JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Effect of Mashing on Malt Endoproteolytic Activities

BERNE L. JONES*,†,‡ AND LAURIE MARINAC†

U.S. Department of Agriculture, Agricultural Research Service, Cereal Crops Research Unit, Madison, Wisconsin 53705 and Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706

During malting and mashing, the proteinases of barley (*Hordeum vulgare* L.) and malt partially hydrolyze their storage proteins. These enzymes are critical because several aspects of the brewing process are affected by the soluble proteins, peptides and/or amino acids that they release. To develop improved malting barleys and/or malting and brewing methods, it is imperative to know whether and when the green malt endoproteinases are inactivated during malting and mashing. These enzyme activities are totally preserved during kilning and, in this study, we have determined when they were inactivated during mashing. Samples were removed from experimental mashes that mirrored those used in commercial breweries and their endoproteolytic activities were analyzed. The malt endoproteinases were stable through the 38 °C protein rest phase, but were quickly inactivated when the mash temperature was raised to 72 °C for the conversion step. All of the proteinase activities were inactivated at about the same rate. These findings indicate that the soluble protein levels of worts can be varied by adjusting the protein rest phase of mashing, but not by altering the conversion time. The rates of hydrolysis of individual malt proteins probably cannot be changed by altering the mash temperature schedule, since the main enzymes that solubilize these proteins are affected similarly by temperature.

KEYWORDS: ***Hordeum vulgare;*** **protease; protein hydrolysis; mashing; brewing; soluble protein**

INTRODUCTION

During malting and mashing, proteinases partially degrade the barley storage proteins into amino acids and peptides that are critical for brewing high quality beer. We have shown previously that germinated barley (green malt) contains over 40 endoproteinase activities, the majority of which are cysteine class enzymes (*1*). Ungerminated barley contains only small amounts of proteolytic activity, but this activity increases rapidly during the early stages of the malting germination step (*2*, *3*, *4*).

The proteinase activities that formed during malting were totally stable to kilning when methods that mimicked commercial conditions were used, and even increased during the final, 3 h at 85 °C, step (*4*). During the mashing step of brewing, however, the overall malt endoproteinase activity is not stable (reviewed by *2*, *5*, *6*) and preliminary studies have indicated that the proteinases are inactivated at temperatures greater than about 60 °C (*5*).

Even though the mashing protein rest times are very short compared to the several-day malting process, most researchers have reported that relatively high proportions of the wort soluble protein were solubilized during mashing [from 30% (*7*, *8*) to about 47% (*9*, *10*)]. One group has maintained that no protein is enzymatically solubilized during mashing (*11*), but this seems very unlikely, since all of the other researchers have shown that solubilization does occur. Previous research has indicated that, using the American Society of Brewing Chemists (ASBC) Congress mashing method, about 25% of the final wort protein was solubilized during mashing (*5*). The fact that a significant proportion of the protein solubilization occurs during mashing necessitates our understanding how the endoproteinases behave during this period.

In the US, commercial mashes are normally double mashes and are comprised of a hot adjunct mash that is quickly raised to 100 °C to gelatinize the adjunct starch and a malt mash that is initially held at a relatively low temperature (protein rest) to allow the malt enzymes to hydrolyze their malt substrates (*6*). The adjunct and malt mashes are then mixed, raising the temperature of the mash to about 70 °C, at which point the adjunct and malt starches are quickly hydrolyzed to sugars. Enzymes are inactivated or denatured by high temperatures, especially in the presence of water, with most enzymes being inactivated at temperatures between 60 and 70 °C (*12*). It therefore seemed likely that some or all of the malt endoproteinases would be inactivated during mashing even though, while protected within the drying grain, they were stable throughout the kilning process.

Little has been published about the heat stabilities of barley endoproteinases. However, one purified, dissolved, cysteine class barley aleurone proteinase was 80% inactivated when heated

* To whom correspondence should be addressed. 501 N. Walnut St., Madison, WI 53705-2334 telephone (608) 262−4478; fax (608) 264−5528; E-mail: bljones@facstaff.wisc.edu.
† U.S. Department of Agriculture.
‡ University of Wisconsin.

10.1021/jf0109672
Published on Web 01/18/2002

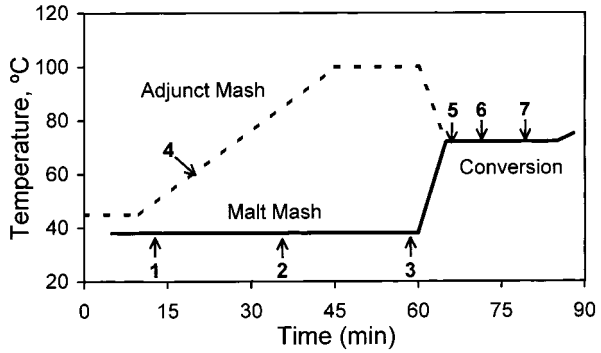

Figure 1. The mash schedule used for these experiments. Aliquots of the mash were removed at the times indicated by the numbers and analyzed for their proteolytic activities. - - -, Adjunct mash; —, malt mash.

to 60 °C (*13*) and dissolved malt proteinases were inactivated within 2 min at 70 °C (*14*). During mashing, the proteinases responsible for releasing soluble nitrogen were inactivated at temperatures of 55−60 °C (*15*). These findings, together with our preliminary studies (*5*), indicated that it was unlikely that very much of the proteolytic activity would survive the final steps of the mashing process. This study was carried out to ascertain quantitatively (with an in vitro assay) when the overall proteolytic activity of a mash declined and to semiquantitatively (with the 2-D assay) determine when the individual proteolytic activities were inactivated.

MATERIALS AND METHODS

Preparation of Kilned Malt. Seeds (170 g aliquots) of both Morex and Harrington barleys (the six-rowed and two-rowed American Malting Barley Association malting quality standards, respectively) were steeped at 16 °C for 36 h, to 45% moisture, with four equally spaced 4-hr couchings. The steeped seeds were germinated in the dark, with slow rotation at 16 °C and 100% humidity, for 4 days. The resulting green malt was kilned to around 4% moisture using a schedule (*4*) that started at 49 °C (10 h), finished at 85 °C (3 h), and that conformed closely to U. S. industry practices. The malt samples produced were stored at room temperature until mashed.

Preparation of Mashes. Kilned malt was ground using the standard ASBC fine grind method (16) and mashed according to a schedule (see **Figure 1**) that is used to make experimental beers in our laboratory, and which generally conforms to U. S. industry practices (*6*). The cooker (adjunct) mash was prepared by adding 295 g of malt and 1,270 g of ground corn grits (ADM/Krause Milling Co, Shawnee Mission, KS) to 6.6 L of 45 °C sparge water (deionized water containing 1.9 mM $CaSO_4$, 1.0 mM $MgSO_4$, and 3.6 mM NaCl) over a period of 5 min, after which the mixture was maintained at 45 °C for a further 10 min. The mash temperature was then raised to boiling (100 °C) at a rate of 1.6 °C/min. It was boiled for 15 min and then combined with the malt mash.

The malt mash was prepared by mixing 2,676 g of ground malt into 9.7 L of 38 °C sparge water over a period of 5 min. This mixture was stirred for an additional 5 min and the protein rest was started by shutting off the mixer. The protein rest was continued for 50 min at 38 °C, after which the temperature was raised to 72 °C over a period of 10 min (3.4 °C/min). During the first 5 min of this temperature rise, the hot cooker mash was transferred into the malt mash tun at a constant rate and sufficient steam heating was used to maintain the 3.4 °C/min temperature rise rate. During the remainder of the temperature rise, only steam heating was used to sustain this rate of heating. Conversion was carried out at 72 °C until all of the starch had been degraded, as measured with the starch-iodide reaction test. After 2 further min at 72 °C, the mash temperature was raised to 76 °C and it was transferred to the lauter tun for filtration.

Collecting Samples from the Mash Subsamples. A total of seven 100-mL aliquots were removed from either the cooker mash, the malt mash, or the converting mash and these were used to prepare the enzyme extracts. Samples were removed from the mash tun at the beginning of the protein rest and 20 and 45 min thereafter. A single sample was removed from the adjunct mash when the temperature had reached 60 °C, and three samples were collected during conversion; 0.5, 6.5, and 16.5 min after the temperature reached 72 °C. These sampling times are indicated in **Figure 1**. The adjunct mash samples used for the one-dimensional (1-D) and 2-D electrophoretic studies were collected as soon as the temperature reached 100 °C, rather than at 60 °C.

Preparation of Enzyme Extracts. Aliquots (1.8 mL) of the mashes were collected and held on ice until the final sample was removed. They were then centrifuged at 10,800*g* for 5 min and the supernatants were decanted and stored in an ice−water bath until analyzed by the *in solution* azogelatin method. Duplicate aliquots were cooled, centrifuged, and concentrated four-fold with Amicon 3,000 molecular weight cutoff centrifuge concentrators (Amicon, Inc., Beverley, MA). The concentrated extracts were stored at −20 °C and were used for the 1-D and 2-D electrophoretic studies.

Detection of Proteolytic Activities Using an Azogelatin *In Solution* Analysis Method. The enzymatic activities of the enzyme extracts prepared from the various mash fractions were analyzed using the in-solution assay method of Jones et al. (*17*). Azogelatin was synthesized, freeze-dried, and dissolved in either 0.1 M ammonium acetate, pH 3.8 or 4.8, or 0.1 M Na citrate, pH 6.0, buffer to make a 1% (w/v) solution. These substrates were stored at 4 °C and reliquified at 40 °C just prior to being used for conducting the assays.

The *in-solution* assays were conducted by mixing 0.67 mL of enzyme extract with 67 μL of 0.1 M cysteine and 1.0 mL of the appropriate buffer. This mixture was incubated for 10 min at 40 °C and the reaction was started by adding 2.2 mL of the 1% azogelatin solution in appropriate buffer. The reactions were terminated by removing 0.6 mL of the reaction mixture and adding it to 0.9 mL of 15% trichloroacetic acid. Typically, aliquots were collected after 0, 10, 25, 45, and 60 min of reaction. After the undigested protein had precipitated for 30 min in an ice−water bath, the tubes were centrifuged for 8 min at 11,500*g*. The absorbances of the supernatants at 440 nm were measured, the 0 min reaction time control values were subtracted, and the data were plotted.

Electrophoresis and Detection of Proteolytic Activities. The proteolytic activities of the extracts were separated and semiquantitatively determined using modifications of the 2-D isoelectric focusing x polyacrylamide gel electrophoresis (IEF x PAGE) separation method of Zhang and Jones (*1*) or the 1-D PAGE method of Wrobel and Jones (*18*). Gelatin or edestin, at a content of 0.1%, was used as the immobilized substrate protein in all PAGE analyses. Both Morex and Harrington mash samples were analyzed by the 2-D and 1-D methods, but only the Morex gel results are shown in this report. After electrophoresis, the gels were incubated in 0.1 M ammonium acetate buffer, pH 3.8 or 4.8, or in 0.1 M Na citrate buffer, pH 6.0, all of which contained 2 mM cysteine. After being incubated at 40 °C for 16 h, the gels were stained with amido black and the enzymatic

activities were detected as clear zones from which the substrate protein had been hydrolyzed by the separated enzymes.

RESULTS AND DISCUSSION

Very little direct information has been published on the changes that occur in barley malt endoproteolytic activities during mashing. Until studies with our 2-D IEF x PAGE endoproteinase activity method showed that malt contained more than 40 endoproteinase activities (*1*), it was believed that only a relatively small number of such enzymes were present, and little work was carried out on separating and characterizing them. To investigate the effect of mashing on the proteolytic activities, we mashed malts prepared from Morex and Harrington barleys using a mash schedule (**Figure 1**) that closely resembled those that are used by commercial brewers. This was a double mash procedure, in which the malt and adjunct were initially mashed separately, and then combined for the conversion step. Aliquots were removed from the mashes at critical points (**Figure 1**) and the overall (*in solution* azogelatin hydrolysis) and individual (2-D analysis) proteolytic activities of each sample were determined. Three samples (numbers 1−3) were removed during the protein rest phase, a single sample (number 4) was collected from the adjunct (cooker) mash when the temperature reached 60 °C (in vitro analysis) or 100 °C (2-D analyses) and three aliquots (numbers 5−7) were removed at the beginning, middle, and latter stages of the conversion period.

In Vitro Assays with the Substrate Azogelatin. When these seven samples were collected from a Morex mash and their overall pH 4.8 and 6.0 endoproteolytic activities were measured in vitro with the substrate azogelatin, the proteolytic activities were strongly inactivated when the temperature of the mash was raised above the 38 °C used for the protein rest step (**Figure 2A, B**).

The analyses were carried out at pH 4.8, because this is the pH of endosperm tissue collected from germinating barley grains, and at pH 6.0, the pH of most American mashes. These two pH values were also chosen because the pH 4.8 analyses measure the activities of the cysteine-, serine- and metalloproteinases, whereas only the serine- and metalloproteinases are active at pH 6.0 (*1*). The overall proteolytic activities were unchanged throughout the protein rest. As expected, the activities were nearly twice as high at pH 4.8 as at 6.0, presumably resulting from the additional cysteine proteinases that are active at pH 4.8. It is apparent, therefore, that in mash suspensions at 38 °C the azogelatin-hydrolyzing cysteine-, serine- and metalloproteinases are entirely stable. This is not surprising, since most enzymes are not inactivated until temperatures approach 60 °C (*12*). These results indicate that it should be possible to enhance the amount of protein solubilized during mashing by increasing the length of the protein rest.

There was a low level of endoproteolytic activity in the 60 °C-heated adjunct mash (**Figures 2A** and **2B**, fr. 4). This probably reflects the fact that only a small amount of malt, (and therefore of proteinases) was added to the adjunct mash, rather than that the proteinases were inactivated at 60°C. Whereas the malt mash contained 27.6% (w/v) of the enzyme-rich malt, the adjunct mash had only 4.5%. The relative enzymatic activities of the protein rest and adjunct mash samples reflect this ratio fairly well, indicating that little of the overall activity had been inactivated.

By the time the adjunct and malt mashes were mixed and the temperature had reached 72 °C, the proteolytic activity had dropped dramatically, by approximately 67% (at pH 4.8) or 59%

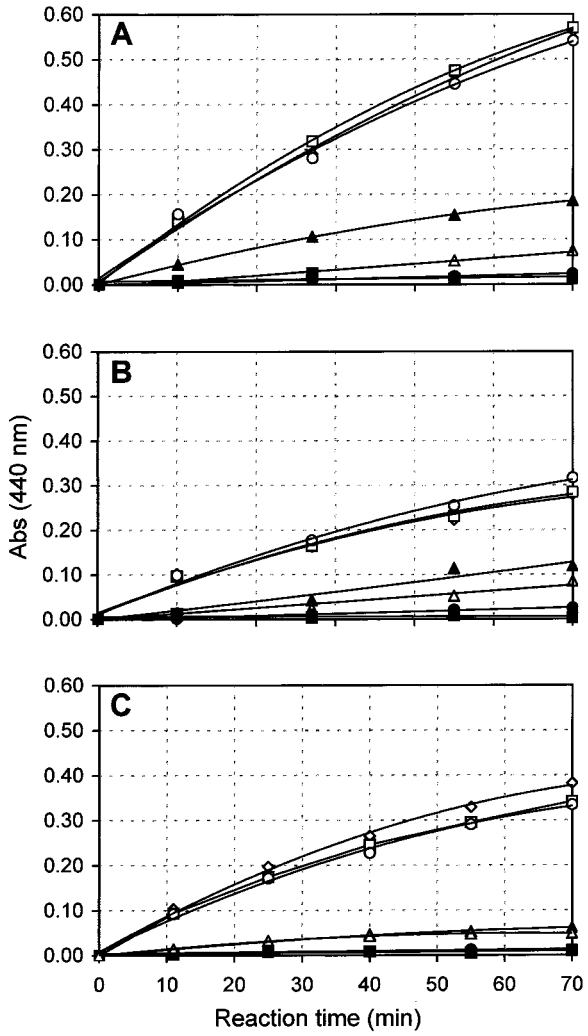

Figure 2. The *in vitro* hydrolysis of azogelatin by the proteinases present in samples removed from: (A, B) Morex or (C) Harrington mashes. The analyses were carried out at: (A) pH 4.8; (B and C) pH 6.0. The samples were removed from the mash at the times indicated in **Figure 1** as: ◇, fraction 1; □, fr. 2; ○, fr. 3; △, fr. 4; ▲, fr. 5; ●, fr. 6; and; ■, fr. 7.

(pH 6.0), as shown in **Figures 2A** and **2B**, fr. 5. This activity drop was not due to the dilution of the malt mash with adjunct mash, since this would only have diluted the malt concentration of the sample by 1/3, from 27.6% to 18.2%. Six minutes into the conversion, the proteinases were nearly inactive (**Figure 2**, fr. 6). These findings indicate that the bulk proteolytic activity was relatively stable at 60 °C, but that it was rapidly inactivated at 72 °C. The experiments of **Figure 2A** and **2B** were carried out with Morex (six-rowed barley) malt, and corresponding studies with Harrington (two-rowed) malt gave essentially the same results (**Figure 2C**). The proteinases were stable throughout the protein rest but were quickly inactivated when the conversion temperature was reached.

Electrophoretic Assays with Gelatin or Edestin Substrates. The studies reported in **Figure 2** indicated how the bulk endoproteolytic activity of a mash changed during processing, but told nothing about how the individual enzymatic activities were affected. We have shown previously that there are over 40 individual endoproteinase activities in green malt, including representatives of all four proteolytic types (*1*). We have also shown that the aspartic-, cysteine- and metalloproteinases likely play roles in solubilizing the storage proteins of barley and malt, whereas the serine proteinases apparently do not (*5*). To

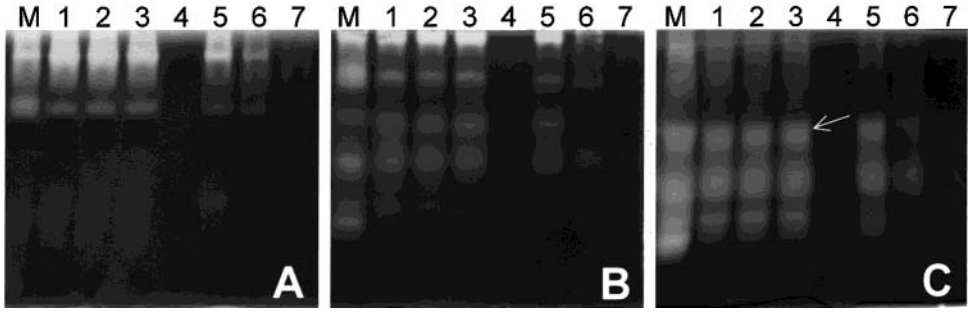

Figure 3. 1-D PAGE separations of endoproteinases removed from Morex malt samples that were undergoing mashing: (A) the PAGE gel contained incorporated gelatin and, after separation, the activities were developed at pH 6.0; (B) the gel contained gelatin, developed at pH 4.8; (C) gel contained edestin, developed at pH 4.8. The fractions 1 to 7 were removed from the mash as indicated in **Figure 1**, except that fr. 4 was collected when the temperature reached 100 °C, not 60 °C. Lane M was loaded with an extract prepared from green (unkilned) malt. The arrow in C indicates the area occupied by the aspartic proteinases.

determine whether these various activities were inactivated at the same time during mashing, it was necessary to separate them from each other before measuring their activities. This is most easily done by separating them by electrophoresis, using either 1-D (to get an overall impression of how the enzymes of the different fractions compared) or 2-D (to measure the activities of the individual enzymes) systems, and then measuring their activities while they were still localized in the electrophoretic gels (*1*). In these experiments, the enzymes were separated in gels that contained incorporated substrate proteins and were detected by their abilities to hydrolyze those substrates

Assays with 1-D PAGE Gels. **Figure 3** shows photos of the results that were obtained when the proteinases of the various fractions were separated by gel electrophoresis and their abilities to hydrolyze the substrate gelatin at pH 6.0 (**Figure 3A**) and 4.8 (**Figure 3B**) or the substrate edestin at pH 4.8 (**Figure 3C**) were measured. The shading of the various bands indicates their relative enzymatic activities, the lightest indicating the highest activity. These results corroborate those found with the in vitro azogelatin hydrolyses. With both pH levels and substrates, the enzyme patterns remained constant throughout the protein rest (frs. 1, 2, 3; **Figures 3A**, **B**, **C**), the enzymatic activities were somewhat lowered by the start of the conversion phase (fr. 5, all 3 gels) and were essentially destroyed after 16.5 min of conversion (fr. 7). There was no activity left in the various **Figure 3** adjunct mash samples, since these samples were collected after the temperature reached 100 °C, instead of 60 °C. As expected from our previous studies (*1*), different proteinases were active at pH 4.8 and 6.0 (**Figures 3A** and **3B**). At pH 4.8, the hydrolysis patterns obtained with the substrates gelatin and edestin (**Figures 3B** and **3C**) were similar, but different. This was not surprising, since we have shown previously that some of the malt enzymes hydrolyzed the gelatin and edestin substrates differently. For example, the aspartic proteinases hydrolyze edestin, but not gelatin (*1*), so the activity in **Figure 3C** that migrated about 40% of the way through the gel (arrow) is apparently the result of the multiple aspartic proteinases that occur in malt. Like the activities of the other proteinase class enzymes, the aspartic proteinase activities were stable throughout the protein rest, but were rapidly inactivated when the conversion temperature was reached. The first lane of each of these gels was loaded with a green malt extract and the differences between the green malt and kilned malt proteinase activities (as shown by the first protein rest fractions) were relatively small. This conforms with our previous findings (*4*) that indicated that there was very little alteration or inactivation of the proteinases during the kilning phase of malting.

2-D IEF x PAGE Analyses. The results obtained from the 1-D gels (**Figure 3**) indicated, in general, the fates of the different groups of proteinases during mashing, but did not yield data about how the individual enzymes behaved. To get this information, the proteinases from the mash fractions were separated with a 2-D acrylamide gel separation system that consecutively utilized isoelectric focusing (IEF) and polyacrylamide gel electrophoresis (PAGE) separations. This system also uses the hydrolysis of substrate proteins incorporated into the second dimension gels to detect the proteolytic activities and the method is capable of separating over 40 different proteinase activities that are present in malt. The results obtained with this system using gelatin substrate are shown in **Figures 4** (activities were developed at pH 4.8) and **5** (activities at pH 6.0). For these experiments the enzyme extracts were concentrated four-fold to ensure that even the weak activities could be detected. In each 2-D gel, a sample of the original (not subjected to IEF) extract was applied to the lefthand section of the PAGE gel so that the 2-D activity spots could be correlated with the 1-D activity bands of **Figure 3**.

Proteinases Active at pH 4.8. At pH 4.8 and with the substrate gelatin, five metalloproteinase activity spots were obvious (**Figure 4**, indicated by M), together with 3 serine enzymes (S) that migrated to two distinct areas and a group of about a dozen cysteine-class (C) proteinases. The proteinase classes of the separated enzymes were assigned on the basis of our previous in-depth study of the proteinases in green malt (*1*). These are minimal values for the numbers of enzymes present, since there is no evidence that each activity spot was due to only a single enzyme form. The aspartic proteinases could not be detected with this system, since they do not hydrolyze gelatin (**Figures 3B** and **C**; *1*). The activities of all three of the protein rest fractions were identical and are diagramed in **Figure 4A**. These diagrams are really negatives of the real gels, since in that system the activities show up as white spots on a blue background. In contrast to **Figure 3**, the highest relative proteolytic activities are indicated by the darkest spots. The adjunct mash fraction contained no enzymatic activity, and its gel is not shown. By the time the mash reached the 72 °C conversion temperature, the enzymatic activities were already partially inactivated (**Figure 4B**). All were still present, but at lower levels. Six and a half minutes into the conversion (**Figure 4C**) the activities of the remaining enzymes had decreased greatly and some of them (the upper right-hand serine activities, several of the cysteine ones) had disappeared. Ten minutes later, essentially all of the proteolytic activity had vanished from the mash (**Figure 4D**), with only one very weak cysteine activity being still visible. These results indicate that the individual

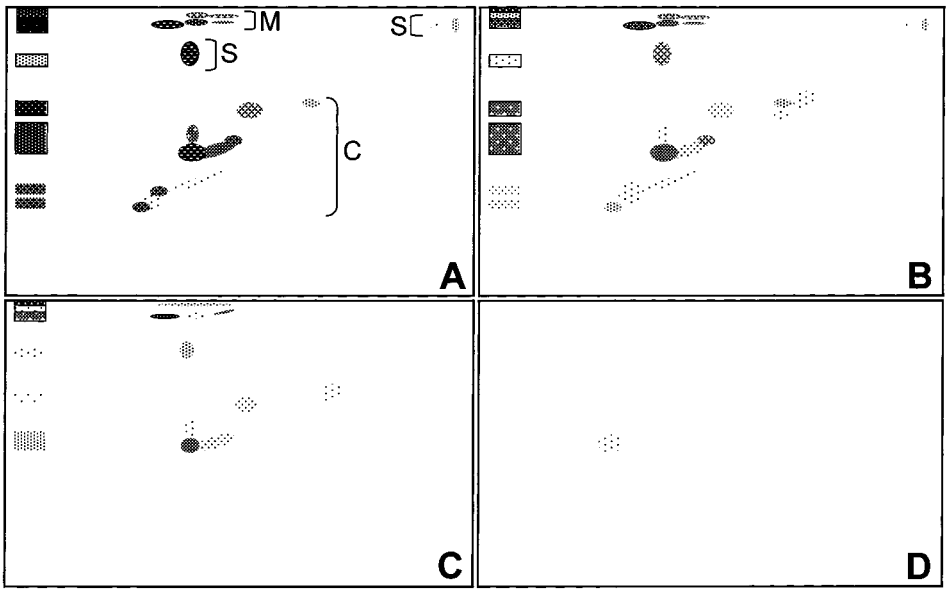

Figure 4. 2-D IEF x PAGE separations of endoproteinases removed from Morex malt samples undergoing mashing: (A) the enzymes present in the protein rest fractions 1, 2 and 3, all of which gave identical patterns; (B) enzymes of fr. 5 (conversion temperature, 72 °C); (C) proteinases of fr. 6, after 6 min at 72 °C; (D) proteinases of fr. 7, after 16 min at 72 °C. The activities were developed at pH 4.8. The activities indicated by M, S, and C comprised, respectively, the metallo-,serine- and cysteine endoproteinases.

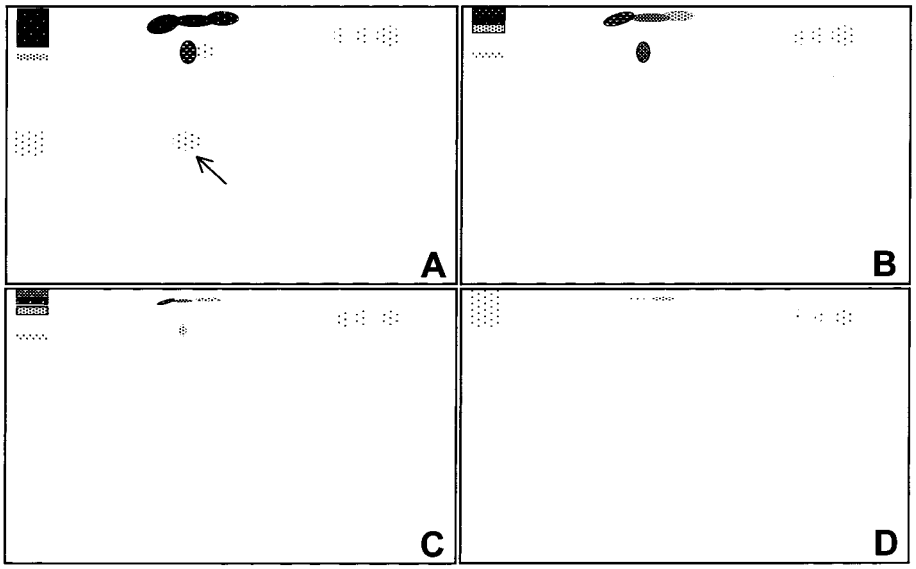

Figure 5. 2-D IEF x PAGE separations of endoproteinases removed from Morex malt samples undergoing mashing: (A) the enzymes present in the protein rest fractions 1, 2, and 3, all of which gave identical patterns; (B) enzymes of fr. 5 (conversion temperature, 72 °C); (C) proteinases of fr. 6, after 6 min at 72 °C; (D) proteinases of fr. 7, after 16 min at 72 °C. The activities were developed at pH 6.0. The arrow indicates the low-level, relatively heat-stable cysteine proteinase activity.

proteinases have quite similar temperature stabilities in mashes. They were stable at 38 °C, throughout the protein rest, but were inactivated rapidly as soon as the temperature of the mash was raised for conversion. All were degraded at roughly the same rate, so that those that were most prevalent at the beginning of mashing were still discernible even after the less active ones had disappeared.

Proteinases Active at pH 6.0. The results at pH 6.0 were very similar to those seen at pH 4.8. However, the activities observed were quite different because nearly all of the cysteine proteinases were inactive at pH 6.0. Only three metalloproteinases were obvious at this pH, but that may result from the fact that they were considerably more active than at pH 4.8, and their expanded activities probably caused some of the bands to overlap and thus show up as single activities. One cysteine activity was present in the fraction 1−3 protein rest samples (arrow, **Figure 5A**). This cysteine protease activity, while faint, persisted throughout the protein rest. In 2-D experiments that were carried out at pH 3.8 with the substrate azogelatin, where only the cysteine class enzymes are generally active (*1*), this activity spot was present and also persisted (albeit at a low level) through fraction 7 (results not shown). As at pH 4.8, the serine- and metalloproteinase activities were readily inactivated during conversion, although a trace of metalloproteinase activity was still present 16.5 min into the conversion step (**Figure 5D**). The multiple serine protease activities in the upper right-hand section of the gel were apparently impervious to heating, and persisted throughout mashing, although they were slightly diminished in the fraction 7 sample (**Figure 5D**). This stability and the fact that they were not apparent in our earlier study of the effect of

malt kilning on proteolytic activities (*4*) may indicate that these were artifacts and did not truly represent proteolytic activities. More likely, they showed up in this study, and not in the previous kilning work, because the samples were concentrated four-fold for this research, whereas the previous work was with unconcentrated samples.

When corresponding 2-D studies were carried out with PAGE gels that contained the substrate edestin instead of gelatin (results not shown), the results were similar. However, at pH 4.8, the aspartic proteinase activities were also obvious and at pH 6.0 little activity was observed. With this substrate also, at pH 4.8, the unique heat stable cysteine activity (**Figure 5A**, arrow) was still obvious in the fraction 7 sample.

CONCLUSIONS

When barley malt was mashed under conditions that mimic the commercial process (**Figure 1**), the overall endoproteolytic activity of the malt mash remained constant during the 55 min mixing and protein rest stages as measured at pH 4.8 and 6.0 (**Figure 2**). However, as soon as the temperature of the mash was increased to 72 °C to speed the conversion of carbohydrates into sugars, the proteinases were quickly inactivated and were almost totally inactive within 16 min (**Figure 2**). These results were verified for individual enzyme groups by separating these groups by electrophoresis and allowing the separated enzyme fractions to hydrolyze the substrates gelatin and edestin (**Figure 3**). These results showed that even after 16 min of conversion, very small amounts of both pH 4.8 and pH 6.0 proteolytic activity were still present. When the proteinases were further separated by a 2-D IEF x PAGE method (**Figures 4** and **5**), the same pattern emerged. The enzymes were all stable until conversion, when most were totally inactivated. Even the ones that retained some activity were only very slightly active 16 min into the conversion step. One group of three weak pH 6.0-active enzymes (upper-right corner of **Figure 5**) retained most of their activities throughout mashing.

Our earlier studies of the effects of kilning on green malt endoproteinases indicated that kilning had only a very marginal effect on these enzymes (*4*). This work shows further that the proteolytic enzyme profile of the mash through the protein rest phase is quite similar to that of kilned malt and that the changes in those activities are minimal. From these studies it is obvious that the majority of the protein degradation that occurs during malting and mashing must occur either during malting (when the enzymes and their substrates are maintained in a strictly ordered system) or during the protein rest phase of mashing. Thus, if brewers want to increase the amount of protein in a wort, they need to either lengthen (or speed up) the germination stage of malting or the protein rest period of mashing. Extending the length of the conversion period would have little effect on the final protein solubilization, due to the rapid inactivation of the enzymes at 72 °C.

The activities of all of the detected proteinases dropped at about the same rate during conversion, except possibly for those of the enzymes that migrated to the top right-hand corner of the gel. We have shown previously (*1*) that the malt proteinases that migrate to that corner of the gel are serine class enzymes that apparently do not solubilize storage proteins, so they probably have very little effect on the level of soluble protein in the final mash. It therefore seems unlikely that it will be possible to alter the relative amounts of the individual proteins that are degraded during mashing by altering the mashing conditions since, even if the different proteinases degrade different proteins, they all appear to be affected uniformly by changes in the mash temperature scheme. It might, however, be possible to make some changes by introducing a hold at some intermediate temperature where only a portion of the proteinases have been partially inactivated. Since the rate of inactivation of the various enzymes is apparently fairly constant, it seems unlikely that such an approach would have a very significant effect.

Samples of the Harrington malt mashes that were used to carry out the experiments of **Figure 2** were also used to conduct 1-D and 2-D electrophoresis experiments such as those of **Figures 3**−**5**. As in **Figure 2**, the results obtained with the Harrington malts were the same as those gotten with Morex malts, so these results have not been shown. The fact that the Morex (six-rowed) and Harrington (two-rowed) mashes yielded very similar results implies that these conclusions will probably hold for all or most malting barleys.

ABBREVIATIONS USED

1-D, one-dimensional; 2-D, two-dimensional; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

We thank Christopher Martens, Doug Braun and Debora Fontanini for conducting many of the enzyme analyses and Eddie Goplin for carrying out the mashing.

LITERATURE CITED

(1) Zhang, N.; Jones, B. L. Characterization of germinated barley endoproteolytic enzymes by two-dimensional gel electrophoresis. *J. Cereal Sci*. **1995a**, *21*, 145−153.

(2) Harris, G. The enzyme content and enzymatic transformation of malt. In *Barley and Malt;* Cook, A. H., Ed.; Academic Press: New York, 1962; pp 635−642 and 666−677.

(3) Zhang, N.; Jones, B. L. Development of proteolytic activities during barley malting and their localization in the green malt kernel. *J. Cereal Sci*. **1995b**, *22*, 147−155.

(4) Jones, B. L.; Marinac, L.; Fontanini, D. A quantitative study of the formation of endoproteolytic activities during malting and their stabilities to kilning. *J. Agric. Food Chem*. **2000**, *48*, 3898−3905.

(5) Jones, B. L. Malt endoproteinases and how they affect wort soluble protein levels. *Proceedings of the 9th Australian Barley Technical Symposium*, *Barley − Pathway Into the 21st Century*, Melbourne, Australia, 1999; Organizing Committee: Melbourne, 1999; pp 2.39.1−2.39.8.

(6) Rehberger, A. J.; Luther, G. E. Brewing. In *Handbook of Brewing*; Hardwick, W. A., Ed.; Marcel Dekker: New York, 1995; pp 271−274.

(7) Burger, W. C.; Schroeder, R. L. Factors contributing to wort nitrogen. I. Contributions of malting and mashing, and effect of malting time. *J. Am. Soc. Brew. Chem*. **1976a**, *34*, 133−137.

(8) Burger, W. C.; Schroeder, R. L. Factors contributing to wort nitrogen. II. Effects of malting time and gibberellic acid on endopeptidase and exopeptidase activities. *J. Am. Soc. Brew. Chem.* **1976b**, *34*, 138−140.

(9) Barrett, J.; Kirsop, B. H. The relative contributions to wort nitrogen of nitrogenous substances solubilized during malting and mashing. *J. Inst. Brew*. **1971**, *77*, 39−42.

(10) Clapperton, J. F. New aspects of the composition of beer in relation to nitrogenous compounds. *Proc. Eur. Brew. Convention Congr., Estoril*. **1971**, *13*, 323−332.

(11) Lewis, M. J.; Robertson, I. C.; Dankers, S. U. Proteolysis in the protein rest of mashing- an appraisal. *MBAA Technical Quarterly* **1992**, *29*, 117−121.

(12) Lehninger, A. L. *Biochemistry*, 2nd ed.; Worth Publishing: New York, 1975; p 62.
(13) Koehler, S.; Ho, D. T.-H. Purification and characterization of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. *Plant Physiol*. **1988**, *87*, 95−103.
(14) Kringstad, H.; Kilhovd, J. Weitere studien uber die proteolytischen enzyme von gerste und malz. *Proc. Eur. Brew. Convention Congr*. **1957**, *6*, 67−71.
(15) Schoenfeld, F. *Handbuch der Brauerei und Malzerei*; Verlag Paul Parey: Berlin, Germany, 1935; Vol. 3 (reproduced in Briggs, D. E.; Hough, J. S.; Stevens, R.; Young, T. W. *Malting and Brewing Science, Vol 1., Malt and Sweet Wort;* Chapman and Hall: London, U.K., 1981; p 285.
(16) American Society of Brewing Chemists. Malt-4 Extract. *Methods of Analysis*, 8th ed. The American Society of Brewing Chemists: St Paul, MN, 1992.
(17) Jones, B. L.; Fontanini, D.; Jarvinen, M.; Pekkarinen, A. Simplified endoproteinase assays using gelatin or azogelatin. *Anal. Biochem*. **1998**, *263*, 214−220.
(18) Wrobel, R.; Jones, B. L. Appearance of endoproteolytic enzymes during the germination of barley. *Plant Physiol.* **1992**, *100*, 1508−1516.

Received for review July 24, 2001. Revised manuscript received October 30, 2001. Accepted November 21, 2001. We thank the American Malting Barley Association and Anheuser-Busch, Inc. for partially funding this research. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

JF0109672