

Malt Endoproteinases and How They Affect Wort Soluble Protein Levels

Berne L. Jones

USDA, ARS, Cereal Crops Research Unit, 501 N. Walnut St., Madison, WI, 53705, USA and Department of Agronomy, University of Wisconsin- Madison.

The endoproteinases that form during the malting of barley play important roles in determining the soluble protein levels of worts prepared from the malt. The soluble protein levels, in turn, can affect many aspects of the beers made from these worts, including their haziness, foam characteristics, extent of fermentation, etc. We have been characterizing these endoproteinases and their endogenous inhibitors for several years and are now trying to determine which ones play important roles in solubilizing proteins during mashing and how the endogenous barley and malt proteinase inhibitors affect this solubilization.

The Synthesis of Malt Endoproteinases

Unmalted barley contains very little endoproteolytic activity, nearly all of which is due to the aspartic class enzymes (Wrobel and Jones, 1992). However, there is copious activity in 4-day germinated malt, with representatives of all four protease classes being present among the approximately 45 activities that are readily detected (Zhang and Jones, 1995). We have used two very different methods to measure the proteolytic activities of barley samples that were undergoing malting. The first method was an 'in solution' assay that used the substrate azogelatin (Jones, et al., 1998) to quantitatively measure the overall endoproteolytic activity of samples and the second was a 2-dimensional (2-D) IEF x PAGE method that detected the activities of the individual proteases (Zhang and Jones, 1995). The 2-D method gives only a semiquantitative indication of the proteinase activity levels.

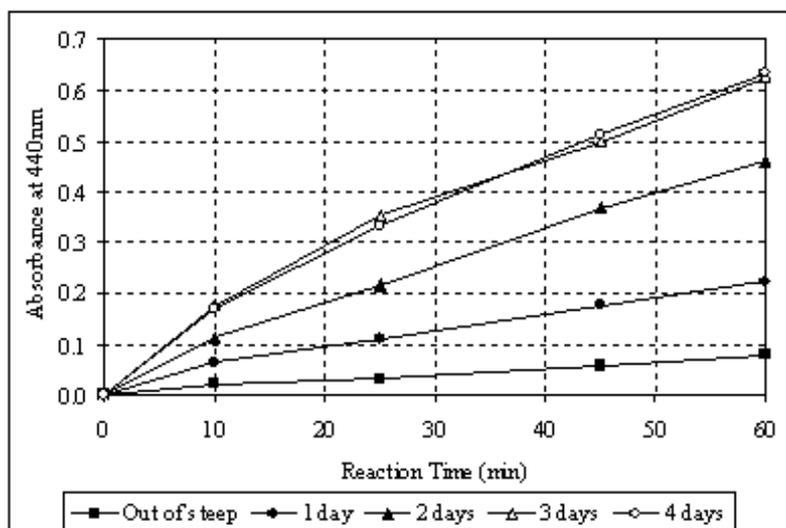


Figure 1. The proteinase activities of extracts prepared from Morex barley that was undergoing malting. pH 4.8, 4 mM cysteine added.

Malts were prepared from Morex and Harrington barleys by steeping the samples to 45% moisture, germinating for 4 days at 100% humidity and 17°C with intermittent sample rotation, and kilning the green malt with temperatures that were raised from 49 to 85°C over 24 hrs. Samples of unmalted barley and fractions removed at the end of steeping and after 1, 2, 3, and 4 days of germination were extracted and their proteolytic activities were measured. The results (Fig. 1) indicated that, as expected, there was little activity in the out-of-steep sample. The activities began to rise after 1 day of ‘germination’ and reached a maximum on the third day of germination. These results are consistent with what would be expected of enzymes that formed *de novo* in response to plant hormones or by the activation of preexisting enzymes.

The Stabilities of Green Malt Proteinases During Kilning

To explain certain anomalous results, some researchers have postulated that some green malt proteinases are inactivated during malt kilning. During this process, the green malt is dried in a current of warmed air whose temperature normally is slowly raised from 49°C to about 85°C. We have produced malt from both 2- and 6-rowed barleys and subjected these malts to the kilning procedure that is shown in Figure 2. This kilning schedule is very similar to

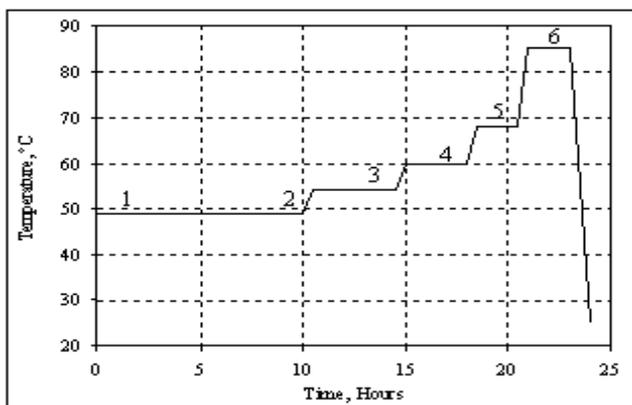


Figure 2. Kilning schedule used to prepare samples. Samples were removed for analysis after each step, as indicated by the numbers.

those that are normally used by U.S. commercial maltsters. Samples were removed during the kilning process at the end of each temperature step, as indicated on Figure 2. The proteases were extracted from each of these samples and their overall activities were measured using the quantitative azogelatin ‘in solution’ assay and the individual activities were analyzed using the 2-D IEF x PAGE method. Both of these methods indicated that there was no measurable inactivation of the green malt proteinases during kilning to 85°C. It appeared, however, that some of the proteinases were slightly affected at the higher temperatures. Even though their activities were not affected, their migration on the 2-D gel indicated that they were possibly slightly denatured, since the individual activity spots of the samples heated to 68 and 85°C formed more diffuse activity spots than those that were only subjected to lower temperatures.

The Inactivation of Kilned Malt Proteases During Mashing

Since the proteases within the malt kernels were stable to the 85°C kilning temperatures, we tested their stabilities during the mashing process, after they were solubilized. The mash

schedule used is shown in Figure 3. This is the schedule that we use when preparing laboratory brews and is similar to those used commercially in the U.S. It is a double mashing process, in which the malt and adjunct (corn grits) are mashed separately and then combined.

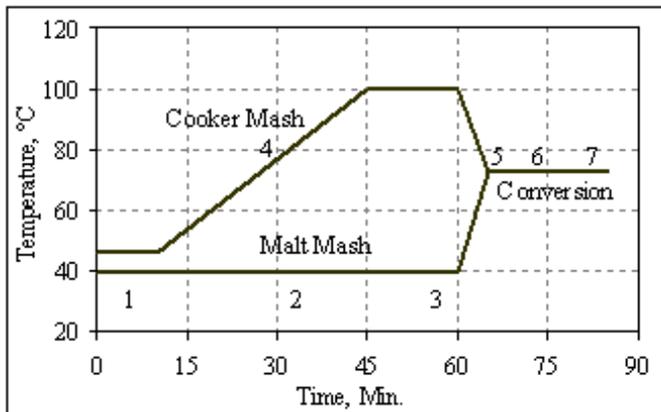


Figure 3. Mashing schedule used to prepare samples. Samples were removed for analysis at the times indicated by the numbers.

Samples were removed from the mash at the points indicated by numbers on Figure 3; three from the ‘protein rest’ phase of the malt mash, one from the adjunct cooker mash and three from the conversion step, after the mash and adjunct mashes were mixed. The activities of the fractions were determined using both the ‘in solution’ quantitative and the 2-D qualitative methods. The results of both methods agreed; the activities were completely stable throughout the protein rest, there was a little protease activity in the adjunct mash (due to the small amount of malt added to the adjunct mash to keep it soluble) and all of the proteinase activities were quickly inactivated as the mash temperature was raised above approximately 60°C. By the time the mash temperature reached 70°C, the majority of the proteolytic activity was destroyed, and within 10 min nearly all of the activity was gone. The 2-D analyses showed that all of the proteases were inactivated at roughly the same rate. None were stable at 70°C.

It is interesting and instructive to note that the proteases were completely stable at 85°C *in situ*, in the malt kernel during kilning, but were readily inactivated at 60°C in solution during mashing. The environment in which the enzymes were located obviously very strongly affected their stabilities.

The Endoproteinase Classes That Are Involved in Producing ‘Soluble Protein’

It has, in the past, been generally accepted that only the cysteine class malt endoproteinases played a significant role in producing ‘soluble protein’ (solubilized protein, peptides or amino acids) during malting and mashing. It was important to know whether this was correct, because our goal is to determine how researchers can more scientifically and efficiently develop improved malting barley cultivars and/or malting and brewing methods. Before barleys and/or processing methods can be altered to produce worts with better soluble protein profiles, we need to know which of their many malt endoproteinases really contribute to the pool of protein breakdown products that comprise the wort.

To study this process, we have carried out ASBC ‘congress mashes’ in the presence of various chemicals that specifically inhibit the activities of each of the four classes of proteases. These compounds, the proteinase classes whose activities they inhibit, and the concentrations at which they were tested are indicated in Table 1. The ASBC congress mash method (Figure 4) is quite different from commercially used mashing techniques. However, it is the standard method used for measuring malting quality in North America and it provides a good baseline with which we can compare the results obtained when we carry out similar experiments using ‘commercial’ mashing schedules. We mashed samples at pH 3.8 (the pH at which the total proteolytic activity is highest, and where the cysteine proteinases strongly predominate), pH 6.0 (mashing pH) and pH 8.0 (where the cysteine proteinases are inactive and the serine and metalloproteinases predominate). The results of the experiments carried out at pH 6.0 are shown in Table 2, since this, being the standard North American mashing pH, provides the information that will be most useful for understanding what really happens during commercial brewing.

Table 1. Endoprotease classes and their specific chemical inhibitors.

Protease class	Inhibited by:	Inhibitor concentration
Cysteine	E-64	1 μ M
Serine	PMSF	1 mM
Aspartic	Pepstatin A (Pep A)	5 μ M
Metallo-	o-Phenanthroline (o-Phen)	5 mM
	EDTA	5 mM

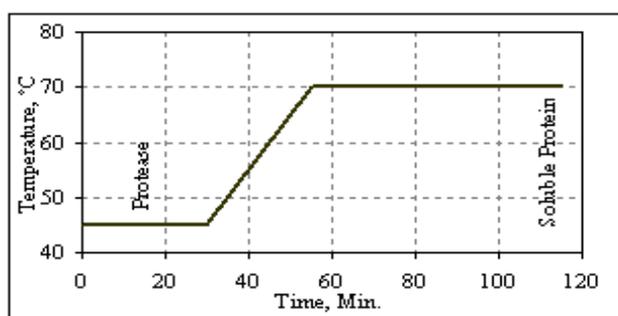


Figure 4. ASBC ‘congress’ mash schedule used for inhibitor studies. Samples for proteinase activity and soluble protein measurements were collected at the times indicated.

The soluble protein results indicate that the cysteine proteinases are apparently not the only ones that produce soluble protein during mashing. While good inhibition was found when E-64 (cysteine proteinase inhibitor) was added to the reactions, the addition of either pep A (aspartic protease inhibitor) or o-phen (metalloprotease inhibitor) also resulted in the inhibition of some soluble protein formation, indicating that proteinases of these classes were also involved in protein solubilization. The addition of PMSF (serine protease inhibitor) had no effect on protein solubilization and adding EDTA (an alternate inhibitor of metalloproteinases) actually caused an increase in the soluble protein level. While it was expected that the two metalloproteinase inhibitors o-phen and EDTA would affect solubilization in the same way, this was not the case. Either the EDTA and o-phen inhibited different sets of metalloproteinases in this system or they affected the same set of enzymes in different ways. Throughout this experiment, the EDTA appeared to behave inconsistently, and it seems

probable that the o-phen results more realistically reflect what actually occurs during mashing. From these results, it appears that the cysteine-, aspartic- and metalloproteinases all contribute to the solubilization of protein during mashing.

Table 2. The effects of adding class-specific inhibitors to pH 6.0 mashes.

	Inhibitor	Soluble	Percent	Protease	Percent	FAN	Percent
	added	protein,%	inhibition	activity ¹	inhibition	ppm	inhibition
Morex							
	None	6.03	-	0.071	-	235	-
	E-64	5.55	8	0.043	39	213	10
	Pep A	5.72	5	0.070	1	227	4
	PMSF	5.99	1	0.061	14	224	5
	o-Phen	5.30	12	0.047	34	227	4
	EDTA	6.28	- 4	0.127	- 79	278	-18
Harrington							
	None	4.93	-	0.041	-	192	-
	E-64	4.26	14	0.030	27	167	13
	Pep A	4.56	8	0.040	2	172	10
	PMSF	4.67	5	0.030	27	168	12
	o-Phen	3.93	20	0.033	20	150	22
	EDTA	4.94	0	0.058	-42	184	4

¹ OD_{440 nm} / 60 min

While these samples were being mashed in the presence of the various inhibitors, samples were removed from the mashes to determine how the endoproteolytic activities of the mashes were affected. As expected, the addition of each of the inhibitors except EDTA and pep A reduced the proteolytic activity. A comparison of the proteolytic activities and the final wort soluble protein levels shows that, in general, when the proteolytic activities were lowered the soluble protein levels also fell. In the presence of pep A, however, protein solubilization was significantly lowered, while the proteolytic activity was unchanged. This is because the aspartic proteases that would have been inhibited do not hydrolyze the azogelatin substrate used to measure the protease activities, and thus would not have been detected. PMSF, while causing a strong lowering of proteolytic activity, has essentially no effect on protein solubilization. This indicates that while there is considerable serine protease activity in mashes, it apparently does not hydrolyze the storage protein into ‘soluble protein’. The soluble protein levels were, of course, measured on the final worts or ‘extracts’, while the samples that were used to assay the proteolytic activities were removed from the mashes after 10 min of ‘protein rest’ to ensure that none of the activity had been destroyed by the higher temperatures that occurred during the ‘ramping’ phase of the mashing.

When the mashes were carried out at pH 3.8 and 8.0 in the presence of the inhibitors (data not shown), the expected results were found. That is, in the pH 3.8 mashes the effect of the cysteine protease inhibitor (E-64) was enhanced, since those enzymes were more active at pH 3.8 than at pH 6.0. At the same time, the effects of pep A and o-phen were minimized, since the aspartic and metalloproteinases show only minimal activity at pH 3.8. On the other hand, in the reactions carried out at pH 8.0 the inhibition of protein solubilization by o-phen and pep A were enhanced and that due to E-64 was almost nonexistent, reflecting the relative activities of these proteinases at pH 8.0.

Since wort free amino nitrogen (FAN) contents are dependent on the exopeptidases, not the endoproteases affected by the added inhibitor, the FAN contents behaved differently from the soluble protein and proteinase levels.

The Formation of Soluble Protein During Malting and Mashing

If maltsters and brewers want to alter their methods to vary the soluble protein levels of worts, they need to know how much of the soluble protein that is present in the final wort forms during the malting process and how much during mashing. Over the years, this subject has been investigated by several laboratories and the results have varied greatly; it was claimed in some reports that about 50% of the protein was solubilized during mashing and in others that none was. After we demonstrated (above) that only the aspartic, cysteine and metalloproteinases were involved in solubilizing protein during mashing, it became possible for us to directly measure the amount of protein solubilization that occurred during this step. By adding the inhibitors E-64, pep A and o-phen to congress mashes, we could inhibit all of the solubilization that occurred during mashing and thus measure how much had occurred during malting. Normally, when ‘malt soluble protein’ levels are measured, the hydrolysis that occurred during both malting and mashing is measured, because the malt is subjected to a mashing process during the ASBC congress wort analysis method.

To measure exactly when the soluble protein content of congress worts developed, we carried out three separate analyses: 1) We extracted ungerminated barley. This gave a measure of the amount of soluble protein that was present in the barley before it was malted. To my knowledge, this value had never been measured previously. 2) We extracted malt using the normal ASBC extract method. This gave a measure of the total soluble protein in the wort, whether it was preformed in the barley or arose during malting or mashing. 3) We extracted malt in the presence of E-64, pep A and o-phen to get a measure of the soluble protein that was present in barley plus that released during malting prior to mashing. By comparing the soluble protein levels of these three mashes, we ascertained how much came from the original barley, from mashing and from malting. The results are shown in Table 3.

Table 3. The soluble protein levels of Morex barley, malt and mashes.

Soluble Protein,%; (% solubilized, this step) ¹			
Extract	Barley ²	Malt ³	Wort ⁴
pH 3.8	3.29; (36)	6.66; (37)	9.14; (27)
pH 6.0	2.21; (33)	5.03; (43)	6.60; (24)

pH 8.0	2.44; (34)	6.19; (61)	6.54; (05)
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¹ The percentage that the soluble protein increased over the previous step.

² Ungerminated barley was mashed.

³ Mashed in the presence of the inhibitor mix.

⁴ Mashed without inhibitor added.

Since the results with Morex and Harrington were similar, only those obtained with Morex are shown. They showed that, at pH 6.0, about 33 % of the wort protein was present in the ungerminated barley, 43 % was released on malting and the remaining 24% was solubilized during mashing. One of the major advantages of using this method for studying this phenomenon is that all of the extractions are carried out under identical physical conditions, so that none of the variation measured is due to temperature or other differences in the mashing procedures. In the past, it has sometimes been alleged that no protein was really hydrolyzed during mashing, but that the appearance of additional soluble protein during mashing was entirely due to the solubilization of previously hydrolyzed proteins. This work shows that this postulation was incorrect.

Varying the Soluble Protein Levels of Mashes by Adding Endogenous Barley/Malt Inhibitors and/or Cysteine

Our rationale for studying the endogenous compounds of barley and malt that inhibit green malt proteinases was that it seemed probable that they might play some role in regulating the levels of soluble protein in worts. In the U.S., commercial brewers have indicated that the soluble protein levels of worts prepared from our leading malting barleys are too high, and that they would like newly developed cultivars to yield lower protein worts. It seems logical that this could be done by developing (using classical breeding methods or molecular biology techniques) either lines that have lower proteinase levels or those with increased amounts of endogenous inhibitors. Since there are over 45 separate endoproteinase activities in green malt and probably only six or fewer inhibitors, it seems likely that it will be easier to alter

the enzymatic activities by changing the levels of the inhibitors, rather than those of the enzymes.

The work reported above, which showed that soluble protein levels correlated well with the amounts of cysteine, aspartic and metalloproteinase activities, indicated that by lowering the activities of any one of these enzyme groups we should be able to alter the wort protein levels. The facts that the endogenous barley and malt inhibitors studied to date have all preferentially inhibited the cysteine class proteinases and that these proteases are also very active in solubilizing protein during mashing, made it even more likely that raising the inhibitor levels would significantly lower the final wort protein concentrations.

For these reasons, we carried out ASBC mashes in the presence of added endogenous malt inhibitor preparations and compared these to normal mashes. While the results of these experiments are still preliminary and have been rather variable, the addition of the proteinase inhibitor preparations always yielded worts that had lowered soluble protein levels. This research is presently being continued and refined.

While carrying out the above experiments, we noticed that when cysteine was added to our mashes, it had a very strong influence on the overall endoproteinase activities of the mashes. This was not particularly surprising, since the presence of the reducing agent cysteine could increase the activities of the malt cysteine class proteinases and these enzymes were known to play the major role in hydrolyzing proteins during mashing and, probably, during malting. It was, however, a bit surprising that the activity was so strongly activated. We therefore tested the effect of adding cysteine to mashes and found that its presence also caused a strong rise in the level of the wort soluble protein, from 6.2 to 8.5%.

Conclusions

- The great majority of the endoproteinases of malt are not present in the ungerminated barley, but form during the ‘germination’ phase of the malting process.
- The activities of the 40 or more green malt endoproteinases are totally stable to kilning, under standard conditions, up to at least 85°C.
- The malt proteinases are stable throughout the ‘protein rest’ phase of mashing, using the standard ASBC mash process, but are inactivated during the ‘ramping’ phase, as soon as the temperature rises above about 60°C. They are very quickly inactivated during the ‘conversion’ stage, so that no activity remains at the end of conversion.
- The addition of class-specific protease inhibitors to mashes shows that the cysteine-, aspartic- and metalloproteinases all play roles in the solubilization of protein during mashing. It was previously thought that only the cysteine proteases were involved in this process. While this is apparently not true, the cysteine enzymes are probably the predominant determinants of how much and which proteins are solubilized.
- About 43% of the soluble protein of a wort is preformed in the barley grain, 32% is solubilized during the malting process and the remaining 25% is released during mashing. This means that it should be possible to vary the final wort soluble protein levels by up to about 57%, either by making genetic changes in the malting barleys or by changing the processing methods used during malting and mashing. Some such changes would, however, probably adversely affect some of the other parameters that are required of an acceptable malting barley.

The soluble protein levels of worts can be lowered by adding endogenous inhibitor preparations to mashes or enhanced by adding cysteine (or certain other reducing agents).

Acknowledgments

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