Quantitative Study of the Formation of Endoproteolytic Activities during Malting and Their Stabilities to Kilning

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The proteinases of germinating barley (*Hordeum vulgare* L.) hydrolyze storage proteins into amino acids and small peptides that can be used by the growing plant or, during brewing, by yeast. They are critical for the malting and brewing processes because several aspects of brewing are affected by the amounts of protein, peptide, and amino acids that are in the wort. This study was carried out to quantitatively measure when endoproteinases form in green malt and whether they are inactivated at the high temperatures that occur during malt kilning. Little endoproteolytic activity was present in ungerminated barley, but the activities began forming 1 day into the "germination" phase of malting, and they were nearly maximal by the third germination day. Quantitative studies with azogelatin "in solution" assays showed that the green malt endoproteolytic activities were not inactivated under commercial kilning conditions that use temperatures as high as 85 °C but that some actually increased during the final kilning step. Qualitative (2-D, IEF × PAGE) analyses, which allow the study of individual proteases, showed that some of the enzymes were affected by heating at 68 and 85 °C, during the final stages of kilning. These changes obviously did not, however, decrease the overall proteolytic activity.

Keywords: *Barley; Hordeum vulgare; malting; protease; protein hydrolysis; kilning*

INTRODUCTION

During malting, barley storage proteins are partially degraded by proteinases into amino acids and peptides that are critical for obtaining high-quality malt. Several years ago, we developed a two-dimensional isoelectric focusing × polyacrylamide gel electrophoresis (2-D IEF × PAGE) analysis system that showed that germinated barley (green malt) contained >40 endoproteinase activities, the majority of which were cysteine class enzymes (Zhang and Jones, 1995a). Using relatively qualitative methods, we (Zhang and Jones, 1995b) and other researchers (Harris, 1962) have shown that ungerminated barley contains only small amounts of proteolytic activity and that this activity increases during the early stages of the "germination" step of malting. Until lately, there was no good method for quantitatively measuring the activities of the multiple malt endoproteinases and correlating this activity with the presence of the individual enzymes.

Recently, we described an improved "in solution" protease assay method that is based on the hydrolysis of the substrate azogelatin (Jones et al., 1998). In this paper, this in solution assay method will be termed the "in vitro" method. Even though the 2-D separation method is also an in vitro method, it will be referred to as the "2-D method" to distinguish it from the in solution one. By using this in vitro assay to measure the activities of solubilized malt proteinases and concomitantly separating and analyzing those activities with the 2-D IEF × PAGE system using the gel-incorporated substrate azogelatin [or gelatin, which detects the same enzymes (Zhang and Jones, 1995a)], it is possible to quantitatively and qualitatively measure the proteinases and to see how changes in the activities of individual enzymes affect the overall proteinase activity. In this study, we have used the in vitro and 2-D assays to measure the increase in proteolytic activity that occurs during the initial stages of malting and to examine the extent of the proteinase activity decrease that occurs during malt kilning.

In the United States, commercial malts are normally kilned to final temperatures of ∼85 °C (185 °F). Enzymes are inactivated or denatured by high temperatures, especially in the presence of water; most are inactivated at temperatures of between 60 and 70 °C (Lehninger, 1975). For example, barley carbohydrate-degrading enzymes (α-amylase, *β*-amylase, and limit dextrinase) were reportedly all degraded at temperatures between 65 and 75 °C (Inkerman et al., 1997), and wheat amylases were 50% inactivated at 75, 64, and 70 °C (Tkachuk and Kruger, 1974; Walden, 1955). Most enzymes are more stable in biological systems that contain other proteins and biochemicals than they are in water alone.

Little has been published about the heat stabilities of barley endoproteinases. One purified, dissolved, cysteine class barley aleurone proteinase maintained ∼20% of its activity when heated to 60 °C (Koehler and Ho, 1988), and dissolved malt proteinases were inactivated within 2 min at 70 °C (Kringstad and Kilhovd, 1957). During mashing, the proteinases responsible for releasing soluble nitrogen were inactivated at temperatures

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of 55−60 °C (Schoenfeld, 1981). The 85 °C temperature to which malts are subjected during kilning is, therefore, considerably higher than the temperatures at which most of their proteases would be inactivated if dissolved. However, in malt that is undergoing kilning, the enzymes are not in solution but are intimately associated with other compounds in a matrix that drops from about 45 to 4% moisture as the temperature is slowly increased. By the time the temperature reaches 85 °C, the moisture level has already dropped to ∼4%, which would be expected to retard the rate at which the proteinases are inactivated.

It has been stated fairly often that green malt proteinases are susceptible to inactivation during kilning (Lewis and Young, 1995, for example), but most scientific studies have indicated that these enzymes are fairly stable to kilning conditions (Kringstad and Kilhovd, 1957). In this study we have quantified the total proteinase activity of green malt throughout the kilning process using the in vitro analysis method and have followed the activities of the individual protease activities with the 2-D system.

MATERIALS AND METHODS

Preparation of Kilned Malt and Partially Kilned Green Malt Samples. Seed [170 g (db) aliquots] of the barley cultivars Morex and Harrington (the six-rowed and two-rowed American Malting Barley Association malting quality standards, respectively) were steeped at 16 °C to 45% (w/w) moisture (∼36 h), by alternating 4 h of wet steep with 4 h air rests. The steeped seeds were germinated in the dark for 5 days at 17 °C and near 100% humidity, in cans that were rotated for 3 min every 30 min. The resulting "green malt" was then kilned using a schedule (from 49 to 85 °C, see Results and Discussion) that mimicked those used commercially by U.S. malting companies. Six Morex and six Harrington green malt fractions (170 g of dw each) were put into the kiln, and a seventh sample of each was kept as the green malt specimen. Partially kilned fractions were obtained by removing one sample of each cultivar just prior to each kilning temperature change. The last sample (kilned malt) was collected at the end of the final, 85 °C, step. The collected malt fractions were lyophilized, the kernel rootlets were removed, and the grains were ground in a Brinkmann ZM-1 centrifugal grinding mill (Westbury, NY) to pass a 0.5 mm screen. The ground malts were dried under vacuum, after which they were stored in a desiccator at 4 °C until extracted.

Preparation of Protease Extracts From Malts. The ground, lyophilized malt samples (8 g) were extracted by stirring for 30 min with 24 mL of 4 °C buffer [0.1 M sodium acetate (NaAc), pH 4.7]. The extracts were centrifuged at 12100*g* for 20 min, and the supernatant was strained through two layers of cheesecloth, frozen, and stored at −20 °C until analyzed. Immediately prior to analysis, the samples were thawed and centrifuged for 5 min at 11500*g* to remove any residual haziness.

Separation and Detection of Extract Proteolytic Activities Using a 1-D PAGE or a 2-D IEF × PAGE System. The proteolytic activities of the extracts were separated and detected using either a modification of the 2-D IEF × PAGE separation method of Zhang and Jones (1995a) or the 1-D PAGE method of Wrobel and Jones (1992). Azogelatin, at a final concentration of 0.2% (w/v), was used as the immobilized substrate protein in all PAGE analyses. After electrophoresis, the gels were incubated in 0.1 M NaAc buffer adjusted to either pH 3.8 or 4.8 or in 0.1 M sodium citrate buffer, pH 6.0. The incubation buffers all contained 2 mM cysteine. The gels were incubated at 40 °C for 16 h, during which time the separated proteinases hydrolyzed the adjacent incorporated azogelatin. The remaining azogelatin was then stained with amido black, leaving clear zones on the gel where the substrate protein had been hydrolyzed.

Detection of Proteolytic Activities Using the In Vitro Analysis. The enzymatic activities of extracts prepared from green malt, kilned malt, and the partially kilned green malts were analyzed using an in vitro assay that utilized the substrate azogelatin (Jones et al., 1998). Lyophilized azogelatin was dissolved in warm 0.1 M NaAc, pH 3.8, 0.1 M ammonium acetate (NH_4Ac), pH 4.8, or 0.1 M sodium citrate, pH 6.0, buffer to make 2% (w/v) substrate solutions. The substrate solutions were stored at 4 °C and heated at 40 °C prior to use to liquify the azogelatin for pipetting into the assays. The assays were conducted both in the presence and in the absence of 4 mM cysteine. Those performed in the presence of cysteine were carried out by mixing 0.12 mL of 100 mM cysteine with 0.60 mL of enzyme extract and 0.54 mL of the appropriate buffer. After the mixture had incubated at 40 °C for 10 min, the reactions were started by adding 1.80 mL of the azogelatin solution. The reactions were terminated by removing 0.50 mL of the reaction mixture and adding it to 0.70 mL of 25% (w/v) trichloroacetic acid. Typically, sample aliquots were removed after 0, 10, 20, 30, and 40 min of reaction. After sitting for 15 min in an ice−water mixture, the tubes were centrifuged for 10 min at 11500*g*, and the 440 nm absorbances of the supernatants were measured. For measuring reactions in the absence of cysteine, an extra 0.12 mL of buffer was added to compensate for the absence of the cysteine solution.

RESULTS AND DISCUSSION

Formation of Endoproteinases during the Malting Process. We reported previously that, as assayed with a qualitative gel electrophoretic analysis method, there was little endoproteolytic activity in resting barley seeds (Zhang and Jones, 1995b). The proteolytic activity began rising strongly after 1−2 days of the "germination" step of malting and was maximized after ∼3 days of germination. When these experiments were carried out, however, we did not have a good method for quantitatively determining how much the activities changed during each stage of the malting process. The in vitro analysis method can now be used to quantify the amounts of endoproteolytic activity in the various fractions (Jones et al., 1998). The substrate used, azogelatin, has several characteristics that make it very useful, including the fact that its structure renders it susceptible to hydrolysis by most endoproteinases (Jones et al., 1998). This means that when it is used to measure the activities of mixtures of proteases, such as exist in malt, the results obtained are more reliable than those obtained with substrates (such as azocasein) that are hydrolyzed by only a few of the malt endoproteinases (our studies, results not shown).

In vitro analyses were conducted on extracts that were prepared from resting barley seeds and from barley that was removed from the malting process at the end of the steeping procedure and after 1, 2, 3, and 4 days of germination. The analyses were carried out at pH 3.8, 4.8, and 6.0. The results, shown in Figure 1, confirm our earlier qualitative findings; there was little endoproteinase activity in out-of-steep barley, the activity had begun to rise in 1-day-germinated barley, it rose steeply during the second day of the germination process, and the 3- and 4-day-germinated samples had achieved their maximal proteolytic activities. There was little difference in how the protease activities increased with time, whether they were measured at pH 3.8, 4.8, or 6.0. As expected from our previous findings (Zhang and Jones, 1995a), which showed that the majority of the malt proteinases were maximally active at pH values of 3.8−4.5, there was only about half as much activity at pH 6.0 as at either pH 3.8 or 4.8. These pH

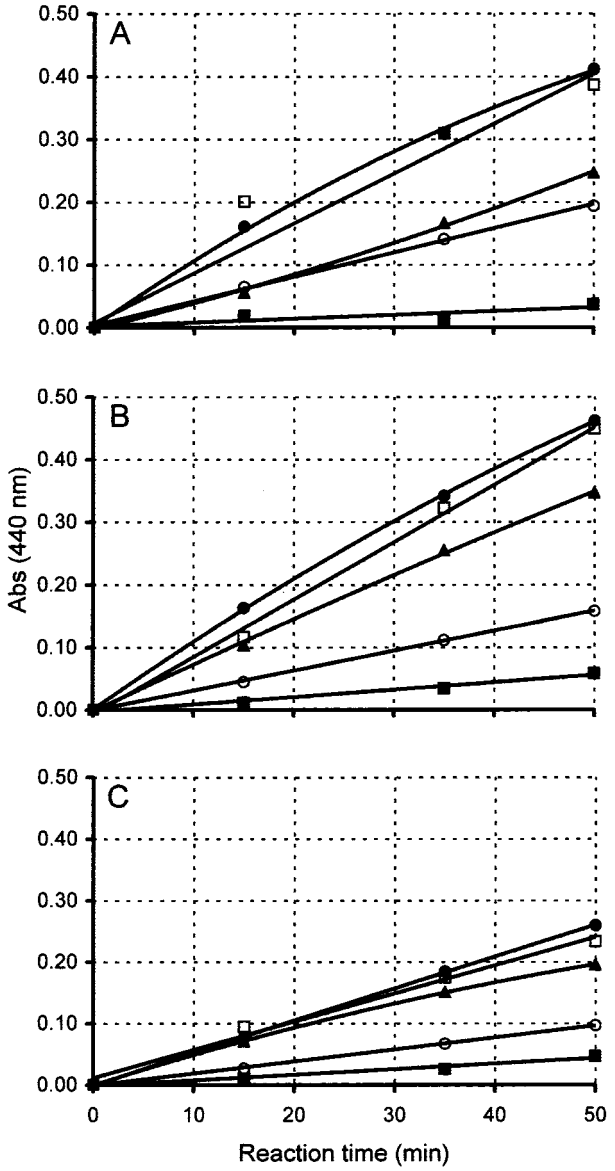

Figure 1. Hydrolysis of azogelatin by extracts of Morex barley seeds undergoing malting: (A) analyses at pH 3.8; (B) analyses at pH 4.8; (C) analyses at pH 6.0. The reactions contained 4 mM cysteine. The samples extracted were (■) out-of-steep, (○) 1 day of germination, (▲) 2 days of germination, (■) 3 days of germination, and (●) 4 days of germination (green malt).

values were chosen for the analyses because only the cysteine class proteinases (which are thought to be the main ones involved in storage protein degradation) are active at pH 3.8, the pH of germinating barley seed endosperm tissue is ~4.8 (so this is likely the pH at which storage proteins are degraded during malting), and the pH levels of North American brewing mashes are generally close to 6.0.

We have shown previously that the green malt endoproteinases that are active at pH 3.8 (only cysteine class proteases) are very different from the pH 6.0-active ones (serine and metalloproteases) and that at pH 4.8 some proteases of each of the classes are active. It is, therefore, interesting that the activities of all three of the enzyme groups increased at the same times during malting. Such behavior would, however, be expected if all of the proteases being measured were synthesized in response to gibberellins that formed during the germination process.

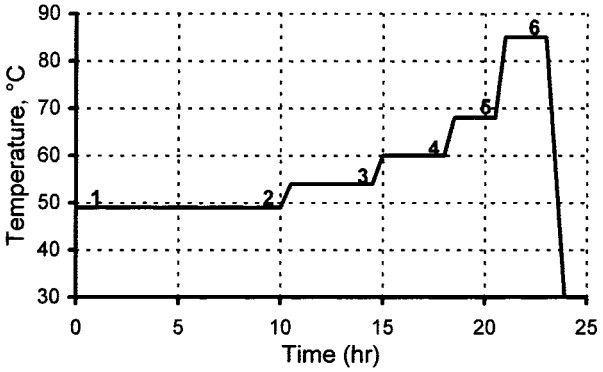

Figure 2. Kilning program used to prepare samples for analysis. Samples were removed at the beginning of kilning (green malt, 1) and at the end of each heating step, as indicated by the numbers 2−6 (kilned malt).

Inactivation of Proteinases during Kilning. Little solid information has been published on the changes that occur in endoproteolytic activities during kilning. It has been stated without providing proof that "kilning substantially inactivates proteolytic enzymes (endopeptidases, mostly sulfhydryl-dependent enzymes)..." (Lewis and Young, 1995) and, conversely, that the activities of the protein-degrading enzymes actually increase during kilning (Kunze, 1996). We have reported previously that there are >40 endoproteinase activities in green malt extracts, including representatives of all four of the protease classes (Zhang and Jones, 1995a). It seemed unlikely that all, or even a majority, of these endoproteolytic activities would be inactivated during kilning under conditions that did not destroy the carbohydrate-degrading enzyme activities.

To investigate the fate of the endoproteinase activities during kilning, we subsampled portions of Morex (six-rowed) and Harrington (two-rowed) malting barleys that were undergoing kilning. The kilning schedule that was used was designed to mimic commercial kilning conditions; it started at 49 °C (10 h) and finished with a 3 h, 85 °C step (Figure 2). Samples were removed for analysis at the beginning of the kilning process and immediately prior to each temperature increase, as indicated by the numbers 1−6 of Figure 2. The removed samples were lyophilized, ground, and dried under vacuum to a constant moisture content. The weight losses due to removal of the dried rootlets varied from 6.2 to 7.2% and averaged 6.8%. The moisture contents of the various fractions are listed in Table 1. By grinding the samples and then drying under vacuum, we were able to reduce all of their moisture levels to between 0.5 and 2.3%, which ensured that each extract contained the enzymes that were originally present in equal weights of malt. Freeze-drying of the Morex and Harrington whole kernel samples 1 and 2 lowered their moisture levels to only about 10 and 8%, respectively, indicating that when very dry samples of green malt are wanted, the kernels need to be ground prior to the final vacuum-drying. The whole kernel samples 1 and 2 were freeze-dried to ensure that they would grind nicely. The other samples were all dry enough to grind when they were removed from the kiln.

In Vitro (Quantitative) Proteolytic Activity Measurements. *Activities in the Presence of 4 mM Cysteine.* To quantify the amounts of proteolytic activity that were present in the various Morex and Harrington malt kilning fractions, they were finely ground and equal weight samples were extracted with equal volumes of

Table 1. Moisture Contents (Precent Dry Weight) of the Kilned Fractions

sample	kilned malt[a]	vacuum-dried whole kernels[b]	vacuum-dried ground grain[c]
Morex			
1[d]	49.8	10.6	0.8
2	11.8	7.8	0.9
3	6.8		0.5
4	6.0		0.9
5	5.2		1.5
6	3.8		1.5
Harrington			
1	49.1	10.1	1.5
2	11.8	8.0	1.7
3	6.8		1.4
4	5.8		1.6
5	5.4		2.3
6	3.8		2.1

[a] Moisture when removed from the kiln. [b] Only samples 1 and 2 were vacuum-dried as whole kernels. [c] From which the endoproteinases were extracted. [d] See Figure 2.

NaAc buffer. The extracted proteinases were incubated with azogelatin, and its hydrolysis was followed spectrophotometrically (Jones et al., 1998). Figure 3 shows the hydrolysis that occurred at pH 3.8, 4.8, and 6.0 in the presence of proteinases that were extracted from Morex malt when 4 mM cysteine was added to the reaction mixtures. The cysteine was added to ensure that all of the cysteine class proteinases were activated. Under these conditions, the rates of azogelatin hydrolysis with the enzyme preparations 1−6 (green malt to kilned malt, see Figure 2) were identical at each pH. Only the activities of the sample 1 and 6 extracts were analyzed at pH 3.8, but it follows that because these activities were identical, those of the intervening samples were, too. These data show that there was no detectable decrease in the proteinase activities during kilning, indicating that the proteinase enzymatic activities were totally stable to these conditions. Neither the pH 3.8-active nor the pH 6.0-active proteinases showed any decrease in activity during the kilning, and because essentially all of the green malt endoproteinases are active at one or the other of these two pH levels (Zhang and Jones, 1995a), it appears that none of the >40 green malt endoproteinases that hydrolyze azogelatin (Zhang and Jones, 1995a) is inactivated by kilning to 85 °C when the schedule of Figure 2 is used. The azogelatin assay does not, however, measure the activities of some of the aspartic class proteinases (Zhang and Jones, 1995a), and some of these activities may have been reduced by the kilning.

When the corresponding experiments were carried out with Harrington malt, the results were identical. The individual analyses were not quite as reproducible as those obtained with the Morex samples (results not shown), but it was obvious that there was no measurable decrease in the proteinase activity during kilning. Figure 4 shows the most important Harrington malt analyses, which compare the endoproteinase activities of the kilned malt (sample 6 of Figure 2) with those of green malt (sample 1 of Figure 2). The activities were measured at pH 3.8, 4.8, and 6.0, in the presence of 4 mM cysteine. At each of these pH levels the kilned and green malt activities were identical. Also, as in Figure 3, the activities were highest at pH 4.8, although the Harrington extract proteolytic activities were uniformly lower than those of their Morex counterparts. This activity difference probably accounts for the fact that, during brewing, the soluble protein levels of Morex malt worts are significantly higher than those prepared with Harrington malts.

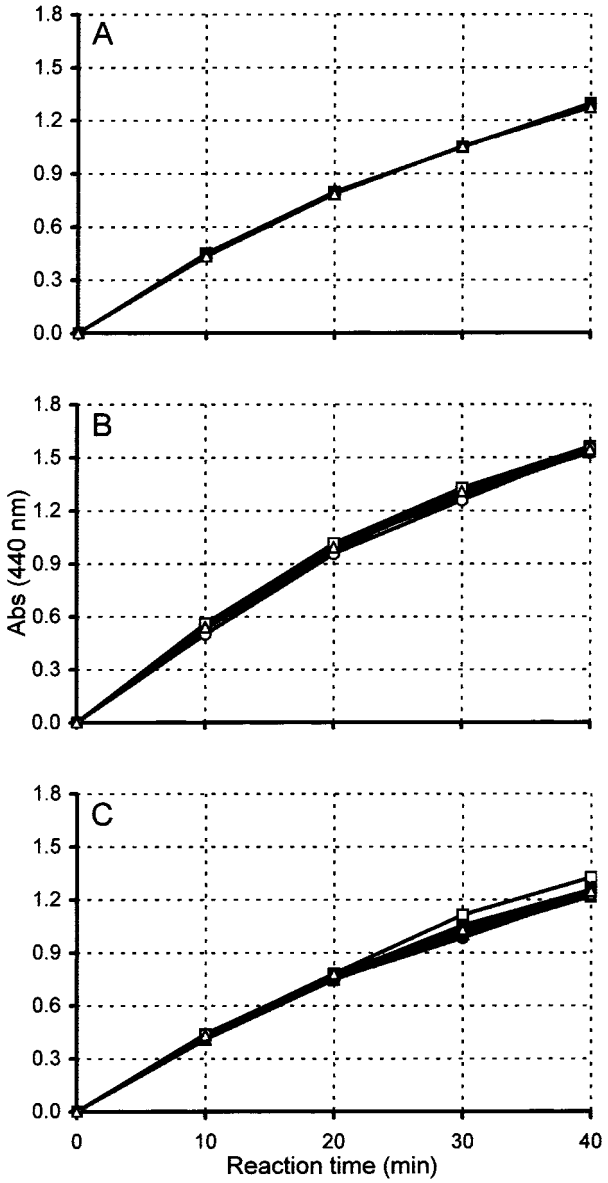

Figure 3. Hydrolysis of azogelatin by extracts of Morex green malt that was undergoing kilning: (A) analyses at pH 3.8; (B) analyses at pH 4.8; (C) analyses at pH 6.0. All assays were carried out in the presence of 4 mM cysteine. The samples extracted were removed from the kiln at the points indicated on Figure 2 and were fractions (■) 1, green malt; (●) 2; (▲) 3; (□) 4; (○) 5; and (△) 6, kilned malt.

Figures 3 and 4 show that with both Morex and Harrington malts the proteinase activities varied little with pH. The greatest variations were only ∼20%, compared to the 45% differences that were found between the pH 4.8 and 6.0 activities of the 3- and 4-day extracts (Figure 1). This may be have been due to the fact that, to ensure that no dilution of the extracts occurred, the experiments of Figures 3 and 4 were carried out with extracts that were not subjected to dialysis, whereas those of Figure 1 utilized enzyme extracts that were dialyzed against 40 volumes of 5 mM NaAc, pH 5.0, for 16 h.

Activities in the Absence of Added Cysteine. We had found previously (results not reported) that the malt endoproteinase activities were strongly activated by the presence of cysteine and, presumably, by other reducing

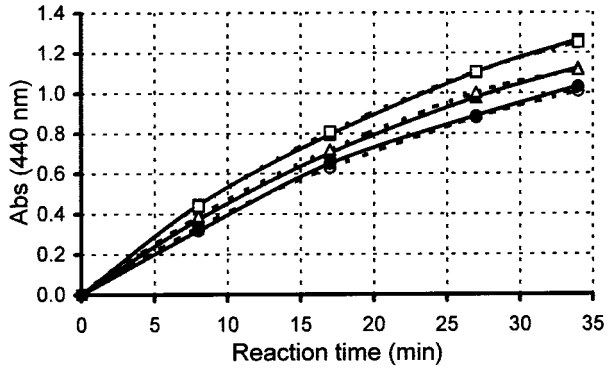

Figure 4. Hydrolysis of azogelatin by extracts of Harrington green malt and kilned malt. All assays were carried out in the presence of 4 mM cysteine. The samples were extracted from fractions 1 and 6, as indicated in Figure 2, and were analyzed at the pH values indicated: (■) green malt, pH 4.8; (□) kilned malt, pH 4.8; (●) green malt, pH 6.0; (○) kilned malt, pH 6.0; (▲) green malt, pH 3.8; (△) kilned malt, pH 3.8.

agents. For this reason it seemed to be important to compare the activities of the various samples in the presence and absence of added cysteine. A second set of Morex malt extracts was, therefore, prepared, and the activities of the partially kilned samples were measured in the absence of added cysteine. The results are shown in Figure 5. Preliminary experiments using the 2-D IEF × PAGE method discussed below had indicated that there might be subtle differences among the enzymes that were removed from the kilning process at step 5 and those that were subjected to the 85 °C step. We therefore analyzed the extracts that were prepared with green malt (fraction 1), malt kilned to 68 °C (fraction 5), and completely kilned malt (fraction 6) at pH 4.8 and 6.0. Only the sample 1 and 6 extracts were analyzed at pH 3.8. As expected, the proteinase activities in the absence of cysteine were 65−80% lower than they were in its presence (Figure 5 versus Figure 3). This makes sense in the case of the pH 3.8 and 4.8 reactions because most of the activities at these pH values (especially at pH 3.8) are known to be due to cysteine class proteinases which would normally be activated by cysteine. However, it is not clear why the pH 6.0 reactions were activated by cysteine because very little cysteine class proteinase activity is normally detected at this pH (Zhang and Jones, 1995a). It may be that when cysteine is added to the reactions, it partially reduces the substrate protein, azogelatin, rendering it more susceptible to hydrolysis. In any case, the overall picture was similar to that seen in the presence of cysteine, with the activity being highest at pH 4.8 and 3.8 and considerably lower at pH 6.0. Also, as in the cysteine-containing reactions, there was no loss of proteolytic activity during the kilning process. In fact, at all three assay pH values the proteolytic activities of the samples increased significantly after the samples were treated at 85 °C (Figure 5). This finding fits well with the report that, during kilning, the activities of the protein-degrading enzymes increase by 10−30% (Kunze, 1996).

1-D and 2-D (Semiquantitative) Proteolytic Activity Measurements. *1-D PAGE Studies.* Using a 1-D PAGE nondenaturing proteinase analysis system (Wrobel and Jones, 1992) that contained incorporated azogelatin in the gel matrix, we compared the endoproteolytic activities that were present in green malt to those that occurred in extracts prepared from Morex and

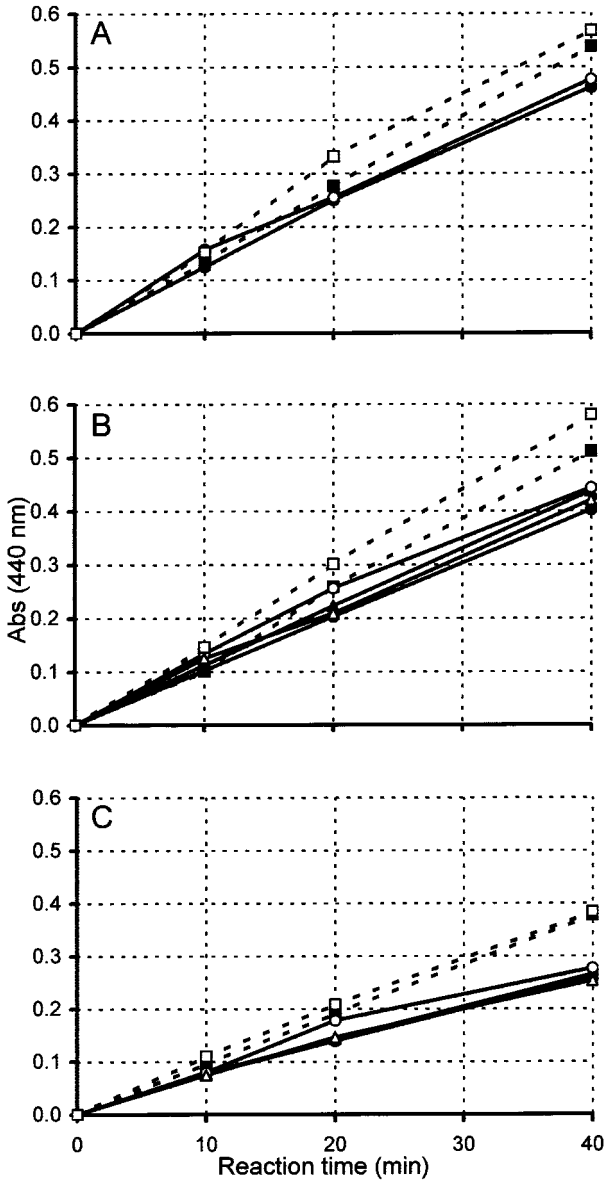

Figure 5. Hydrolysis of azogelatin by extracts of Morex and Harrington green, partially kilned, and kilned malts: (A) analyses at pH 3.8; (B) analyses at pH 4.8; (C) analyses at pH 6.0. The activity assays were carried out in the absence of added cysteine. The samples extracted were, as indicated in Figure 2, (○, ●) fraction 1 (green malt); (△, ▲) fraction 5 (partially kilned, to 68 °C); and (□, ■) fraction 6 (kilned malt); (open symbols) Harrington samples; (solid symbols) Morex malts.

Harrington barleys that had undergone the various kilning temperature changes that are shown in Figure 2. After the electrophoresis was finished, the gels were incubated in pH 3.8 and 6.0 buffers that contained 2 mM cysteine for 16 h at 40 °C. Figure 6 shows the gels that resulted from analyzing the Morex barley as it underwent kilning. The gels developed at pH 3.8 indicate when the cysteine class endoproteinases were most active, whereas the serine and metalloproteinases are best observed in the gels that were developed at pH 6.0 (Zhang and Jones, 1995a). The first lane in each gel was loaded with an extract of unkilned (green) malt. Extracts prepared in the same fashion from malt samples removed from the kiln at the end of each stage of kilning (Figure 6, lanes 2−6) yielded patterns that were identical to those of green malt, indicating that

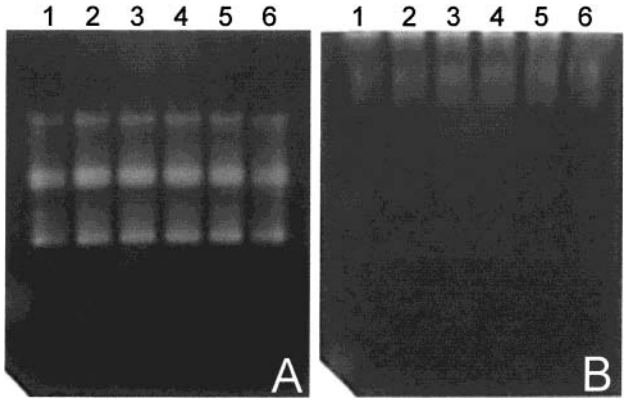

Figure 6. 1-D PAGE analysis of endoproteinase extracts prepared from Morex malt undergoing kilning. Lanes 1−6 were loaded with equal amounts of extract prepared from each of the subsamples indicated in Figure 2. Azogelatin substrate was incubated for 16 h in buffer containing 2 mM cysteine. The enzyme activities were developed at pH 3.8 (A) and pH 6.0 (B).

there was little or no change in the amount of azogelatin digested by the various samples at either pH 3.8 or 6.0. This implied that raising the temperature from 49 to 85 °C had very little effect on the malt endoproteinase activities.

2-D IEF × PAGE Studies. The Morex or Harrington extracts were analyzed using the more discriminating nondenaturing 2-D IEF × PAGE method (Zhang and Jones, 1995a) so that the individual proteolytic activities could be scrutinized more closely. In Harrington gels that were developed at pH 4.8, the pH of germinating barley endosperm tissue (Figure 7), no significant changes were found in the cysteine proteinase activities [all of the activities that migrated more than a third of the way down the gel in Figure 7 (Zhang and Jones, 1995a)] until the samples had been heated for 3 h at 68 °C, the fifth kilning stage (results of stages 2−4 are not shown). At that point, one of the proteases had lost some of its activity (Figure 7B, arrow) and some of the other activity spots had become more diffuse. After the samples had been heated at 85 °C for 3 h, several further cysteine class proteinase activity spots had become more diffuse (Figure 7C). From the data shown in Figure 3C, however, it is obvious that the phenomenon that caused the activities to diffuse (possibly a "loosening" of the physical structures of the enzymes) either did not lower their activities or else the activities of some of the other proteinases increased enough to cancel the losses of the diffuse ones. The kilning had less effect on the serine and metalloproteinases [migrated less than a third of the way down the gel (Zhang and Jones, 1995a)] that were active at pH 4.8, but those that migrated to the top right corner of the gel were affected.

When a similar experiment was carried out with Morex enzymes and the gels were developed at pH 3.8, conditions under which the cysteine activities strongly predominate (Zhang and Jones, 1995a), the results shown in Figure 8 were obtained. Except for the fact that the Morex patterns differed slightly from those of Harrington and that the serine and metalloproteinases were only very slightly active at this pH, the patterns were very similar to those of Figure 7. Only small enzymatic changes occurred prior to the end of kilning stage 5, and during the 85 °C final step some of the cysteine class proteases were altered so that they

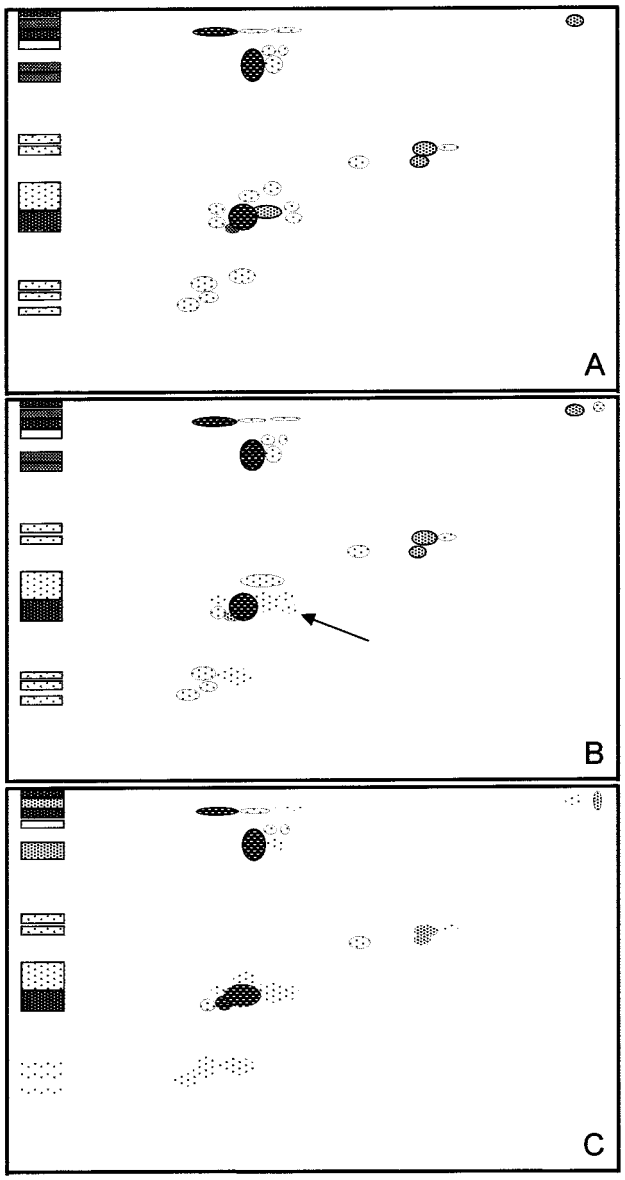

Figure 7. IEF × PAGE analysis of the pH 4.8-active endoproteinases in samples removed from Harrington green malt that was undergoing kilning. Fractions 1 (green malt), 5, and 6 (kilned malt) as indicated in Figure 2 were extracted, and their endoproteinases were separated and analyzed at pH 4.8. The samples analyzed were (A) fraction 1, (B) fraction 5, and (C) fraction 6. The sample on the left side of each gel was subjected to only the PAGE separation. The arrow indicates a cysteine class proteinase the activity of which was apparently lowered during the 68 °C kilning step.

migrated to give diffuse activity areas. As was also seen in Figure 7, the serine protease activity that migrated to the top right corner of the gel was partially inactivated after heating at 68 °C and totally inactive after the 85 °C step.

The activities of the serine and metallo-endoproteinases are best studied at pH 6.0 using the 2-D analysis system (Zhang and Jones, 1995a). Little change occurred in the pH 6.0 proteinase activity pattern during kilning, except in the activities of the high-p*I* enzyme forms that migrated to the upper right-hand corner of the gel (arrow, Figure 9A). These proteinases were strongly affected when the kilning temperature was raised to 85 °C (Figure 9C). In the fraction 5 sample (Figure 9B), they migrated as tightly circumscribed

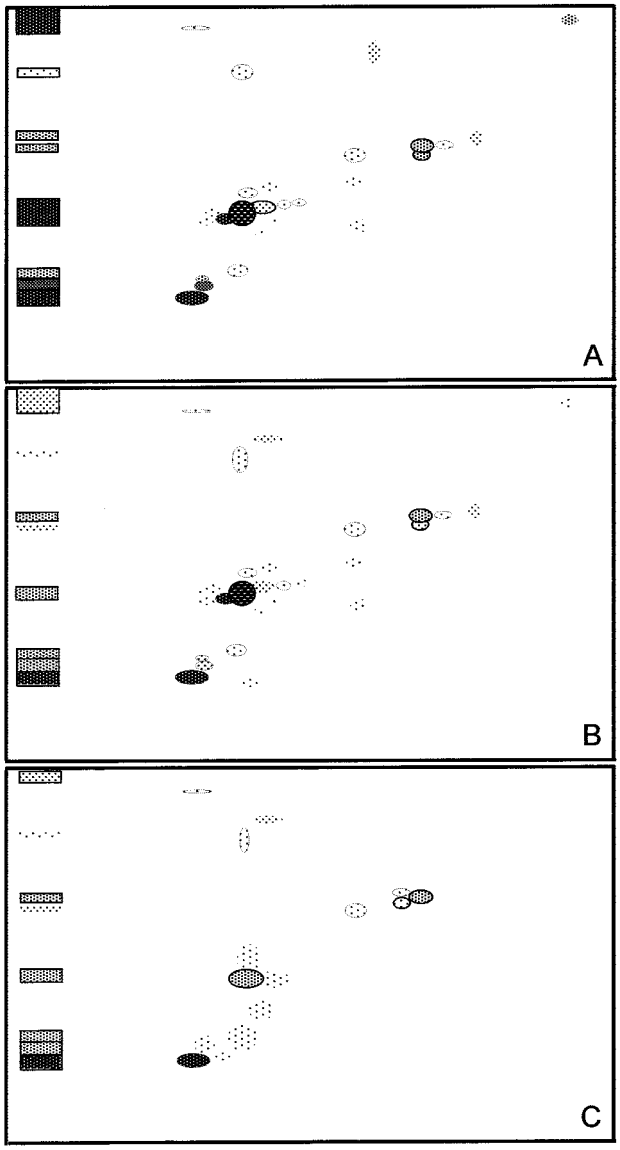

Figure 8. IEF × PAGE analysis of the pH 3.8-active endoproteinases in samples removed from Morex green malt that was undergoing kilning. Fractions 1 (green malt), 5, and 6 (kilned malt) as indicated in Figure 2 were extracted, and their endoproteinases were separated and analyzed at pH 3.8. The samples analyzed were (A) fraction 1, (B) fraction 5, and (C) fraction 6. The sample on the left side of each gel was subjected to only the PAGE separation.

activity spots that moved nearly to the right-hand edge of the gel, whereas in the fraction 6 samples (Figure 9C), they did not move as far in the first dimension and they migrated as a very diffuse set of bands. We have shown previously that these activities were due to serine class proteinases (Zhang and Jones, 1995a), and we reported recently (Jones, 1999) that the malt serine proteinases apparently are not directly involved in solubilizing proteins during mashing. It therefore seems likely that there would be little or no effect on the soluble protein level in wort, even if this group of enzymes was totally inactivated during kilning. Kilning had no detectable effect on the activities of the metalloproteinases, which migrated to the top center of the 2-D gel (Zhang and Jones, 1995a). As opposed to the malt serine proteinases, it appears that the metalloproteinases probably do play a role in solubilizing proteins during mashing (Jones, 1999).

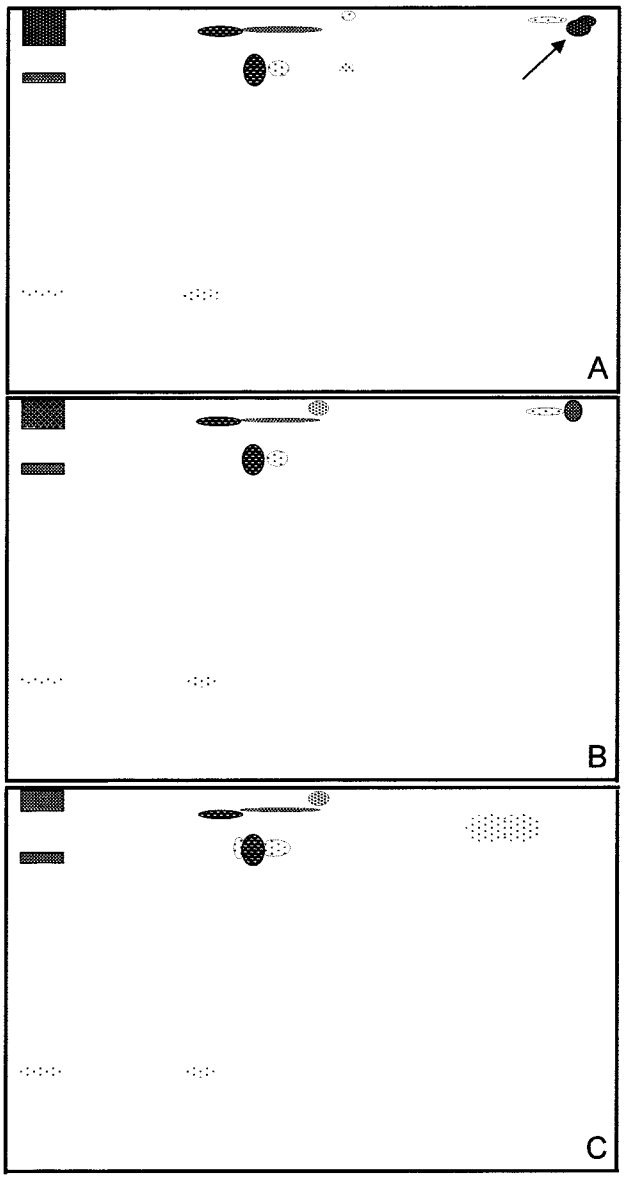

Figure 9. IEF × PAGE analysis of the pH 6.0-active endoproteinases in samples removed from Morex green malt that was undergoing kilning. Fractions 1 (green malt), 5, and 6 (kilned malt) as indicated in Figure 2 were extracted, and their endoproteinases were separated and analyzed at pH 6.0. The samples analyzed were (A) fraction 1, (B) fraction 5, and (C) fraction 6. The sample on the left side of each gel was subjected to only the PAGE separation. The arrow indicates a serine class proteinase the activity of which was strongly altered during the 85 °C kilning step.

Summary. This work demonstrates how quantitative in vitro and semiquantitative (2-D IEF × PAGE) enzymatic analytical methods can be used together to obtain an increased understanding of how complex biological phenomena occur. Use of the in vitro method confirmed our earlier electrophoretic results (Zhang and Jones, 1995b), which had indicated that little proteolytic activity was present in ungerminated barley and that the majority of the proteinases formed after 1−2 days of the germination phase of malting (Figure 1).

When enzyme extracts were prepared from Morex or Harrington green malts that were undergoing kilning and the proteolytic activities of the extracts were measured in the presence of 4 mM cysteine, at pH 3.8 (cysteine class proteinases are active), 4.8 (pH of

germinating barley endosperm tissue), or 6.0 (serine and metalloproteinases are active), the kilning process had no measurable effect on the overall proteolytic activities (Figures 3 and 4). The overall in vitro proteolytic activity remained constant in extracts that were prepared from grain samples that were collected throughout the kilning process. In the absence of added cysteine, the activities of the extracts remained constant throughout the first five kilning steps and then increased during the final, 85 °C step (Figure 5). This activity increase might be due to some of the proteinase activities being activated by alterations in their structures, but this seems unlikely because no similar activity increase was seen when cysteine was present. It may be, however, that this activity increase reflects the formation of "reducing power" in the malt at 85 °C and that this reducing power further activated the predominant (cysteine class) malt proteinases. If so, the protease activity increase that was obvious in the absence of cysteine reducing power might have been overwhelmed and rendered unnoticeable in the presence of the strongly increased proteolysis that occurred in the presence of 4 mM cysteine.

The 1-D electrophoretic semiquantitative analysis method (Figure 6) indicated that little or no change occurred in either the pH 3.8-active or pH 6.0-active endoproteinases throughout kilning. However, when the activities were separated more clearly with the 2-D IEF × PAGE method, it was obvious that the structures of the constituent proteinases were affected by heating at 68 and 85 °C. Several of the activities that previously migrated as tightly constrained activity areas moved as more diffuse areas after heating. This was particularly true of the pH 4.8-active cysteine proteinases and the pH 6.0-active serine class enzymes that migrated to the upper right corner of the PAGE gels. Our recent work (Jones, 1999) indicates that whereas the activities of these cysteine class enzymes might affect the rate of protein solubilization during mashing, it seems unlikely that the changes of the serine proteinases would have any significant affect on the wort soluble protein levels.

In any case, when green malt is kilned using normal commercial temperature and time schedules, the endoproteolytic activity of the malts is not lowered. In the absence of added cysteine, as is the case in commercial brewing, the proteolytic activity of the malt actually increased during the final, 85 °C, kilning step. For specialty dark malts, however, this is probably not the case because they are heated to much higher temperatures.

ABBREVIATIONS USED

2-D, two-dimensional; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; NH_4Ac, ammonium acetate; NaAc, sodium acetate.

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