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Effect of Reducing and Oxidizing Agents and pH on Malt Endoproteolytic Activities and Brewing Mashes

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The activities of the four endoproteinase classes of malted barley are known to vary with pH, and it seemed likely that the cysteine enzyme activities could be altered by redox agents. This study determined how altering the pH and adding redox agents to mashes influenced the worts that were produced during the brewing process. The reducing agents cysteine·HCl, dithiothreitol, and β-mercaptoethanol increased the proteolysis that occurred in malt extracts and mashes. This increased proteolysis was negated by the addition of the oxidizing agents diamide or hydrogen peroxide. The addition of reducing agents to mashes increased the soluble protein, free amino nitrogen (FAN), and extract values of their resultant worts, and this effect was abolished by the concomitant addition of oxidizing agents. Raising the pH values of the mashes strongly reduced their proteolytic activities, soluble protein, FAN, and extract values, but not their β-glucan levels. These results show that several of the major aspects of malting and brewing quality can be adjusted by varying the pH and redox qualitites of mashes, which could be helpful to brewers. These results also strengthen the previous proposal made by Buchanan et al. that the redox status of plants may play a significant part in controlling their physiology.

KEYWORDS: ***Hordeum vulgare*; barley; proteinases; soluble protein; cysteine; oxidizing agents; reducing agents; diamide**

INTRODUCTION

Our laboratory has been studying the processes whereby the proteins of barley and malt are solubilized and hydrolyzed during the malting and mashing phases of brewing, and we have shown that this is a complicated procedure involving multiple proteinases and several endogenous proteinase inhibitors. It is generally accepted by the U.S. brewing industry that the "soluble" or "wort" protein levels of most malt extracts prepared from recently developed malting barleys are higher than preferred, so it would be helpful to determine how these soluble protein levels can be reduced in the barley lines that are now being developed. While carrying out studies on how the various endoproteinase enzymes of barley (*Hordeum vulgare* L.) malt affect the soluble protein levels of brewing worts, we noticed that the addition of the amino acid cysteine (cys) to mashes strongly increased their proteolytic activities (*1*). In the past, it had generally been accepted that the cys class endoproteinases in mashes were responsible for most of the protein solubilization that occurred during barley germination (*2*). However, we have recently shown that both aspartic and metalloproteinase activities also affect the mash soluble protein levels (*1*). Still, the cys class proteases of malts are probably the most important group of enzymes that contribute to the wort soluble protein levels. The activities of these cys proteinases are maximized when they are in their reduced forms, so it is reasonable that the proteolytic activities of mashes would increase in the presence of reducing agents, unless all of the cys proteinases were already completely reduced throughout the mashing process.

Studies by Buchanan, Kobrehel, and their collaborators have indicated that there may be a system within cereal grains whereby the reduction states of various proteins in the grain are controlled by the presence of a system composed of NADPH, thioredoxin, and the enzyme NADP-thioredoxin reductase (*3*, *4*). They isolated a storage-protein-hydrolyzing serine-class proteinase from durum wheat that was activated by calcium, but this activation occurred only after the thiol groups of the enzyme had been reduced (*5*). Additionally, they have shown that thioredoxin reduces the abilities of a barley α-amylase-subtilisin inhibitor (*6*) to inhibit the activity of subtilisin and of certain soybean protease inhibitors to inhibit trypsin activities (*7*). These findings indicate that the abilities of grain proteinases to operate in vivo might also be controlled by whether they are in a reduced or oxidized state.

It was thus important to determine in detail how reducing agents such as cys, dithiothreitol (DTT) and β-mercaptoethanol (βME) and the oxidizing compounds 1,1′-azobis(*N*,*N*-dimethylformamide) (diamide, Sigma-Aldrich no. 19,315-1) and hydrogen peroxide (H_2O_2) affected the proteinase activities and the soluble protein levels and other brewing quality aspects of

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malt extracts and mashes. H_2O_2 is a strong general oxidizing agent, whereas diamide is apparently more specific, in that it can oxidize the sulfhydryl groups of proteins without interfering with the other cell functions (*8*). It also has the advantage of being readily soluble in water and of resisting hydrolysis in aqueous systems.

In this study the effect of varying concentrations of reducing and oxidizing agents on the endoproteinase activities of green malt (germinated, but not kilned, barley) and kilned malts was determined. In addition, to directly ascertain how these compounds affected the brewing process, they were added to American Society of Brewing Chemists (ASBC) mashes of barley malts to ascertain whether and how they altered the soluble protein and free amino nitrogen (FAN) levels of the worts from these mashes. During the cys addition study, it was noticed that the protein characteristics of mashes were strongly affected by their pH levels, so this phenomenon was also investigated.

EXPERIMENTAL PROCEDURES

Materials. The barley used was cv. Morex, the U.S. six-rowed malting barley quality standard, grown in Idaho and supplied by Dr. D. Wesenberg. It was malted using our standard methods (*9*), which include steeping to 45% moisture and germinating for 5 days at 17 °C. The green malt samples contained ~45% moisture and were removed prior to kilning, whereas the kilned samples were dried to ~4% moisture by a 24 h process that finished with a 3 h, 85 °C step (*9*). The diamide, DTT, L-cys hydrochloride (cys·HCl), and other chemicals were purchased from Sigma (St. Louis, MO) except that the 30% H_2O_2 was from Fischer Scientific, Fair Lawn, NJ.

Preparing Green and Kilned Malt Endoproteinase Extracts. *Kilned Malt.* The rootlets were removed from kilned malts. The malts were ground with a U/D mill (UDY, Ft. Collins, CO) to pass a 0.5 mm screen, and 29.0 g was stirred at room temperature with 100 mL of 50 mM, pH 5.5, ammonium acetate buffer for 30 min. The suspension was then centrifuged at 12100*g* for 10 min, and the supernatant was filtered through four layers of cheesecloth and used as the enzyme source.

Green malt was from the same germination batch but had not been kilned, and its rootlets were still present. Fifty grams of the 45% moisture green malt was frozen in liquid N_2, crushed with a mortar and pestle, added to 100 mL of the pH 5.5 buffer, stirred at room temperature, and centrifuged and filtered in the same way as the kilned malt.

Mashing. *ASBC "Congress" or "ASBC" Mashes.* Fine-grind malt samples were prepared using a Miag laboratory cone mill (Buhler, Inc., Plymouth, MN) adjusted as specified in the Malt-4 method of the ASBC analysis methods manual (*10*). These samples were extracted according to the ASBC Malt-4 procedure except that all weights and volumes specified for the method were halved. The compounds being tested were added to the mashes immediately after the initial water. During the Malt-4 procedure, the mash is maintained at 45 °C for the first 30 min, and this is called the "protein rest" phase. At the end of the protein rest the temperature is raised to 70 °C. The pH values of the mashes were measured with a pH meter 5, 10, 20, and 30 min after the protein rest phase started. The pH values were thus measured at 45 °C. Two-milliliter aliquots were removed from the mashes 10 and 30 min into the protein rest. After clarification by centrifugation in a microfuge, the endoproteolytic activities of these samples were measured (see below). Proteolytic activity assays were not carried out on the mashes after the temperatures were raised to 70 °C because previous studies have shown that the endoproteinases were very quickly inactivated at this temperature.

Endoproteinase Assays. *In Vitro, Quantitative Analyses.* The proteolytic activities of the various samples were analyzed using the "in solution" azogelatin proteolysis method of Jones et al. (*11*). Lyophilized azogelatin was dissolved in warm 0.1 M ammonium acetate, pH 5.5, buffer (extract assays) or 0.1 M citrate−phosphate buffer, pH 6.0 (mash analyses), to make a 2% (w/v) substrate solution. The substrate solution was stored at 4 °C and heated at 40 °C prior to use to liquify the azogelatin for pipetting into the assay tubes.

Extract Assays. A typical assay was carried out by diluting 0.6 mL of malt enzyme extract (described previously) to 1.88 mL with the pH 5.5, 50 mM ammonium acetate buffer or a combination of buffer and reducing or oxidizing agent. When needed, appropriate volumes of 50 mM cys·HCl, dissolved in the 50 mM ammonium acetate buffer and either "as is" or adjusted to pH 5.5 with ammonium hydroxide, were added and the solutions were incubated at 40 °C for 10 min. An appropriate volume of 21.5 mM diamide (or H_2O_2), dissolved in the ammonium acetate buffer, was then added, and another 10 min incubation was allowed. Azogelatin (1.5 mL of 2% solution in 5 mM, pH 5.5, ammonium acetate) was added to start the reactions. Aliquots (500 μL) of the reaction mixtures were removed after appropriate periods (normally 0, 10, 20, 30, and 50 min), put into 700 μL of cold 25% trichloroacetic acid, and incubated in an ice−water bath. After the last sample was in the ice bath for at least 10 min, the precipitated samples were centrifuged for 10 min at 11000 rpm in a microfuge, and the absorbances of the supernatants were measured at 440 nm. The resulting data were graphed, and the activities of the various reactions (change in A_{440nm}/min) were calculated using the linear portions of the reaction curves. The activity value was multiplied by 1000 to make it easier to compare values. When only cys or an oxidizing agent was being tested, the other reagent was omitted and only a single 10 min incubation was used prior to adding the substrate.

Mash Assays. Mashes were prepared that contained varying levels of cys, DTT, diamide, and/or H_2O_2 and, after 10 min of the protein rest phase, 2.0 mL of the mash was removed and used as the enzyme source for endoproteinase analyses. A second 2.0 mL sample was removed after 30 min of protein rest. The mash samples that were collected were centrifuged for 10 min, at 11000 rpm, in a microfuge, and the supernatant was decanted. A 0.60 mL aliquot of the supernatant was added to 1.17 mL of 50 mM, pH 5.5, ammonium acetate that contained enough cys, DTT, H_2O_2, and/or diamide to maintain the concentrations of those compounds at the levels in which they occurred in the mash. The reactions were started by adding 1.5 mL of 2% azogelatin in 5 mM, pH 5.5, ammonium acetate. Samples (500 μL) were removed from the reaction after 0, 10, 20, 30, 40, and 50 min, mixed with 700 μL of 25% trichloroacetic acid, and held in an ice−water bath. After the last sample had been in the ice water for at least 30 min, they were centrifuged in a microfuge at 11000 rpm for 15 min, and the absorbances of the supernatants at 440 nm were read.

Two-Dimensional IEF × PAGE Semiquantitative Analyses. The malt endoproteinases were separated by subjecting extracts to a two-dimensional separation on acrylamide gels, using isoelectric focusing (IEF) from pH 3.2 to 6.8 in the first dimension and polyacrylamide gel electrophoresis in 11% acrylamide gels that contained 0.1% azogelatin in the second, as described by Zhang and Jones (*12*). The enzymatic activities were then analyzed by developing the gels in the presence or absence of inhibitor fractions, as described previously (*13*).

Determining the Malting Quality Characteristics of the Mashes. Variations of standard ASBC methods (*10*) were used to measure the extract, wort soluble protein, free amino nitrogen (FAN), and β-glucan levels of the various samples.

Extract. The densities of filtered mashes (worts) were measured with an Anton/Parr DMA 5000 density meter (Anton Parr USA, Ashland, VA). The density data were used to calculate the amount of soluble material present in the filtrate and, thus, the percentage that had been extracted from the malt (ASBC Wort-2B) (*10*).

Soluble Protein. The soluble protein levels of the worts were determined using two methods. In the first, the UV absorbance method (ASBC Wort-17), the absorbances of the worts were measured at 215 and 225 nm, and the differences in these absorbance values were used to calculate the amount of protein present (*10*). For the second method, 0.25 mL wort fractions were pipetted into tin foil capsules, weighed, and dried overnight at 70 °C in an oven. The nitrogen remaining in the capsules was then measured in a LECO 528 nitrogen analyzer (LECO Corp., St. Joseph, MI) using the Dumas (ASBC Wort-10B) method (*10*). The protein values measured by both the UV and Dumas methods were adjusted to account for the background absorbances and nitrogen

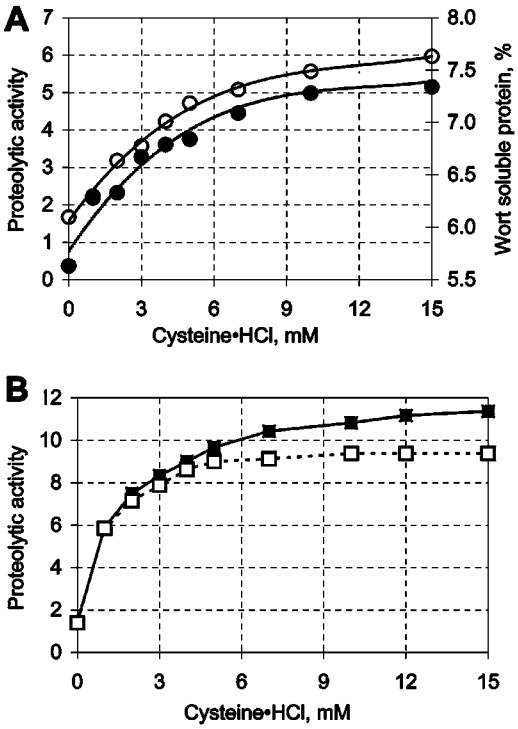

Figure 1. Effect of cysteine•HCl concentration on (A) the proteolytic activity and soluble protein levels of a mash and (B) kilned and green malt extracts: (○) proteolytic activity, $Abs_{440\ nm}$/min × 1000; (●) wort soluble protein; (□) green malt extract; (■) kilned malt extract.

contents of the reagents. The nitrogen levels measured by the Dumas method were multiplied by 6.25 to convert them into "soluble protein" (SP) values.

FAN Values. The FAN values of the worts were measured using an automated version of the ASBC Wort-12 method (*10*).

β-Glucan levels were determined by diluting a sample of the fine-grind extract with 17 volumes of 0.2 M glycine buffer, pH 10.0, mixing the diluted sample with a fluorescing agent, calcofluor, and reading the fluorescence of the mixture (ASBC Wort-18 method, ref *10*).

RESULTS AND DISCUSSION

Adding Cys·HCl to Malt Mashes. Because cys is often added to the extraction media used for studying the endoproteinases of barley and malts and to protease activity analyses, its effect on the endoproteolytic activity of Morex malt extracts was investigated first. Cys·HCl was added to ASBC mashes at concentrations between 0 and 15 mM, and the proteolytic activities of the mashes were measured after 10 min of stirring. The proteolytic activities of the mashes increased with the cys·HCl concentration, but not linearly (**Figure 1A**). The activities increased quickly up to ~5 mM cys, after which they continued to grow but at a much lower rate of increase. After the mashes had proceeded to completion, their SP levels were measured. The soluble protein value is the percentage of the original malt that ends up as dissolved "protein" (measured nitrogen × 6.25) in the final filtered mash. It is an indication of how well the malt had been "modified" and strongly affects the quality of the final beer. The effect of the mash cys·HCl levels on the soluble protein levels of the worts is also graphed in **Figure 1A**. Not surprisingly, the increase in endoproteolytic activity that occurred in the presence of increased cys·HCl levels was reflected in a parallel increase in the wort soluble protein levels. Neither the proteolytic activities nor soluble protein levels fell to zero in the absence of added cys. The activity of the mash was quite low in the absence of added cys, but the soluble protein level was over two-thirds as high as it was with 15 mM cys. This is due to the fact that only a small percentage of the soluble protein present in a wort is released during the mashing process—most of it is either released during malting or was already present in the unmalted barley grain (*1*).

Adding Cys·HCl to Green and Kilned Malt Extracts. The experiments shown in **Figure 1A** were carried out by mashing kilned malt that had been stored frozen for several months. When similar studies were carried out by measuring the proteolytic activities of freshly prepared buffer extracts (not mashes) of green (unkilned) and kilned malts, the results in **Figure 1B** were obtained. The kilned malt extracts behaved similarly to the mashes (**Figure 1A**), except that the activities were slightly higher. These higher kilned malt activities reflect the fact that the extract contained more malt per milliliter than the mash. The green malt activities also increased as cys·HCl was added, up to ~5 mM, but after that they remained constant. Repeated experiments confirmed that the green malt proteolytic activities remained constant at cys·HCl concentrations >5 mM, whereas those of the kilned malt continued to rise slowly with the increase in cys concentration. The fact that both the mashes and extracts showed the same general responses to added cys·HCl indicates that results obtained with the extracts are representative of what can be expected with real mashes. The green malt extract activities were measured to determine whether the characteristics of the endoproteinases were affected by the kilning process, during which the malt was subjected to high temperatures. This heating was not expected to greatly affect the results, because we have shown previously (*9*) that the kilning process had little or no effect on the malt proteolytic activities. The fact that the green and kilned malt results were similar, and differed only at high cys levels, indicates that the findings that were obtained with kilned malts probably also apply generally to germinating seeds.

Effect of pH on Protein Hydrolysis. Measurement of the pH values of the mash worts of **Figure 1A** indicated that the addition of the cys·HCl solution, even at the low levels used and in the presence of the buffering action of the malt, lowered the pH of the mashes from 5.97 (no addition) to 5.73 (15 mM cys·HCl). We had shown previously that the cys class proteinases of malted barley were maximally active at pH 4.5 and that the activities had dropped off strongly at pH 6.0 (*12*), so it seemed possible that some of the proteolysis activity increase that occurred as the cys·HCl levels increased was due to the concomitant lowering of the mash pH.

To test this, enzyme extracts were prepared from kilned Morex malt with 50 mM, pH 5.5, ammonium acetate buffer. Two sets of aliquots of this extract were incubated with cys at levels that varied from 0 to 9 mM. The cys that was added to these extracts was cys·HCl that was dissolved in 50 mM, pH 5.5, ammonium acetate. In one set, the pH of the cys·HCl solution had been adjusted to pH 5.5 with NH_4OH, whereas in the other the pH of the cys·HCl solution was not adjusted. The pH of the untitrated cys·HCl solution, dissolved in the 50 mM ammonium acetate, was 2.8. The proteolytic activities of these extracts were measured, together with the pH values of the extract−cys mixtures that were left over after samples were removed for the analyses.

The results are shown in **Figure 2**. The pH values of the extracts that contained pH-adjusted cys all stayed constant at 5.45, whereas those of the extracts that received the non-pH-adjusted cys·HCl dropped from 5.45 (0 mM cys·HCl) to 5.08 (9 mM cys·HCl) as the cys concentration increased. This relatively small change strongly affected the proteolytic activi-

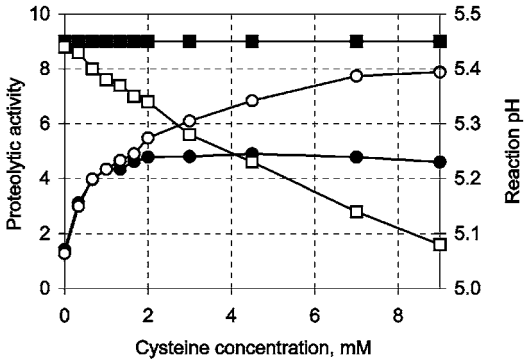

Figure 2. Effect of adding cysteine·HCl or cysteine adjusted to pH 5.5 on malt extract pH levels and proteinase activities: (■) pH of pH-adjusted cysteine reactions; (□) pH of nonadjusted cysteine·HCl reactions; (●) proteolytic activities with pH-adjusted cysteine; (○) proteolytic activities with nonadjusted cysteine·HCl.

Table 1. Effect of Treatments on the Proteolytic Activities of Green and Kilned Malt Extracts

	endoproteolytic activity[a]	
treatment	green malt	kilned malt
none	1.19	0.96
2 mM diamide	0.83	0.92
1 mM cysteine	4.85***	4.85***
1 mM serine	1.12	1.19
1 mM alanine	1.09	1.19
1 mM cystine	1.06	1.12
deaerated buffer[b]	1.58***	1.35***
deaerated, 1 mM diamide	1.29	1.19
deaerated, 1 mM cysteine	3.63***	5.64***
air, bubbled 5 min[c]	1.12	1.19
air, 2 mM diamide	0.92	0.91
air, 1 mM cysteine	4.65***	4.75***
nitrogen, bubbled 5 min[c]	1.19	1.16

[a] $\Delta Abs_{440\ nm}$/min × 1000. ***Values differed from the control at the 0.05 level. ($f = 15.68$; df = 12,12; $p < 0.05$. Nonparametric completely randomized ANOVA based on ranks using treatment and enoproteolytic activity as factors. All treatments were compared against the control using Dunnett's *t* test.). [b] Extract was prepared with vacuum-deaerated buffer. [c] Air or nitrogen was bubbled through the extract for 5 min.

ties. The activity of the non-pH-adjusted cys·HCl sample continued to rise up to at least 9 mM, whereas that of the pH-adjusted samples coincided with that of the nonadjusted samples only up to 1 mM cys; thereafter, it rose slightly with cys concentration up to 2 mM and then remained steady. From this, it appears that the presence of 1−2 mM cys increased the proteolytic activity of the extract by ~3-fold, and the activity increase that was observed thereafter was entirely due to the pH change.

Inhibition of the Endoproteinase Activities by Oxidizing and Other Reagents. To determine whether the activation of the endoproteolytic activities of extracts was due to the reducing characteristics of cys and to ensure that it was not an artifact of the extraction process, activity measurements were carried out in the presence of an oxidizing agent, diamide, and with other amino acids (**Table 1**). In all cases, the addition of diamide reduced the endoproteinase activities of both malt extracts slightly, but not enough to show as statistically significant at the 0.05 level. The amino acids serine, alanine, and cystine had no discernible effect on the activities of either the green or kilned malt enzymes. The activation was thus specific to cys and, therefore, was probably due to the fact that it is a reducing agent and not because it is an amino acid. The addition of cys activated the enzyme preparations strongly, indicating that these preparations were similar to those studied previously.

Table 2. Effect of Oxidizing Agents and Cys on Proteolytivc Activities

	endoproteolytic activity[a]	
treatment	green malt	mash
none	5.5	1.75
1 mM diamide	4.6***	1.67***
1 mM H_2O_2	2.5***	1.07***
1 mM cys	11.3***	5.10***
1 mM cys, 1 mM diamide	4.3***	1.67***

[a] $\Delta Abs_{440\ nm}$/min × 1000. ***All treatment values differed from those of the controls at the 0.05 level. Statistical analyses as for **Table 1**, but $f = 15.68$; df = 12,12; $p < 0.05$.

To ensure that the enzymatic activities of the enzymes had not been altered by air oxidation during their extractions, extracts were prepared with buffer that was vacuum degassed and other extracts were saturated with air or nitrogen gas. The bubbling with nitrogen was carried out to ensure that the enzymes were not simply being inactivated by the bubbling process. There was no indication that the proteolytic activities were affected by bubbling air or nitrogen through their solutions, for up to 5 min, because the activities of all of the samples were essentially identical to those of the nonbubbled controls (**Table 1**). In the presence of diamide both the degassed and oxygenated extracts had slightly lowered proteolytic activities, and the addition of cys strongly enhanced the activities in both cases. The activities of the deaerated buffer extracts were slightly enhanced, but they behaved like the control samples in that their activities were slightly inhibited by diamide and were activated by the addition of cys. Statistical analyses indicated that there were no statistically significant differences between the green and kilned malt extract hydrolyses ($f = 0.26$; df = 1,12; $p = 0.6206$).

Adding Additional Oxidizing and Reducing Agents to Malt Extracts. Because the addition of the reducing agent cys to extracts strongly increased their endoproteolytic activities, it seemed likely that oxidizing agents might also affect those activities, but in the opposite direction. To test this, ammoniuim acetate extracts of green malts and mash samples were treated with 1 mM diamide or hydrogen peroxide (H_2O_2) (**Table 2**). The compound diamide has been shown to specifically oxidize the disulfide bonds of certain proteins without affecting their other characteristics (*8*). H_2O_2, on the other hand, is a general, strong oxidizing agent.

In **Table 1**, when diamide was added to kilned malt extracts, it lowered the proteolytic activities, but in that experiment these differences were not statistically significant. However, when either diamide or H_2O_2 was added to either green malt extracts or malt mashes, they significantly and reproducibly lowered their endoproteolytic activities (**Table 2**). As before, the addition of 1 mM cys strongly activated the reactions, and this activation was nullified by the addition of an equal concentration of diamide. The diamide decreased the activities of the samples containing cys to essentially the same levels they were in the absence of cys. The activations/inactivations were thus apparently due to the reduction and oxidation of the endoproteinase enzymes. In a separate experiment (not shown), it was found that 1 mM DTT, a strong reducing reagent, increased the proteolytic activity of mash fractions and that this activation was also neutralized by 1 mM diamide. A second strong reducing agent, βME, however, did not act as a proteinase activity enhancer in this analysis system (discussed in detail

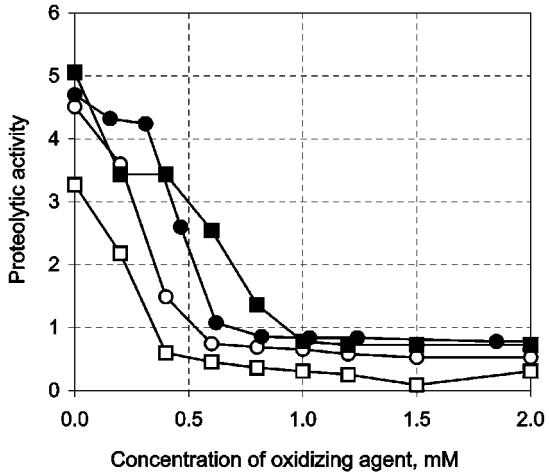

Figure 3. Inhibition by oxidizing agents of the activation of malt endoproteinases by cysteine and dithiothreitol: (○) H_2O_2 inhibition of 1 mM cysteine activation; (●) diamide inhibition of 1 mM cysteine activation; (□) H_2O_2 inhibition of 0.75 mM DTT; (■) diamide inhibition of 0.75 mM DTT.

below). Statistically, all of the green malt extract values were significantly different from those of their mash counterparts, due to the fact that the extract enzyme levels were higher than those of the mashes ($f = 361$, df = 1.4, $p < 0.05$). However, the correlation between the green malt and mash values was 0.984, indicating that the reactions behaved very similarly. When a second analysis was carried out with smaller amounts of the green malt extract, the high correlation, 0.998, again indicated that the green malt and mash enzymes behaved very similarly.

Stoichiometry of the Cys and DTT Activation Reactions with Diamide and H_2O_2. To measure how well the oxidizing agents diamide and H_2O_2 overcame the activation of the mash endoproteinases by cys and DTT, reactions were carried out in which various amounts of diamide and H_2O_2 were added to reactions that contained either 1 mM cys or 0.75 mM DTT (**Figure 3**). These cys and DTT concentrations had given nearly maximal activation of the endoproteinases in previous experiments. As the concentrations of the oxidizing agents were increased, the proteolytic activities decreased and then stabilized. It took more H_2O_2 to overcome the action of cys than of DTT, but with diamide this order was reversed. Nonlinear regression analyses indicated that the oxidant/reductant ratios needed to neutralize the activation of the enzymes were as follows: H_2O_2/cys, 0.86; H_2O_2/DTT, 0.73; diamide/cys, 1.20; and diamide/DTT, 1.46. The H_2O_2 was thus more effective at overcoming the activation by reducing agents than diamide was under these conditions. As was noted in previous experiments, the proteolytic activity in the presence of saturating levels of H_2O_2 was lower than it was with diamide. The activities remained at these levels up to at least 5.6 mM oxidizer, which was the highest concentration tested. Part of this H_2O_2 effect may be due to the fact that it is a stronger oxidizing agent than diamide and thus may have reduced some non-active-site disulfide bonds, which may have helped to lower the enzyme activities. It was not possible to test the slopes of the lines shown in **Figure 3** statistically to prove which inhibitions were most effective, but in a preliminary experiment that tested the ability of diamide to counteract the activation by 1 mM cys and 0.46 mM DTT, it also took about twice as many moles of diamide to overcome the effect of DTT as it did to cancel the cys effect (results not shown).

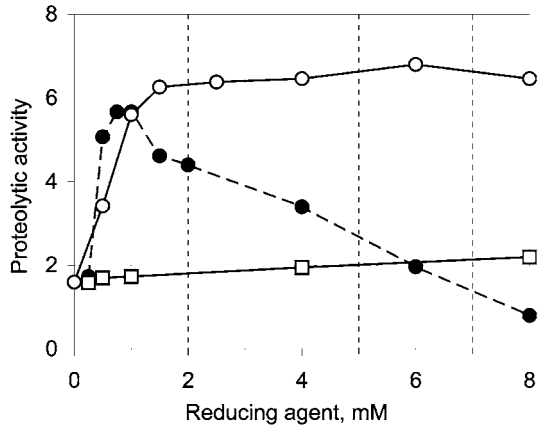

Figure 4. Effect of three reducing agents on the proteolytic activities of a kilned malt extract: (○) cysteine·HCl adjusted to pH 5.5; (●) dithiothreitol; (□) β-mercaptoethanol.

Control reactions for this experiment (no added redox agents) had an average of 1.18 "proteolytic activity units", which was lowered to 0.78 unit in the presence of excess diamide. This indicates that there was ~0.40 unit of activity in the controls that was destroyed by the diamide, above that which was due to the addition of cys or DTT. From this, and from the fact that adding 1 mM cys normally resulted in an ~4 unit activity increase (**Tables 1** and **2**), it appears that the reducing power present in the extracts that did not contain added reducing agent was the equivalent of only ~0.1 mM cys.

Comparison of the Effects of the Reducing Agents Cys, DTT, and βME. *In Vitro Assays.* To compare the activation effects of cys with those of the stronger reducing agents DTT and βME, each was added to mash samples at concentrations up to 8 mM, and the endoproteolytic activities of the mashes were measured. Neither DTT nor βME affected the pH values of the reactions, so they did not need to be neutralized. The cys solution that was tested was neutralized prior to being added. **Figure 4** shows the results. As seen previously (**Figure 2**), cys increased the activities of mashes at concentrations up to ~1.5 mM, above which the activity remained constant. As with cys, the enzymatic activity increased rapidly at the lower DTT concentrations (up to 0.75 mM). From that point on, however, adding more DTT resulted in a leveling off and then a lowering of the proteinase activity, until in the presence of 8 mM DTT the activity was even lower than it was in the control reactions. βME had almost no effect on the activity, as measured with this in vitro assay.

Two-Dimensional (2D) IEF × PAGE Assays. These in vitro assay results contrasted sharply with those obtained using a semiquantitative 2D gel method (**Figure 5**) and with the results obtained with mashes (**Table 3**). The 2D gel analyses have the advantage that they partially separate the component proteinases of the extract and therefore give an indication of which proteinases are affected by the various treatments. **Figure 5** consists of drawings of selected 2D gels. These drawings are negative images of stained, substrate−protein containing gels, in which the blue-staining proteins were hydrolyzed by the separated enzymes, leaving white areas. Thus, the darker spots on the drawing indicate where the higher proteolytic activities were.

Figure 5A shows the normal pattern of the endoproteinases of malt extracts. Previous studies have shown that, except for the light activity indicated by the arrow, the characteristics of which are ambiguous, the enzymes that occupy the top quarter of the gels all belong to the serine or metalloproteinase classes

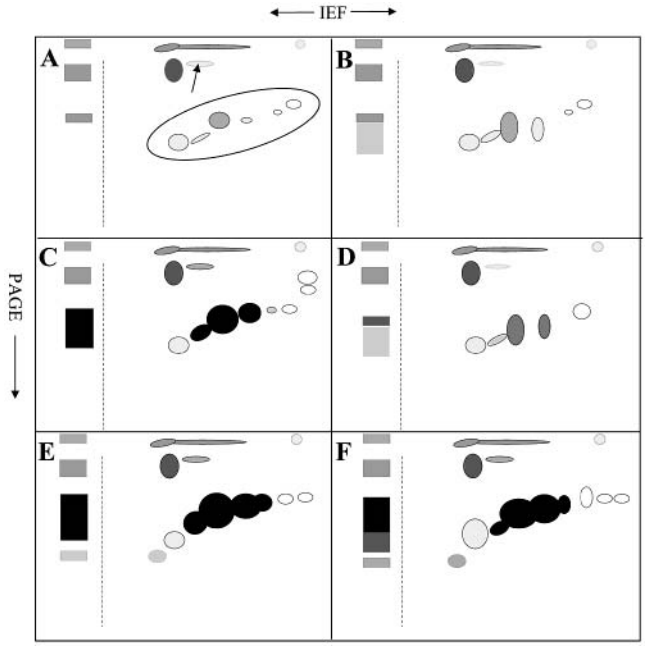

Figure 5. Diagrams of the IEF × PAGE separated endoproteinase activities developed at pH 4.8 in the presence of various reducing and oxidizing compounds. The activities were developed in the presence of (A) no added redox agents, (B) 1 mM diamide, (C) 1 mM cysteine, (D) 1 mM cysteine + 1 mM diamide, (E) 0.75 mM dithiothreitol, and (F) 1 mM β-mercaptoethanol. The arrow indicates the "ambiguous" endoproteinase activity. The cysteine endoproteinases migrated to the area indicated by the circle. The sample on the left side of each gel was subjected only to the PAGE separation. The darker shadings indicate higher proteolytic activity levels.

(*12*). All of the proteinases that migrated further down the gel (circled in **Figure 5A**) were cysteine class proteases. There was no detectable inactivation of any of the separated extract proteinases when the activities were developed in the presence of 1 mM diamide (**Figure 5B**). Developing the gel in a 1 mM cys solution, however, strongly activated three of the cysteine class enzymes (**Figure 5C**). This activation was almost entirely negated when 1 mM diamide was added to the developing solution together with the cys (**Figure 5D**). Development of the activities in 0.75 mM DTT strongly activated the same three cysteine class enzymes, together with an additional one (**Figure 5E**). These same four enzymes were highly activated in the presence of 1 mM βME, but the ratios of the activities of the four enzymes differed slightly from those activated by DTT (**Figure 5F**). The activations by DTT and βME were also completely negated by the addition of 1 mM diamide to the developing solution (results not shown). None of the proteinases belonging to the serine or metalloproteinase classes was affected by any of the redox compounds, except that the "ambiguous" enzyme may have been slightly activated by all three of the reducing agents.

These 2D experiment gels were developed at pH 4.8 because, with this technique, the cysteine proteinase activities do not show up well at pH 5.5, at which the majority of the in vitro assays were conducted. As discussed below, these 2D analysis results correlated very well with those obtained on analyzing the characteristics of brewing mashes. All three of the reducing agents strongly increased the activities of the cysteine class proteinases, in contrast with the results of the in vitro experiments, in which βME never caused any activation. Also, in contrast to the in vitro analysis results, when identical 2D gels were developed in the presence of 4 mM DTT or 4 mM βME, the activities in both cases were as high or higher than those seen with 0.75 mM DTT and 1 mM βME. It would have been hard to detect any increased activities with these higher reducing agent concentrations, because essentially all of the substrate protein had been hydrolyzed from the gel in the presence of the 0.75 mM DTT and 1 mM βME. It would, however, have been easy to detect any diminution of activity, as suggested by the in vitro analysis results, and none occurred. The cys proteinases of gels developed in 8 mM cys had slightly more activity than those in 1 mM cys. Again, these results agreed with the mash composition data, but not with the in vitro analysis results. Both the in vitro and 2D results were very reproducible, which makes it appear that the differences were probably due

Table 3. Effect of Reducing and Oxidizing Agents on Mashes

	wort pH	extract, %	soluble protein, %	FAN,[a] ppm	β-glucan, ppm
avg from analysis of three mashes[b]					
control	5.78	80.3	6.02	297	48
1 mM cysteine[c]	5.73	81.1	6.43	338	44
3 mM cysteine	5.68	81.9	6.91	378	45
8 mM cysteine	5.62	82.5	7.35	412	42
1 mM cysteine + 1 mM diamide	5.77	80.3	5.92	272	54
1 mM diamide	5.80	79.5	5.53	248	64
1 mM βME	5.76	80.6	6.25	314	54
4 mM βME	5.74	81.0	6.55	333	50
1 mM βME + 1 diamide	5.73	80.3	5.79	264	54
avg from analysis of two mashes					
control	5.77	80.5	6.06	294	48
0.75 mM DTT	5.75	80.8	6.43	327	51
4 mM mM DTT	5.71	81.5	7.01	372	64
0.75 mM DTT + 1 mM diamide	5.76	80.4	5.79	263	54
values from analysis of a single mash					
control	5.79	79.6	5.88	295	48
1 mM cys	5.76	80.5	6.59	346	44
1 mM cys + 1 mM H_2O_2	5.77	80.0	5.92	294	54
1 mM H_2O_2	5.80	79.6	5.69	267	45
1 mM cys, unadjusted[d]	5.70	80.9	6.48	338	51
3 mM cys, unadjusted	5.58	82.0	7.11	386	47
8 mM cys, unadjusted	5.23	83.3	7.85	453	49

[a] Free amino nitrogen. [b] Some treatments were carried out with three separate mashes, some with two, and some only once. All β-glucan measurements were made with only one mash. [c] pH of cysteine·HCl adjusted to pH 5.5. [d] pH of cysteine·HCl not adjusted.

to some quirk of one of the analysis systems, presumably the in vitro one, because the 2D results correlated much better with the real mashing results.

Adding Redox Agents to Mashes. When ASBC mashes (*10*) were carried out in the presence of various reducing and oxidizing agents, the results listed in **Table 3** were obtained. The "control" values for the parameters listed are very characteristic of those from well-malted Morex barley. When cys·HCl was added, the pH of the mash dropped, as expected, and the extract, SP, and FAN levels all increased with the amount of cys. When the pH effect was removed by adding cys that had been adjusted to pH 5.5, these parameters still increased, but not as strongly. The addition of diamide or H_2O_2 alone led to moderately reduced extract, SP, and FAN levels, and when they were added together with 1 mM cys, they negated its ability to increase the malt quality parameters. The addition of either DTT or βME caused changes that were similar to those seen with cys. Both raised the extract, SP, and FAN levels, with more reagent yielding higher increases, and, in both cases, this effect was negated by the presence of 1 mM diamide. These results agree with those expected from the results of **Figure 5**, but not with those of **Figure 4**, which indicated that the addition of the high levels of DTT and βME should have led to lowered SP and FAN levels.

Except for those of **Figure 4**, these results all make sense. Under conditions whereby the endoproteinase activities increased, more of the water-insoluble malt storage proteins were apparently hydrolyzed and thus rendered water soluble. In turn, these solubilized proteins then became more vulnerable to the exoproteinases that are present in malt and were more readily hydrolyzed into the very short peptides and amino acids that comprise the FAN levels. Because the "extract" values represent the percentage of the malt that dissolves under a particular set of conditions, a portion of its value is contributed by dissolved protein, so as the soluble protein percentage increased, the extract values increased in tandem. Apparently the enzymes that hydrolyze malt carbohydrates to release β-glucans were unaffected by either reducing agents or pH, because the experimental conditions had no effect on the β-glucan levels of the mashes.

Effect of Mash pH on Wort Characteristics. To measure exactly how changing the pH of a mash affected its biochemical characteristics in the absence of the added reducing agent cys, a series of room temperature "mashes" were carried out to which varying amounts of either acetic acid or NaOH were added. A study of the effect of these additions on the mash pH values was conducted by adding the acid or base to mashes, stirring them for 30 min, and measuring their pH at 0, 5, 20, and 30 min. These initial "mashings" were done at room temperature to ensure that there was no effect of elevated temperatures on the pH measurements. The results are shown in **Figure 6**. The normal components of the mashes were fairly effective buffers, in that the effect of the added acid and base was partially mitigated as the malt components dissolved during the first few minutes of stirring. In the absence of added HAc or NaOH, the pH held steady at 5.8, and when either acid or base was added, the pH tended toward that pH, indicating that this is the natural pH of this ASBC mash. To better ascertain how quickly the pH equilibria were attained, the pH levels of the mashes that contained the highest amounts of acid and base were measured every 30 s during the first 10 min of mixing. The initial pH change was rapid, half of it occurring within 1.5 (NaOH) to 2.5 (HAc) min (**Figure 6**). The effective pH values of the mashes were readily adjusted to cover the range from 4.8 to 6.4.

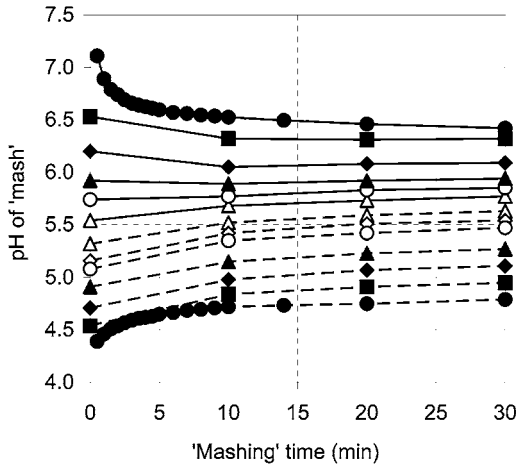

Figure 6. Alteration of the pH values of mashes by the addition of acetic acid or NaOH. Mashes were at room temperature. Solid lines indicate that NaOH was added to a final concentration of (△) 0 mM, (○) 0.92 mM, (▲)1.84 mM, (◆) 3.68 mM, (■) 5.52 mM, or (●) 9.20 mM. Broken lines indicate that acetic acid was added at (△) 1.09 mM, (◇) 2.18 mM, (○) 3.26 mM, (▲) 6.53 mM, (◆) 9.79 mM, (■) 15.23 mM, or (●) 21.75 mM. The pH levels of the mashes having the highest levels of acid and base added were measured every 30 s between 0 and 10 min.

When normal, 45 °C, mashes were carried out at final wort pH values that varied from 5.09 to 6.61 (addition of HAc or NaOH), many of the wort characteristics varied strongly (**Figure 7**). The proteolytic activities of all of the mashes were identical whether measured after 10 or 30 min (**Figure 7A**), indicating that, as shown previously (*14*), these enzymes were completely stable at 45 °C. As expected, the overall activities decreased greatly as the pH increased, presumably because the more vigorous, low-pH-active cys endoproteinases became less active and their less dynamic, higher-pH-active, metalloproteinase and aspartic proteinase kin became predominant (*12*). This change in proteolytic activities was reflected in the malt FAN and soluble protein levels, which both dropped in nearly identical, sigmoidal, fashions as the pH was raised (**Figure 7B**). Although the FAN and SP characteristics dropped in the same way, the FAN levels dropped by 47% between pH 5.1 and 6.2, whereas the soluble protein levels fell by only 30%. This probably reflects the fact that the exopeptidases play a major role in the release of FAN, whereas only endoproteinases significantly contribute to the SP levels. The "extract" percentage of the mashes also dropped as the pH rose (**Figure 7C**), part of its 4.4% decline presumably reflecting the 2.1% lowering of its "SP" component. The wort soluble β-glucan content, on the other hand, increased slightly with the pH, indicating that this carbohydrate was more readily solubilized at the higher pH levels. This change in β-glucan content was not very important, because even a difference of 100 ppm of β-glucan corresponds to a low amount (~0.006% of the malt). In terms of malting quality, the U.S. brewing industry would, at this time, like to have six-rowed barley malts with the following characteristics: SP = 5.2−5.7%, β-glucan < 140 ppm, and extract values of >79% (*15*). No specific value has been specified by the industry, but it is generally accepted that the FAN level should be at least 150 ppm to ensure good yeast growth (*16*). The variations in the SP, extract, and FAN values that were caused by the addition of redox agents and/or the varying of the pH in these

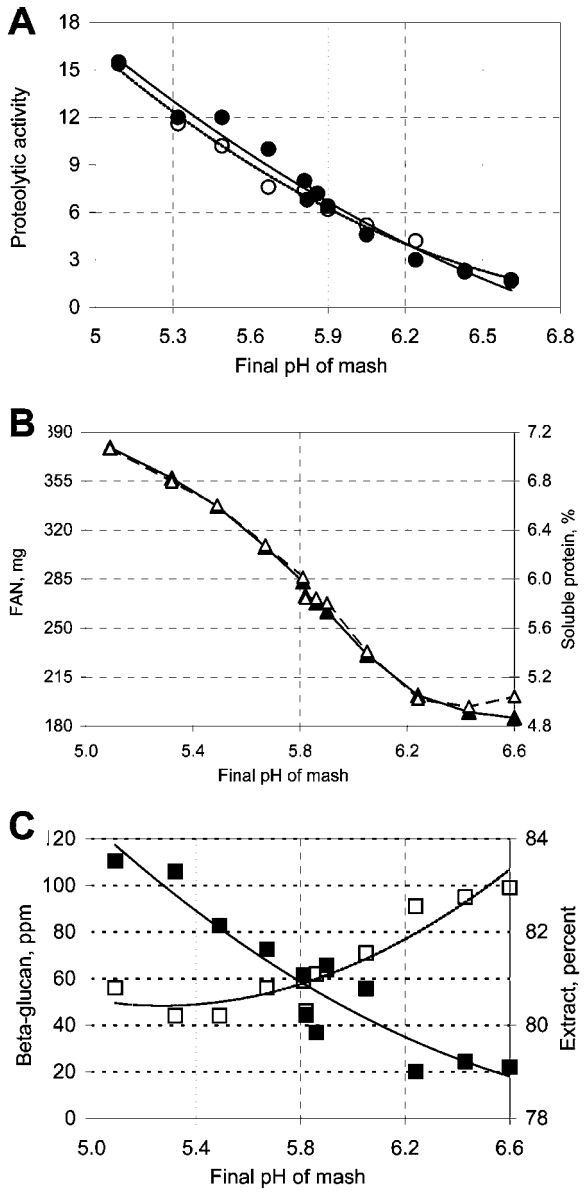

Figure 7. Characteristics of worts prepared at pH levels varying between 5.1 and 6.6: (A) proteolytic activities after 10 and 30 min of mashing; (B) free amino nitrogen and soluble protein levels; (C) β-glucan and extract concentrations; (●) activities after 10 min; (○) activities after 30 min; (△) soluble protein; (▲) free amino nitrogen; (□) β-glucan; (■) extract levels.

experiments were large enough that they would have strongly affected the brewing process. The industry currently considers worts that are prepared from six-rowed malts and that have SP values that fall outside the range of 4.8−6.0 to be unacceptable for brewing (*15*). They would also probably be suspicious of using worts having FAN values that are not between 150 and 250 ppm, and almost no commercial six-rowed malting barleys have extract values >82%. It is obvious from **Figure 7** that compositions outside these values can easily be obtained by simply adjusting the pH values of the mashes, and **Table 3** shows that the same results can be effected by the addition of oxidizing and reducing agents. It would be interesting to use these methods to produce some nontraditional worts and to investigate how they behave during brewing and how the resulting beers compare to conventional ones. Such studies might result in worts that can be used to produce new and useful products.

Summary. From these data, it is obvious that several important aspects of the mashing process of brewing can be altered considerably, simply by adjusting either their pH levels or their redox characteristics. This means that brewers, if they wish, can use these methods to adjust the compositions of worts to produce altered and/or improved beers. If it is correct, as proposed by Buchanan and his collaborators (*6*), that a system already exists in barley whereby specific protein disulfide bonds can be reduced, then this study shows that any such change really could have very substantial effects on the biochemistry of the plant. This study also points out that researchers need to be cautious in conducting research on these complex systems. Although the in vitro method of measuring barley and malt proteolytic activities with the substrate azogelatin (*11*) worked fine for studies with cys and the oxidizing agents that were tested, the results obtained with it obviously did not reflect what actually happened in mashes in the presence of the strong reducing agents DTT and βME, although the 2D experiments, which used the underivatized form of the same substrate, did. Several of these same experiments were carried out with Harrington barley, a very good malting quality North American two-rowed barley. The results of those studies indicated that Harrington extracts and mashes behaved similarly to those of their six-rowed Morex counterparts in terms of the effect of cys, DTT, and βME on their endoproteolytic activities and in the ability of diamide to reverse the activation of its proteinases by cys (results not shown). The effects of redox agents and pH on the proteinases of Harrington extracts and malts did not differ significantly from those of Morex in any of the characteristics that were tested (data not shown).

ABBREVIATIONS USED

ASBC, American Society of Brewing Chemists; βME, β-mercaptoethanol; cys, cysteine; DTT, dithiothreitol; FAN, free amino nitrogen; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; SP, soluble protein; 2D, two-dimensional.

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