ± standard deviation from four replicate assays. Where error bars are not visible, they are less than the size of the plotted symbols.](#)
results are also seen when the Z-Phe-Arg-AMC pH 4.5 assay of Davy et al (1998) is adapted to a microplate format (data not shown).

In production assays (high-throughput assays of malting barley submissions), rates calculated for individual wells generally showed $r^2$ values $>0.9$ for the initial time points used in the rate calculation. Reactions showing correlation coefficients of $<0.8$ were seen at times, particularly for samples with very low proteinase activities. However, occasional rates with relatively low $r^2$ fits were not used in calculating the mean reaction rates for those lines because replicate assays usually showed several fits with $r^2$ values $>0.9$.

**Properties of Proteinase Assays**

Use of fluorogenic substrates greatly simplifies the assay of proteinase activity, allowing activity surveys across much larger populations of malting barley. In this study, over 2,200 lines from 2004 crop year were analyzed for the two proteinase activities. The two assay systems showed minimal variability across replicate treatments. The pH 4.5 Z-Phe-Arg-AMC proteinase assay (run in triplicate) showed an average coefficient of variation (CV) of 9% (± 5%) for 2,235 lines, while the pH 6.0 DQ-Gelatin proteinase assay (run in quadruplicate) showed an average CV of 1.8% (± 1.1%) for 2,331 lines. The low (<2%) CV for quadruplicate assays using DQ-Gelatin suggests that fewer replicates than the four routinely used may have been adequate to obtain reliable estimates of the activity.

Although Davy et al (1998) used the Z-Phe-Arg-AMC peptide as a substrate for measuring the kinetic properties of the two major cysteine endoproteinases EP-A and EP-B, the peptide has also been used for assaying other endoproteinases. Barrett (1980) used the fluorogenic peptide to measure activity of Cathepsin B, a cysteine-class proteinase. However, the peptide has also been used to assay activity of plasma Kallikrein, a serine endoproteinase (Morita et al 1977). Thus, while the substrate is readily hydrolyzed by purified preparations of the two major malt cysteine proteinases, it may also be subject to hydrolysis by other proteinases as well. To examine the specificity of the fluorogenic dipeptide derivative Z-Phe-Arg-AMC as a substrate for malt cysteine proteinases, we assayed the same 2,235 lines for Z-Phe-Arg-AMC hydrolysis in the presence of 10 μM E-64, an inhibitor accepted as a diagnostic for cysteine-type proteinases. Triplicate assays of extracts from the 2,235 lines in the presence of E-64 showed that the activities were generally significantly inhibited by E-64 (85 ± 11% [mean ± SD], 85% [median], and 100% [mode], suggesting that hydrolysis of this substrate at pH 4.5 by malt extracts is primarily due to actions of cysteine proteinases.

Gelatin and its derivatives are readily hydrolyzed by a number of malt proteinase classes (Zhang and Jones 1995; Jones et al 1998). At pH 6.0, the proteinase activities in a Harrington green malt extract appear to be predominantly of the serine mechanistic class because approximately 75% of the activity can be inhibited by PMSF (Fig. 2). Serine-class proteinase activity in barley malt is of particular interest to us because recent results from our laboratory have shown that several serine-class proteinases are capable of degrading barley β-amylase (Schmitt and Marinac, in press). Unfortunately, achieving maximal levels of inhibition with PMSF required use of inhibitor concentrations beyond the limit of solubility, resulting in the formation of significant amounts of precipitate in the aqueous assay system. Experiments using another inhibitor of serine-class proteinase activity 4-(2-Aminomethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Roche Applied Science), which has been touted as demonstrating greater potency and water solubility than PMSF, had consistently less inhibition of proteolysis than did PMSF. As a result, such technical difficulties in quantifying inhibition by serine-class inhibitors precluded us from confirming the contribution of that mechanistic class to pH 6 proteolysis across the malts tested, as we had done with Harrington. Although not reported here, preliminary experiments using inhibitors of asparyl proteinase activity (Pepstatin A) and metallo proteinase activity (α-phenanthroline) found no barriers to their use to assess the relative contributions of those mechanistic classes to overall proteolytic activity in the high-throughput fluorogenic proteinase assays.

**Distributions of pH 4.5 and 6.0 Proteinase Activity Classes in 2004 Crop Year Samples**

Although the lines assayed in this study do not come from a defined structure population, these analyses may provide some insight into the range and distribution of the two activity classes in breeding lines currently under development in the United States. The distributions of the two activities appear quite different (Fig. 3). A few lines have relatively high pH 6 proteinase activities compared with the bulk of the lines, with the majority clustered at the low end of the activity distribution. In contrast, the distribution of cysteine proteinase activities appears more widely dispersed. Ear-
lier work (Zhang and Jones 1995) suggested that there are a large number of endoproteolytic activities in green malt that are inhibited by E-64 (suggesting cysteine-class activity) and fewer endoproteolytic activities that are inhibited by PMSF (indicating serine-type mechanisms). The activity distributions in Fig. 3 are consistent with population models of a relatively larger number of segregating cysteine proteinase genes and a relatively smaller number of genes responsible for the pH 6 (putative serine) proteinase activities. Alternatively, an unknown number of genes responsible for the pH 6 activity could be fixed in the population. However, these simplistic interpretations should be tempered by the recognition that there are additional factors that could potentially complicate the interpretation of the proteinase activity distributions. Definitive analysis of the distribution of proteinase activities across genotypes and the underlying genetic structure responsible for the activities found will require appropriately structured populations, rather than the snapshot of the current breeding lines presented here.

Associations Between Proteinase Activity and Malting Quality Attributes

The size and breadth of this data set containing both proteinase activity data and malting quality assessments provides an opportunity to examine current malting barley breeding programs for associations between the activity and malting quality data. Table 1 lists 11 malting quality parameters that are routinely determined on lines submitted to our program for malting quality assessment, and reports both the magnitude (r) and significance (P) of any correlations found between the malting quality attributes and the proteinase activities in the individual lines measured. While the magnitude of the correlations in this study between malting quality and proteinase activity measurements was relatively low (maximum |r| = 0.2 – 0.4), each proteinase activity showed a number of highly significant (P < 0.0001) correlations with the malting quality parameters. The combination of high significance (P) and low strength (r) of many of these correlations indicates that there is a real association between the two parameters but that there are also other uncontrolled factors that contribute to sample variability, thus reducing the strength of the correlation. In a data set such as this, where there is no defined population structure, no control on sample pedigree, and where the constituent lines were produced in a wide range of environments, one would expect to find larger sample variability than would be found in a structured population, with consideration for pedigree and environmental effects included in the experimental design. As a result, the strengths of the correlations reported here may represent a minimal estimate of the actual correlations that could be found in a defined population. In addition, because the lines tested here have all been subjected to strong selection during line development, unfavorable associations may have already been reduced or eliminated from the populations before their evaluation in this data set.

Not surprisingly, the strongest, most consistent correlations found were those between proteinase activity and malting quality parameters relating to protein mobilization. Specifically, both proteinase activities showed positive significant correlations with wort-soluble protein and with the Kolbach index (fraction of total protein that is solubilized). Although both activities were correlated with wort-soluble protein and Kolbach index, the correlation was twice as strong for the pH 4.5 activity as it was for the pH 6.0 activity (Table 1). Neither proteinase activity was correlated with total barley protein levels. Consistent with these results, Kihara et al (2002) found a strong correlation between proteinase activity and both wort-soluble protein and Kolbach index in a collection of 43 barley lines grown in a number of environments. In a second experiment, the authors examined cysteine, aspartic, and other (residual activity not inhibited by E-64 or Pepstatin, presumably a sum of serine and metallo-class activities) proteinase activities across a collection of 27 barley lines grown in a common environment. In their analysis, there were highly significant correlations between cysteine-class proteinase activities and both soluble protein and Kolbach index, but not between either the aspartic and other proteinase activities and wort-soluble protein or Kolbach index.

Both studies point to a significant impact of cysteine-class proteinase activities on protein solubilization during malting and mashing, consistent with reports of cysteine-class proteinase hydrolases of the major barley storage proteins (Wallace and Phillips 1989; Davy et al 1998). However, our results also show a weaker, but still highly significant, effect of the pH 6.0 (presumptive serine-class) proteinase activities on wort-soluble protein and the Kolbach index. An earlier study (Jones and Budde 2005) of the effects of inhibitor treatments during mashing of Harrington and Morex malts reported no significant contribution of serine-class proteinases to wort-soluble protein while confirming the major role of cysteine proteinases in protein solubilization. Two advantages of utilizing large-scale germplasm surveys to address such questions include the ability to examine responses from a wider range of genotypes (2 vs. 2,200), and the ability to study integrated processes extending through both malting and mashing without needing to employ exogenous proteinase inhibitors.

There were also highly significant (P < 0.0001) positive correlations between the pH 4.5 (cysteine) proteinase activity and both measures of carbohydrate mobilization (diastatic power and alpha amylase activity), but only weak negative correlations between the pH 6.0 (putative serine) proteinase activity and the carbohydrate mobilization parameters.

The positive correlations of the pH 4.5 proteinase activities with the carbohydrate mobilization malting quality parameters confirm

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Z-Phe-Arg-AMC, pH 4.5</th>
<th>DQ-Gelatin, pH 6.0</th>
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<tr>
<td>Extract</td>
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<td>Total barley protein</td>
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<td>Color</td>
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<td>0.282 &lt; 0.0001</td>
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</table>

* Parameters established using standard ASBC methodology and terminology except for Quality Score (Clancy and Ullrich 1988), where values are derived according to values listed in Appendix B at http://www.ars.usda.gov/sp2UserFiles/Place/36351000/barleyreports/2005MVPreliminaryRpt.pdf
earlier reports (Kihara et al 2002; Collins et al 2004). Neither of those reports examined proteolysis at values above pH 5.0 for comparison with the pH 6.0 DQ-Gelatin hydrolysis reported here.

In addition to the effects of cysteine-class proteinases on storage protein mobilization discussed above, Sopanen and Laurière (1989) described the solubilization, partial proteolysis, and activation of barley β-amylase by cysteine proteinases. This solubilization and activation of β-amylase by cysteine proteinases could then be linked to increased β-amylase activity and higher DP values.

There is less information about any potential linkages between pH 6.0 proteinase activity and quality traits. This information largely consists of the observations that the two malt serine proteinases studied in detail to date do not hydrolyze hordein (Terp et al 2000; Fontanini and Jones 2002), and the report that associations of serine-class inhibitors to malts did not affect overall protein solubilization (Jones and Budde 2005).

For malting quality parameters related largely to carbohydrate mobilization (diastatic power, α-amylase), both protease activities showed correlations that were significant at the 99% level or better, but with opposite directionality. That is, the cysteine proteinase activities (pH 4.5) were positively correlated with DP and α-amylase, while the putative serine proteinase activities (pH 6.0) showed weak negative correlations with those attributes.

Mechanistically, the positive correlation of cysteine protease activities with extract, DP, and α-amylase activity can also be related to the degradation of hordein by cysteine proteinases. It is well accepted that hordein levels are negatively correlated with extract values, such that malting barley crops with high hordein levels (strongly correlated with high grain N) will generally give poor starch conversion efficiency. One hypothesis for this correlation is that starch granules are embedded in a protein/hordein matrix and are inaccessible to carbohydrases. Making the starch grain more accessible to seed carbohydrases by proteolytically removing the hordein coat surrounding it could easily increase extract and DP values.

Little information has been published that could provide a mechanistic explanation for a negative association between pH 6.0 endoproteinase activity and extract, DP, and α-amylase measurements. However, recent work from our laboratory may provide the basis for a potential mechanistic link between barley malt serine endoproteinases and DP in that we have observed multiple serine endoproteinase activities that are capable of degrading barley malt β-amylase (Schmitt and Marinac, in press). Such potential hydrolysis of one of the principal enzymes for carbohydrate mobilization could provide a theoretical mechanistic explanation for a negative link between serine endoproteinase activity and DP/extract reported in Table I. The very low correlation coefficient between the pH 6 endoproteinase activity and DP seen in Table I could indicate that the linkage was inherently weak or, alternatively, that a stronger negative association tending to reduce a key malting quality attribute has been selected out of the current malting barley lines tested in this collection. Examination of the proteinase and malting quality attributes in a population derived from wild unselected and barley parents could address these two possible explanations for the low strength linkage between pH 6 proteinase activity and carbohydrate malting traits.

Little data is available to provide a facile mechanistic explanation for the highly significant and relatively strong, albeit opposite direction, correlations between the two proteinase activities and wort β-glucan.

Recently, researchers using gene expression analysis instead of proteinase activity measurements to seek correlations with malting quality have identified two cysteine proteinase genes linked to quantitative trait loci (QTL) for extract and Kolbach index (Potokina et al 2004). Kihara et al (2006) recently expanded their earlier work and have also identified a QTL linked to cysteine proteinase activity and reported negative associations of total and cysteine proteinase activities with wort β-glucan.

In a recent publication, Potokina et al (2006) reported a positive association between a serine carboxypeptidase gene (Csp/P) and a diastatic power QTL in barley. This is an interesting observation because most recent emphasis on protein mobilization in germinating barley has emphasized the role of endoproteinase activities as rate-limiting (Sopanen et al 1980), rather than activities of exopeptidases such as Csp/P. This report of a connection between an exopeptidase activity and DP provides a strong impetus for reconsidering the current view emphasizing the importance of endopeptidase activity over that of exopeptidinases in protein mobilization during cereal germination and malting.

What relationships might exist between the pH 6.0 (putative serine) endopeptidase activities reported here and the serine carboxypeptidase activities of Csp/P identified by Potokina et al (2006), and the mechanisms by which they might affect starch hydrolysis and production of reducing sugars in diametrically opposed directions is not obvious. Nonetheless, these two observations about serine-type peptidase/proteinase activities and how they are correlated with starch mobilization in the germinating seeds emphasize how much still needs to be clarified regarding protein and carbohydrate mobilization in germinating seeds. Key information includes details regarding identity, substrates, and regulation of the various proteinases involved, the structure and expression of their genes, and how each may be linked to malting quality.

CONCLUSIONS

Use of high-throughput methods for malt homogenization (Mini BeadBead-96), coupled with fluorogenic proteinase substrates (Z-Phe-Arg-AMC at pH 4.5 for cysteine proteinases; DQ-Gelatin at pH 6.0), and 96-well microplate format sample handling and assay protocols, greatly simplifies surveys of proteolytic activity in malting barley populations. Application of the methodologies to >2,200 breeding lines analyzed for malting quality from the 2004 crop year shows correlations of malting quality parameters with proteinase activities and allows a first examination of potential proteinase activity distributions in large-scale studies designed to link genotypic and phenotypic data to develop improved tools for enhancing selection efficiency in malting barley breeding programs.

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LITERATURE CITED


Fontanini, D., and Jones, B. L. 2002. SEP-1—A subtilisin-like serine endopeptidase from germinated seeds of *Hordeum vulgare* L. cv.

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