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How various malt endoproteinase classes affect wort soluble protein levels

Berne L. Jones*, Allen D. Budde

USDA, ARS, Cereal Crops Research Unit, 501 N. Walnut Street, Madison, WI 53726, USA

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Abstract

During the germination of seeds, storage proteins are degraded and the resulting amino acids are utilized by the growing seedling. In barley, this process is commercially important because it forms the basis for the malting and brewing industries. In this study, barleys and malts were mashed in the presence of compounds that specifically inhibited the four common proteinase classes. The efficacies of the proteinases in solubilizing proteins were in the order cysteine ≈ metallo > aspartic > serine ≈ 0, which roughly reflected how the inhibitors affected the mash endoproteolytic activities. It was previously believed that only the cysteine enzymes were involved. All four enzyme classes affected the free amino nitrogen concentration but none altered any of the other measured wort characteristics. With either single inhibitors or inhibitor mixtures, the effect of pH was as expected, based on earlier studies that indicated that cysteine and aspartic proteinases were most active at low pH values and the metalloproteinases were only active at high pH. At the North American commercial mashing pH of 6.0, about one third of the soluble protein of a typical wort came from ungerminated barley, half was solubilized during malting and the remaining 22% was released during mashing.
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Keywords: Hordeum vulgare; Barley; Proteinases; Soluble protein; Free amino nitrogen; Malting; Brewing; Germination

1. Introduction

During malting and the mashing phase of brewing, a portion of the insoluble proteins of barley must be converted into 'soluble protein' (SP) if good brews are to be obtained. This SP fraction comprises a mixture of amino acids, peptides and dissolved proteins, and a major portion of it arises by proteolysis of barley proteins. To more efficiently produce malting barleys that have improved SP quality and to develop more effective malting and brewing methods, we need to ascertain which proteolytic enzymes are involved and how they operate. The same processes presumably occur during naturally occurring seed germination. Several researchers have shown that the endoproteinases, not the exoproteinases, are the rate limiting enzymes for the formation of soluble protein (Burger and Schoeder, 1976b; Sopanen et al., 1980). These, then, are the enzymes whose activities will need to be altered to vary the SP levels in the final brewing worts.

Until recently, it was thought that only a few endoproteinases were active during mashing and malting, but we have detected at least 40 different endoproteinases in green malt using a two-dimensional isoelectric focusing×polyacrylamide gel electrophoresis method (Zhang and Jones, 1995a). It was also thought that only the cysteine class of endoproteinases were involved. This seems unlikely, now that it has been shown that multiple enzymes belonging to each of the four classical protease classes are present in green malt (Zhang and Jones, 1995a). There is little or no inactivation of these enzymes during malt kilning (Jones et al., 2000) or the protein rest phase of mashing (Jones and Marinac, 2002) so all have considerable opportunity to hydrolyse the storage proteins and other proteins of the barley.

This study was aimed at determining which of the kilned malt endoproteinase classes were, in fact, involved in solubilizing protein during the mashing phase of brewing

Abbreviations: ASBC, American Society of Brewing Chemists; FAN, free amino nitrogen; *o*-phen, 1,10-phenantholine; HGASBC, high gravity ASBC; PMSF, phenylmethylsulfonyl fluoride; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; SP, soluble protein.

* Corresponding author. Present address: RR1, Box 6, Kooskia, ID 83539, USA. Tel.: +1 208 926 4429.

E-mail address: bhjones@bmi.net (B.L. Jones).

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and to measure what proportion of the SP and free amino nitrogen (FAN) fractions of worts were released during the malting and mashing processes. Compounds that specifically inhibited members of the various classes of proteases were added to mashes. Mashes were made at pH 3.8, 6.0 and 8.0, because it has been shown that there are two groups of proteinases in malt that have widely differing optimal pH values (Zhang and Jones, 1995a) so that by comparing the results obtained with the low and high pH mashes it was possible to deduce which of the enzyme groups was catalyzing the observed protein hydrolyses.

2. Experimental

2.1. Preparation of kilned malt

Seeds (170 g, dry basis), cleaned to remove seeds not retained on a 5/64 in screen, of both Morex and Harrington barleys (the six- and two-rowed American Malting Barley Association malting quality standards, respectively) were steeped at 16 °C for 36 h, to 45% moisture, with four 4-h couchings. The steeped seeds were germinated in the dark, with intermittent (3 min each 30 min) rotation at 17 °C and near 100% humidity, for 5 d. The resulting 'green malt' was kilned to around 4% moisture using a schedule (Jones et al., 2000) that started at 49 °C (10 h), finished at 85 °C (3 h) and that conformed closely to US industry practices. The malt samples produced were stored at room temperature until mashed.

2.2. Mashing

The terms 'mash' and 'extract' are used interchangeably in this paper, because they both refer to essentially the same process; taking a sample of malt or barley and putting it though an aqueous extraction process to obtain a solution of soluble molecules that can be measured. The extraction method applied to the ungerminated barley and ASBC samples would normally result in an 'extract' sample, and it differs somewhat from that used for the 'commercial' samples, whose resultant solution would be called 'wort'.

2.2.1. ASBC 'congress' or 'ASBC' mashes

Fine-grind malt or barley samples were prepared using a Miag laboratory cone mill adjusted as specified in the ASBC Malt-4 (American Society of Brewing Chemists, 1992) method. These samples were extracted according to the ASBC Methods, Malt-4 procedure, except that all weights and volumes specified for the method were halved.

2.2.2. High gravity ASBC congress or 'HGASBC' mashes

HGASBC malt mashes were made using the ASBC Malt-4 method, except that the initial malt concentrations in the extracts were increased from 25 g/100 ml to 60 g/200 ml. It would have been preferable to use a 60 g/100 ml mixture, which would have been closer to commercial mashing conditions, but such mixtures were too viscous to stir. To compensate for this, no water was added when the mash temperature reached 70 °C and the stirrers and containers were rinsed sparingly at the conclusion of mashing. By taking these precautions, the final extract volumes were reconstituted to exactly 200 ml, the normal volume. During extraction, these samples were 1.2 times as concentrated (25 g/100 ml vs 60 g/200 ml) as normal ASBC mashes. The high gravity filtrates that were analyzed were 2.4 (60 g malt vs 25 g/200 ml) times more concentrated than the traditional ASBC mash filtrates, which were diluted to 200 ml before filtration.

2.2.3. 'Commercial' mashes

Kilned malt was ground and mashed according to a schedule that generally conforms to US industry practices (Jones and Marinac, 2002). Since only the malt mash was of interest, no cooker (adjunct) mash was prepared. The malt mash was made by stirring 62 g of ground malt into 200 ml of 50 °C water and mixing it for 30 min at 50 °C. The temperature was then raised to 68 °C over 18 min and held at 68 °C for 30 min. It was then increased to 77 °C at 1.5 °C/min, maintained at 77 °C for 5 min, and cooled to room temperature over 15 min. The cooled samples were adjusted to 200 ml with water, mixed, and filtered though Ahlstrom fluted grade 509, 32 cm, filter paper. The resultant solutions were analyzed for their extract, SP and FAN values.

2.2.4. Adjusting the pH values of mashes

In experiments conducted at pH values that were either higher or lower than normal, the pH was adjusted downward by adding acetic acid or upwards with NaOH as described in Jones and Budde (2003)

2.3. Measuring the malt extract, SP, free amino nitrogen and (1→3,1→4)-β-glucan values

Variations of standard ASBC methods were used to measure the extract, SP, FAN and (1→3,1→4)-β-glucan levels of the various samples.

2.3.1. Extract

The densities of filtered mashes were measured with an Anton/Parr DMA 5000 density meter. The density data were used to calculate the amounts of soluble material present in the filtrates, and thus the percentage of the malt weight that had been dissolved (ASBC method Wort-2B) (American Society of Brewing Chemists, 1992).

2.3.2. Soluble protein

The SP levels of the worts were originally determined using two different methods. In the first, UV absorbance method (ASBC method Wort-17), the absorbances of the worts were measured at 215 and 225 nm and the differences in these absorbance values were used

to calculate the amounts of protein present (American Society of Brewing Chemists, 1992). For the second method, wort fractions were analyzed in a LECO FP-528 Nitrogen analyzer using the Dumas (ASBC Wort-10) method (American Society of Brewing Chemists, 1992). The protein values measured by both the UV and Dumas methods were adjusted to account for the background absorbances and nitrogen contents of the reagents and to account for the differences in the amounts of malt extracted. The nitrogen levels measured by the Dumas method were multiplied by 6.25 to convert them into 'soluble protein' (SP) values. The values obtained using the UV method were not useable for solutions that contained either PMSF or *o*-phen, because these reagents both absorbed the UV light strongly. The Dumas method worked acceptably in the presence of all inhibitors.

2.3.3. FAN values

The FAN values of the worts were measured using an automated version of the ASBC Wort-12 method (American Society of Brewing Chemists, 1992).

2.3.4. (1→3,1→4)-β-glucan levels

The wort (1→3,1→4)-β-glucans were quantified using the ASBC Wort-18 method (American Society of Brewing Chemists, 1992), which used flow injection analysis to measure the fluorescence of a Calcofluor-(1→3,1→4)-β-glucan complex.

2.4. Measuring the proteolytic activities of mashes

Two milliliter samples were removed from the 100 ml, pH 6.0, ASBC mashes 10 min after the 45 °C 'protein rest' phase of the mash was initiated. These aliquots were held in an ice-water bath for 10 min and then centrifuged at 11,600×*g* for 5 min. The supernatants were carefully decanted and the endoproteolytic activities of 10 μL samples were measured using the method of Jones et al. (1998) with gelatin as substrate.

2.5. Inhibiting the activities of the various proteinase classes

The proteinases comprising the four common classes were inhibited, either individually or in concert, by adding chemicals, specific for the classes to the mashes. The compounds that were used, their inhibitory properties and their concentrations are listed in Table 1.

Table 1
Endoprotease classes and their specific chemical inhibitors

Protease class	Inhibited by:	Concentration, individual	Concentration, mixture study
Cysteine, EC 3.4.22	E-64	1 μM	4 μM
Serine, EC 3.4.21	PMSF	1 mM	–
Aspartic, EC 3.4.23	Pepstatin A	5 μM	25 μM
Metallo-, EC 3.4.24	*o*-Phen	5 mM	10 mM
	EDTA	5 mM	–

3. Results

3.1. Effect of endoproteinase inhibitors

To study which of the malt endoproteinases are really involved in producing SP and FAN during mashing, ASBC congress mashes were made in the presence of various chemicals that specifically inhibit the activities of each of the four classes of proteases (Table 1) Preliminary experiments (results not shown) showed that the inhibitor concentrations listed maximized the inhibition of SP release during mashing at pH 3.8, 6.0 and 8.0, although later studies indicated that a slightly higher concentration of *o*-phen might have caused more inhibition. The lowest effective inhibitor concentrations were used to minimize the levels of ethanol (pepstatin A solvent) and isopropyl alcohol (solvent for *o*-phen and PMSF) in the mashes. The inhibitors were dissolved in these alcohols instead of the more commonly used dimethyl sulfoxide because dimethyl sulfoxide caused some proteolysis inhibition, while neither of the alcohols did. For the experiments made with mixtures of E-64, pepstatin A and PMSF, the inhibitor concentrations were increased to 4 μM, 25 μM and 10 mM, respectively, to ensure that maximal inhibition was obtained.

The results of one set of inhibition experiments that were made at pH 6.0 with the Morex and Harrington malts are shown in Table 2. The addition of E-64 (cysteine proteinase inhibitor) to the mashes substantially lowered their SP levels and pepstatin A (aspartic protease inhibitor) reduced them to a lesser extent. The addition of *o*-phen (metalloprotease

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

Inhibitor added	Soluble protein		Endoproteinase		FAN	
	Percent	%Inhibition	Activity[a]	%Inhibition	ppm	%Inhibition
Morex						
None	6.03	–	0.071	–	235	–
E-64	5.55	8	0.043	39	213	10
Pepstatin 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[b]	0.127	−79	278	−18
Harrington						
None	4.93	–	0.041	–	192	–
E-64	4.26	14	0.030	27	167	13
Pepstatin 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	4.90	0	0.058	−42	184	4

[a] $\Delta OD_{440\ nm}$/60 min.
[b] Negative values indicate activation, not inhibition.

Table 3
Effect of individual inhibitors on wort characteristics at various mashing pH levels[a]

Treatment	pH	Soluble protein, %		Extract, %		FAN, ppm	
		Morex	Harrington	Morex	Harrington	Morex	Harrington
Control	3.8	9.08	8.44	63.9	58.1	297	238
+E-64	3.8	8.02 (12)[b]	7.16 (15)	63.0	62.4	259 (13)	201 (16)
+Pepstatin A	3.8	8.67 (5)	6.91 (18)	63.4	61.2	310 (−4)	211 (11)
+PMSF	3.8	9.29 (−2)[c]	7.95 (6)	63.7	59.0	272 (8)	223 (6)
+*o*-Phen	3.8	9.48 (−4)	7.69 (9)	65.3	59.0	288 (3)	234 (2)
+EDTA	3.8	8.83 (3)	9.10 (−8)	63.8	59.5	301 (−1)	252 (−6)
Control	6.0	6.21	5.01	78.1	80.9	246	199
+E-64	6.0	5.49 (12)	4.48 (11)	77.6	79.0	215 (13)	185 (7)
+Pepstatin A	6.0	5.87 (6)	4.70 (6)	78.3	79.1	232 (6)	199 (0)
+PMSF	6.0	6.23 (0)	4.86 (3)	78.4	79.5	208 (15)	206 (−4)
+*o*-Phen	6.0	5.40 (13)	4.21 (16)	78.3	81.2	210 (15)	177 (11)
+EDTA	6.0	6.61 (−7)	5.30 (−6)	80.9	82.2	254 (−3)	222 (−12)
Control	8.0	5.21	4.31	74.7	78.6	158	138
+E-64	8.0	5.23 (0)	4.26 (1)	75.0	78.6	166 (−5)	138 (0)
+Pepstatin A	8.0	4.97 (4)	4.27 (1)	74.9	77.5	171 (−8)	153 (−11)
+PMSF	8.0	5.37 (−3)	4.67 (−8)	75.1	78.6	176 (−11)	140 (−1)
+*o*-Phen	8.0	4.87 (7)	4.13 (4)	74.5	78.1	138 (13)	141 (−2)
+EDTA	8.0	5.19 (0)	4.71 (−9)	73.8	77.6	156 (1)	135 (2)

[a] All values are the averages of three experiments.
[b] Percentage of inhibition.
[c] Negtive values indicate activation, not inhibiiton.

inhibitor) caused an even greater inhibition than E-64. Adding PMSF (serine protease inhibitor) had little or no effect on the protein solubilization and the presence of EDTA (an alternative inhibitor of metalloproteinases) actually caused an increase in the SP level. In this particular experiment, it appeared that PMSF might have caused a slight inhibition in the Harrington mash, but subsequent experiments (Tables 3 and 5) indicated that it normally did not. As expected, the addition of E-64, PMSF and *o*-phen reduced the mash proteolytic activities. Pepstatin A did not affect the measured activity, and in the presence of EDTA, the proteolytic activity was strongly increased. The wort FAN levels were reduced by the presence of each of the inhibitors except EDTA.

3.2. The effect of the mash pH on inhibition

3.2.1. Soluble protein

When mashes were conducted at pH 3.8, the SP levels of the resulting worts were very high (Table 3), reflecting the very high overall proteolytic activity. As expected from previous studies, however, the addition of either E-64 or pepstatin A to the mashes lowered their SP levels. The effects of the other inhibitors were either nonexistent (Morex) or small (Harrington). As in the experiment reported in Table 2, the addition of E-64 and *o*-phen to the pH 6.0 reactions caused strong inhibition of protein solubilization, pepstatin A inhibited less strongly, PMSF had little or no effect and EDTA apparently caused an activation of protein solubilization. In the pH 8.0 reactions, only the *o*-phen addition caused significant inhibition.

3.2.2. Extract

The addition of the various inhibitors to mashes made at the different pH values did not affect their extract values (Table 3). However, the pH of the mashes strongly affected their extract percentages. Mashes at pH 6.0 yielded the highest extract values, there was slightly less extract in the pH 8.0 mashes, and the pH 3.8 values were very low. Both Morex and Harrington malts were affected in the same way, even though the Harrington pH 3.8 extract values were lower than those of Morex and its pH 6.0 and 8.0 values were greater.

3.2.3. FAN

FAN values. The Morex FAN levels were all greater than those of their Harrington counterparts (Table 3) and the amount of FAN formed diminished in the order pH 3.8 > 6.0 > 8.0. Although the FAN values varied somewhat, due to the inherent variation associated with their measurement, at pH 3.8 and 6.0, the inhibitors did affect the FAN levels.

3.3. The effects of varying the malt concentrations of mashes

The conditions used during commercial mashing operations differ from those used for the ASBC experimental mashes. To test how these differences might affect their various proteinase activities, ASBC, ‘commercial’

Table 4
Effect of inhibitors on soluble protein release, malt extract and (1→3,1→4)-β-glucans in ASBC, High gravity ASBC and commercial mashes

Treatment	Soluble protein, %			Extract, %			(1→3,1→4)-β-glucan, ppm	
	ASBC	HGASBC[a]	Commercial	ASBC	HG	Commercial	HG	Commercial
Morex malts								
Control	6.21	8.83	6.71	78.1	83.7	81.2	103	55
+E-64	5.49 (12)[b]	7.66 (13)	5.92 (12)	77.6	83.1	80.5	101	57
+Pepstatin A	5.87 (5)	7.86 (11)	6.45 (4)	78.3	83.3	80.5	102	64
+PMSF	6.23 (0)	8.54 (3)	6.67 (1)	78.4	83.8	81.0	97	63
+*o*-Phen	5.40 (13)	8.23 (7)	6.20 (8)	78.3	83.5	80.5	99	65
+EDTA	6.61 (−6)	8.91 (−1)	6.81 (−1)	80.9	86.2	83.2	91	60
Harrington malts								
Control	5.01	6.92	5.96	80.9	80.8	81.7	172	–[c]
+E-64	4.48 (11)	6.42 (7)	4.83 (19)	79.0	80.6	80.7	179	–
+Pepstatin A	4.70 (6)	6.27 (9)	5.25 (12)	79.1	80.4	80.4	180	–
+PMSF	4.86 (3)	6.81 (2)	5.46 (8)	79.5	80.8	81.1	178	–
+*o*-Phen	4.21 (16)	6.13 (11)	4.99 (16)	81.2	80.6	80.9	181	–
+EDTA	5.30 (−6)	7.40 (−7)	6.20 (−4)	82.2	82.9	83.6	163	–

[a] High gravity ASBC mash.
[b] Percentage of inhibition.
[c] Not determined.

and intermediate 'high gravity ASBC or HGASBC' mashings were made (Table 4).

3.3.1. ASBC congress mashes

These mashes were identical to those reported in Tables 2 and 3, and, not surprisingly, the results were the same.

3.3.2. HGASBC mashes

When the malt concentrations of the mashes were increased by 2.4-fold, the SP levels of both the Morex and Harrington mashes were strongly increased, but they were lowered in the presence of the various inhibitors by about the same percentages as in the regular ASBC mashes. The extract values of the Morex high gravity worts were increased by about 5% points over those of their standard gravity counterparts, whereas those of the Harrington wort remained constant. As with the ASBC mashes, neither the extract values nor (1→3,1→4)-β-glucan concentrations of the HG worts were affected by the addition of inhibitors.

3.3.3. Commercial mashes

When the malts were mashed under commercial conditions, the wort SP levels were also sensitive to inhibitors. The SP concentrations were marginally higher than those of their ASBC counterparts but were considerably lower than those of the HGASBC extracts (Table 4). The extract levels of the malts of the two cultivars were identical, were very acceptable for commercial malting barleys, and were unaffected by the addition of proteinase inhibitors. Those of the Morex samples were nearly intermediate between those of the normal and HGASBC mash samples, while the Harrington values were nearly identical under all thee mashing regimes. There was no

Table 5
The effect on wort compositions of adding a mixture of endoproteinase inhibitors to malt and barley mashes at different pH levels[a]

Treatment	pH[b]	Soluble protein, %	Extract, %	FAN, ppm
Malt extract				
Morex				
Control	3.8	9.14	61.5	289
+ inhibitors[c]	3.8	6.66 (27)[d]	58.5 (5)	220 (24)
control	6.0	6.60	78.0	230
+ inhibitors	6.0	5.03 (24)	76.6 (2)	173 (25)
control	8.0	6.54	74.3	181
+ inhibitors	8.0	6.19 (5)	73.1 (2)	182 (−1)
Harrington				
Control	3.8	7.30	57.8	224
+ inhibitors	3.8	5.50 (25)	58.6 (−1)	177 (21)
Control	6.0	5.14	81.5	217
+ inhibitors	6.0	4.13 (20)	80.2 (2)	156 (28)
Control	8.0	5.55	79.4	146
+ inhibitors	8.0	4.78 (14)	78.7 (1)	149 (−2)
Barley extract				
Morex				
Control	3.8	2.07	–[e]	46
+ inhibitors	3.8	1.59 (23)	–	35 (24)
Control	6.0	2.43	–	37
+ inhibitors	6.0	2.32 (5)	–	39 (−5)
Control	8.0	3.32	–	25
+ inhibitors	8.0	2.27 (32)	–	29 (−6)
Harrington				
Control	3.8	1.88	–	45
+ inhibitors	3.8	1.56 (17)	–	35 (23)
Control	6.0	1.80	–	37
+ inhibitors	6.0	1.48 (18)	–	29 (22)
Control	8.0	3.55	–	31
+ inhibitors	8.0	2.28 (36)	–	40 (−29)

[a] All values are the averages of three experiments.
[b] pH at which extracts were carried out.
[c] A mixture of E-64, pepstatin A and *o*-phen.
[d] Percentage of inhibition.
[e] Not measured.

effect of the proteinase inhibitors on the (1→3,1→4)-β-glucan values of the Morex samples.

3.4. The effect of an inhibitor mixture on worts from 'mashed barley' and malt

By adding a mixture of E-64, pepstatin A and *o*-phen to mashes, it should be possible to inhibit all of the SP release that occurred during the mashing process. In addition, by 'mashing' ungerminated barley in the presence of the inhibitor mixture, the amount of SP that was present in the ungerminated barley prior to malting can be measured. By combining these results with that from a normal uninhibited malt mashing, it can then be calculated how much of the final SP of a wort came from the barley and how much was solubilized during the malting and mashing processes. The results from such experiments are reported in Table 5.

3.4.1. Malt mashings

The SP levels of the malt samples mashed at all thee pH levels were lowered in the presence of the inhibitor mix. The percent inhibition was greatest at pH 3.8, less at pH 6.0 and smallest at pH 8.0, with a maximum of 25–27% inhibition occurring. The extract percentage measurements of the pH 3.8 Morex mashes indicated a slight inhibition may have occurred when the inhibitors were present, but all of the other data indicated that the extracts were unaffected. The FAN levels of the pH 3.8 and 6.0 extracts of both Morex and Harrington were strongly lowered by the inhibitors, but they did not affect the FAN of the pH 8.0 mashes.

3.4.2. Barley mashes

When unmalted barley was mashed in the presence of the inhibitor mixture, the SP concentrations of the resulting extracts were lowered. Because the control SP levels were all quite low, the small SP changes due to the inhibitors showed up as percentage changes that were relatively large, but of questionable significance. The extract levels of the barley samples were not measured in this experiment, and the small FAN level changes that occurred in the inhibited samples were too small to be significant.

3.5. When are the SP protein components worts solubilized?

3.5.1. Protein solubilization

From data obtained by measuring the SP and FAN levels of worts from malt samples mashed in the presence and absence of proteinase inhibitors and those of barley extracted with the inhibitor mixture, it was possible to calculate how much SP was present in barley and at the ends of the malting and mashing steps, and thus when the solubilization occurred (see Section 4). The information obtained from the experiments reported in Table 5 were used to make these calculations for the SP and FAN contents and the results are recorded in Table 6.

The Morex and Harrington results were quite similar and showed that most of the SP and FAN were solubilized during the malting process at each of the three pH levels investigated. At pH 3.8, more SP was released during mashing than was present in the unmalted barley, whereas at the other two pH values, the contribution from the barley was greater than that from the mashing step. At pH 8.0, the contribution of mashing to the final wort SP level was very small. The contribution of the unmalted barley to the final wort FAN concentration was low at all pH values, the majority of the FAN being released during malting. The mashing step contributed an intermediate amount of FAN to the wort at pH 3.8 and 6.0, but even less FAN than SP was

Table 6
The soluble protein and FAN levels of Morex and Harrington barleys, malts and worts[a]

	Measured soluble protein, % (% of wort protein solubilized, this step)			Measured FAN, ppm (% of FAN released, this step)		
	Barley[b]	Malt[c]	Wort[d]	Barley	Malt	Wort
Morex						
pH 3.8[e]	1.59 (17)[f]	6.66 (56)	9.14 (27)	35 (12)	220 (64)	289 (24)
pH 6.0	2.32 (35)	5.03 (41)	6.60 (24)	39 (17)	173 (58)	230 (25)
pH 8.0	2.27 (35)	6.19 (60)	6.54 (5)	29 (16)	182 (84)	181 (−1)
Harrington						
pH 3.8	1.56 (21)	5.50 (54)	7.30 (25)	35 (16)	177 (63)	224 (21)
pH 6.0	1.48 (29)	4.13 (52)	5.14 (20)	29 (13)	156 (59)	217 (28)
pH 8.0	2.28 (41)	4.78 (45)	5.55 (14)	40 (27)	149 (73)	146 (−2)
Average[g]						
pH 3.8	(19%)	(55%)	(26%)	(14%)	(64%)	(22%)
pH 6.0	(32%)	(46%)	(22%)	(15%)	(58%)	(26%)
pH 8.0	(38%)	(52%)	(10%)	(20%)	(78%)	(0%)

[a] Numbers are averages of experiments carried out in triplicate.
[b] Ungerminated barley was mashed in the presence of inhibitor mixture.
[c] Malt mashed in the presence of inhibitor mixture.
[d] Malt mashed in the absence of inhibitors.
[e] pH at which mashings were carried out.
[f] The percentages of the wort soluble protein that was solubilized during this step.
[g] Averages of the percentages of the Morex and Harrington wort soluble protein that was solubilized in the various steps.

solubilized during mashing at pH 8.0. The significances of these data are discussed below.

4. Discussion

4.1. Endoproteinase classes involved in producing wort SP and FAN

It has been generally accepted that only the malt cysteine endoproteinases played any significant role in producing the SP fraction (dissolved protein, peptides and amino acids) of worts during malting and mashing. It was important to know whether this was correct, to guide researchers and brewers more effectively in the development of malting barley cultivars and/or malting and brewing methods that will result in worts with improved SP levels. Because the FAN content of a wort is also critical to its acceptability for brewing and is controlled by the activities of proteinases, its formation was also studied. The FAN values reflect the levels of low molecular mass N-containing molecules (mainly amino acids and small peptides) in the worts, whereas the SP measurements give a better indication of the total amount of peptide-containing material (chiefly proteins) that is present. The ASBC congress mash method differs from commercial mashing techniques, but is the standard method used for measuring the quality of North American malts and provided a baseline with which to compare the results obtained with high gravity ASBC and commercial mashes. Samples were mashed at pH 3.8 (the pH at which the total proteolytic activities of mashes are highest, and where the cysteine and aspartic proteinase activities strongly predominate (Zhang and Jones, 1995a), at pH 6.0, the normal commercial US mashing pH, and at pH 8.0, where the cysteine and aspartic proteinases are inactive and the serine and metalloproteinase activities predominate (Zhang and Jones, 1995a).

The results in Table 2 show that under normal pH conditions, the cysteine proteinases are not the only ones that produced SP during mashing. Pepstatin A and, to an even greater extent, *o*-phen also inhibited the SP formation, indicating that aspartic proteinases and, even more extensively, the metalloproteinases, probably also play major roles in protein solubilization. The addition of PMSF had little or no effect on the protein solubilization and adding EDTA actually caused an increase in the SP level. It was expected that the two metalloproteinase inhibitors, *o*-phen and EDTA, would affect the protein solubilization similarly but this was not the case. Either the EDTA and *o*-phen inhibited different sets of metalloproteinases or they affected the same set of enzymes in different ways. Alternatively, EDTA could have altered some other aspect of the mashing biochemistry that was reflected as an increase in SP. Throughout this study, the behaviour of EDTA was not consistent, usually increasing the protein solubilization, and it seems probable that the *o*-phen results more realistically reflect what actually occurs during mashing. These results showed that the cysteine-, aspartic- and metalloproteinases all contributed to the solubilization of malt proteins during mashing.

The additions of E-64, PMSF and *o*-phen to mashes all reduced their mash proteolytic activities. A comparison of the proteolytic activities and the final wort SP levels shows that, generally, when the proteolytic activities were lowered the SP levels also fell. In the presence of pepstatin A, however, the SP level was significantly lowered, but the measured proteolytic activity was unchanged. This was because, as shown previously (Wrobel and Jones, 1992), the aspartic (pepstatin A-inhibited) proteases do not hydrolyse the gelatin substrate that was used to measure the protease activities, so any inhibition of the activities of these particular enzymes would not have been detected. The aspartic proteinase activity inhibitions could, presumably, have been measured using the substrate edestin (Wrobel and Jones, 1992), but no 'in solution' edestin assay has yet been developed. The addition of PMSF caused a strong lowering of the proteolytic activity, but had essentially no effect on protein solubilization, indicating that even though there was considerable serine protease activity in the mashes, it apparently did not hydrolyse any of the malt proteins into SP. This fits well with the results of studies on two purified malt serine endoproteinases, SEP-1 (Fontanini and Jones, 2002) and hordolisin (Terp et al., 2000). The SEP-1 readily hydrolysed the substrate protein gelatin, but did not degrade any of several isolated barley storage proteins. Hordolisin was also unable to hydrolyse the components of a hordein storage protein preparation (Terp et al., 2000). In addition, the SEP-1 enzyme was present in all malted barley tissues except the endosperm, which is where the grain storage proteins are concentrated. These findings all indicate that the function(s) of the malt serine endoproteinases is to hydrolyse proteins other than the storage proteins, whose hydrolysis contributes most of the protein that ends up in the SP. Generally, the proteinase activities were inhibited much more strongly than the SP levels. This is because 100% of the activity in each proteinase class could theoretically be inhibited but, as discussed later, only about 22% of the total wort soluble protein was solubilized during mashing, and would thus be susceptible to inhibition in this experiment.

The FAN values of the wort generally varied in concert with the SP levels and the proteinase activities. The FAN and SP values did not change proportionately, probably because the wort SPs are released from insoluble storage proteins by endoproteinases while the FAN is produced by exoproteinases that operate predominantly on the SP fraction. It has been proposed that the FAN production is due mainly to the serine-class carboxypeptidases, which should have been inhibited by PMSF. In contrast to its inhibiting of SP formation, PMSF caused the expected inhibition of FAN formation. The FAN-producing exopeptidases do, however, use the SP fraction as substrate, so any inhibitors that lowered

the SP amounts would presumably also lower the amount of FAN produced, as happened in this experiment.

The SP, proteinase activities, and FAN levels of the Harrington malt were all lower than the corresponding Morex values, which is normal for these two cultivars. Although the SP inhibition values of Harrington differed from those of Morex, the order of inhibition was the same. Apparently the inhibition of SP release by PMSF in this experiment was abnormally high, because the values obtained in later experiments (see Tables 3 and 4) indicated that it normally had no, or very little, inhibitory effect.

4.2. Effect of the mash pH on inhibition

4.2.1. Protein solubilization

All four malt endoproteinase classes operate at pH 6.0 (Zhang and Jones, 1995a) However, the proteolytic activity of mashes is greatest at pH 3.8, and for that reason many of the mashing proteolysis studies by other researchers were performed at that pH. The main malt proteases that are active at this low pH belong to the cysteine and aspartic classes (Zhang and Jones, 1995a). Conversely, at higher pH levels, (pH 8.0, in this case) the serine- and metalloproteinases are very active and the aspartic- and cysteine endoproteinases are essentially inactive (Zhang and Jones, 1995a). However, these enzyme characteristics are reflected in the inhibition results listed in Table 3. The cysteine- and aspartic endoproteinases were most active (and therefore most strongly inhibited) at pH 3.8, the metalloproteinases at 8.0, and that the enzymes inhibited by PMSF (serine proteases) played no role in solubilizing malt storage proteins during mashing. Also, these results emphasize that the cysteine- and metalloproteinases probably play the biggest roles in solubilizing proteins during mashing with the aspartic class enzymes playing a lesser role.

4.2.2. Extract effects

The ‘extract’ value of an ASBC mash is a measure of the percentage of the malt that dissolved during mashing. The data in Table 3 show that the endoproteinase inhibitors did not affect the wort extract levels, even though the pH of the mashing did. This variation with pH was obviously not due to the variation in the amount of protein in the extracts, because the pH 3.8 SP levels were generally half again as large as those at either pH 6.0 or 8.0. The pH 6.0 extract values are normal or slightly low for these two cultivars, indicating that these malts were of average quality.

4.2.3. FAN values

The pH 3.8 and 6.0 FAN values were only weakly affected by the presence of inhibitors, and they had no effect at all on the pH 8.0 mashes. However, they were strongly affected by the mashing pH and cultivar (Table 3). All of these FAN values would have been sufficient for producing a good brewing wort, although those of the pH 8.0 Harrington extracts were marginal.

4.3. Effects of varying the malt concentrations in mashes on proteinase inhibitions

The experiments reported in Tables 2 and 3 were all performed using the standard ASBC congress mashing procedure (American Society of Brewing Chemists, 1992). This is the standard method used in North America to compare the malting qualities of various cultivars or malt samples. However, it is significantly different from the mashing techniques used in breweries, especially in that it uses a lower concentration of ground malt in the mash than is common in commercial mashes. ASBC mashes are performed with 25 g of malt per 100 ml of mash while commercial mashes more commonly would be made up with about 65 g of malt per 100 ml of mash. This malt concentration difference means that there will be different endoprotease concentrations in the experimental and commercial worts, and it seemed possible that this disparity might result in differences in their activities or hydrolytic characteristics. Commercial mashes also contain metal ions that are not added to ASBC mashes and are performed under somewhat different temperature conditions. To test for wort differences that might occur due to these variations, a series of experiments were conducted in which the proteinase inhibitors were added to mashes that were made up to simulate the ASBC congress mashes, an ASBC congress mash that contained the same concentration of malt as commercial mashes (high gravity, or HGASBC mash), and a mash that simulated, as closely as possible, a commercial mash. The commercial mashing system used was the malt mashing portion of a double mash procedure that is similar to those used by brewers in the USA (Jones and Marinac, 2002; Rehberger and Luther, 1995) and has been used in our laboratory for many years to test the brewing quality of newly developed barley lines. It has been shown previously that when this mash system is used the individual endoproteinase activities are stable thoughout the protein rest period, but are rapidly inactivated as soon as the conversion step begins (Jones and Marinac, 2002).

The results of these tests, made with Morex and Harrington malts and carried out at pH 6.0, are shown in Table 4.

4.3.1. ASBC congress mashes

As in the experiments reported in Tables 2 and 3, E-64 and *o*-phen strongly inhibited protein solubilization, pepstatin A caused a smaller inhibition and PMSF did not inhibit. The Harrington extract percentages were again slightly higher than those of the Morex mash, and the inhibitors did not diminish the extract values.

4.3.2. High gravity ASBC mashes

These mashes contained 2.4 times as much malt as the normal ASBC congress mashes, but were otherwise the same. Under these conditions, both the Morex and Harrington SP levels were strongly increased, but only by

about 40%, not by 2.4-fold (Table 4). The inhibition effects were similar; E-64, pepstatin A and *o*-phen all inhibited the release of soluble protein, although under these conditions the inhibition by pepstatin A was nearly the same as with the other effective inhibitors, while it was lower under the normal ASBC conditions. Once again, the addition of EDTA activated the overall proteolytic activity, rather than inactivating it. A portion of the HGASBC Morex extract percentage increase may have been due to the large (3.5%) increase in the SP component of the extract value. If so, the almost 2% increase in the Harrington SP value did not have the same effect on its extract values. The Harrington (1→3,1→4)-β-glucan concentrations were almost twice those of the Morex sample, indicating that it had not modified quite as well during malting, but the concentrations in both sets of samples would have been acceptable for brewing purposes.

4.3.3. Commercial mashes

The effects of adding the various inhibitors were as seen previously, with the inhibitions being E-64 ≈ *o*-phen > pepstatinA > PMSF ≈ 0, EDTA activating. The extract levels of the malts of the two cultivars were identical, were very acceptable, and were unaffected by the addition of proteinase inhibitors. Those of the Morex samples were intermediate between those of the normal and HGASBC mash samples, while the Harrington values were nearly identical under all thee mashing regimes. Once again, there was no effect of the proteinase inhibitors on the (1→3,1→4)-β-glucan contents of the Morex samples. Under the commercial mashing conditions the (1→3,1→4)-β-glucan levels were reduced to only about half what they were in the HG mashes, but both the HG and commercial wort (1→3,1→4)-β-glucan levels would have been commercially acceptable.

4.4. The effect of proteinase inhibitor mixes on wort parameters

It was of interest to determine exactly how much of the SP of worts arose during the mashing step, how much was solubilized during the malting process, and how much was already present in unmalted barley grains. From the foregoing studies, it was clear that it would be possible to fully inhibit the protein solubilization that occurs during mashing by conducting the mashing in the presence of a mixture of E-64, *o*-phen and pepstatin A. Because PMSF did not affect the protein solubilization, it was not needed. The difference between the amounts of SP in worts prepared in the presence and absence of the inhibitor mixture should then give a measure of the amount of protein that was solubilized during the mashing process. In the same way, by 'mashing' ungerminated barley grain in the presence of the proteinase inhibitor mix, it would be possible to determine the 'SP' content of the unmalted barley grain without having this value being artificially enhanced by proteins that were solubilized by proteinases during the extraction process. It seemed unlikely that the addition of inhibitors to the mashing of unmalted barley grain would have much effect on the final SP level, because the majority of the endoproteinases of malt do not occur in barley, but are synthesized during the malting process (Zhang and Jones, 1995b).

4.4.1. Malt mashes

Morex and Harrington malt preparations were mashed using the ASBC congress method, in the presence and absence of a more concentrated proteinase inhibitor mixture to ensure that all of the endoproteinase activity would be completely inhibited. Preliminary experiments had shown that even at these higher concentrations the inhibitors did not have any apparent effect on the malt extract values of worts, and thus did not seem to affect any of the aspects of modification except the protein solubilization that occurred during mashing.

While the addition of inhibitors lowered the SP levels at all pH values (Table 5), the effect was greatest at pH 3.8 and smallest at pH 8.0, indicating that the cysteine- and aspartic proteinases together contributed the majority of the activity that was present but that the metalloproteinase also played an important part, as was shown above. Even at these increased inhibitor levels, the extract levels were unaffected. The inhibitor-induced lowering of the FAN levels of the pH 3.8 and 6.0 extracts, but not the pH 8.0 ones indicated that the FAN release was probably not catalyzed by the metalloproteinases.

4.4.2. Barley mashes

When unmalted barley was 'mashed' in the presence of the inhibitor mixture, the extract SP concentrations were lowered. Because the uninhibited SP levels were all quite low, even small changes in the SP levels showed up as relatively large percentage changes, so that undue importance should not be placed on the small differences that were seen. Still, it appears from these data that in ungerminated barley the major inhibition occurred in the pH 8.0 mashes, indicating that the metalloproteinases are probably the predominant protein-solubilizing enzymes in barley grains. The extract levels of the barley samples were not measured in this experiment, and all of the FAN levels were very low, indicating that there are very few soluble amino acids and small peptides in the barley prior to malting.

4.5. When are the soluble proteins in worts formed?

4.5.1. Protein solubilization

For researchers or brewers wishing to know how to improve the SP or FAN contents of worts, it is important that they know at what stage in the process between raw barley and wort the insoluble proteins are solubilized and hydrolysed into peptides and amino acids. The question of

how much of the protein solubilization occurred during the mashing step has been addressed previously by several groups and the answers obtained have varied from none (Lewis et al., 1992) to between 30% (Burger and Schoeder, 1976a,b) and 47% (Barrett and Kirsop, 1971; Clapperton, 1971). By adding proteinase inhibitors to mashes we have been able to study this question by stopping the process at each step. This method was expected to give a better indication of the true SP and FAN situations, because the methods previously used to inhibit the protein solubilization during mashing all involved very vigorous treatments that probably disrupted many of the concomitant biochemical steps involved whereas, as reported here, the addition of inhibitors caused a more directed inhibition that apparently disrupted only the proteinase activities.

The data in Table 6 indicate that, on average, the solubilities of the protein initially present in the unmalted barley were in the order pH 3.8 < 6.0 < 8.0, although the numerical differences were not great. Approximately half of the wort protein was solubilized during the malting process at each pH. Presumably, the protein solubilization during malting was similar at all thee pH values because the actual solubilization would have occurred inside the grain, at the grain pH, which was not affected by the pH of the extraction process.

On the other hand, mashing the malt at pH 8.0 with inhibitors present solubilized little protein, compared to mashing at pH 6.0 or 3.8. This presumably reflects the fact that at pH 8.0 only the metalloproteinases are active, whereas at pH 3.8 both the cysteine- and aspartic proteinases are functioning rapidly, and at pH 6.0, all three proteinase classes are operating, although more slowly than at pH 3.8.

From the averages of the Morex and Harrington values, it appears that at normal mashing pH values (6.0), about a third of the final wort SP was present in the original barley sample prior to malting, approximately half was solubilized during malting and the remaining 22% was released during the mashing. Because different sets of proteinases (with, presumably, different hydrolytic specificities) were active during the mashings at the different pH values, as indicated by their solubilization of varying amounts of protein, the protein + peptide + amino acid compositions of the pH 3.8 and 8.0 worts are almost certainly different, with the pH 6.0 mash probably having an intermediate composition. The differential solubilization of the unmalted barley proteins at the different pH values would also have contributed to the differential protein complements of the different pH worts. This effect might be minimized in malt mashings because the solubilities of the grain proteins probably become more similar as they are partially hydrolysed during the malting process.

4.5.2. Naturally occurring barley/malt proteinase inhibitors

When unmalted barley is used as a brewing adjunct, it will contribute little SP to the wort, compared to an equal weight of malt, and almost no FAN (Table 6). In addition, the added barley would further lower the SP level of the final wort because it contains endogenous endoproteinase inhibitors (Enari et al., 1964; Jones and Marinac, 1991; Mikola and Enari, 1970). These proteinase inhibitors also occur in both barleys and malts (Jones and Marinac, 1995) thus the results shown in Table 6 have been affected by their presence. A significant percentage of the cysteine class proteinase activities are inhibited by these compounds whenever malt is dissolved (Jones, 2001), and this interaction may also occur during malting, depending on whether the inhibitors and proteinases occur at the same locations within the grain. It has been impossible so far to free the active cysteine proteinases from these inhibitors (Jones, 2001), and it has been shown previously that these endogenous inhibitors do strongly inhibit the formation of SP in mashes (Jones and Marinac, 2000 and many unpublished experiments). It must be presumed that if these inhibitors were not present, the amount of protein solubilization that occurred during mashing, as indicated in Table 6, would have been considerably increased.

Although the most potent of these endogenous proteinase inhibitors in barley are the ones that affect the cysteine endoproteinases, it has been demonstrated that proteins from dormant and germinated buckwheat seeds that inhibit a buckwheat seed metalloproteinase (Elpinada et al., 1991). Similar metalloproteinase inhibitors may also occur in malt and/or barley. This metalloproteinase enzyme-inhibitor pair may not play a major role in barley, however, since the substrate for the buckwheat enzyme is a globulin storage protein, whereas the barley storage proteins are hordeins and hordenins, which are chemically very different. Inhibitors of one of the germinated barley serine endoproteinases have also been demonstrated (Jones and Fontanini, 2003), but since the serine endoproteinases play little or no role in forming SP, these inhibitors presumably would have little or no affect on the wort SP levels. They could, however, act to lower the FAN contents of worts.

4.5.3. FAN formation

The pattern of formation of FAN during malting and mashing differed from the protein solubilization. This was not unexpected, since the release of the FAN amino acids and small peptides is primarily catalyzed by the exopeptidases, rather than the endoproteinases that predominately release the SP. However, it seemed likely that there would be some correlation between the SP and FAN levels, because the exopeptidases should operate faster as the amounts of their substrates, the SP molecules, increased, as long as the substrate molecules were not present at enzyme-saturating levels. This is what occurred; the majority of the FAN and SP solubilizations both occurred during malting and the FAN and SP of unmalted barley were both highest at pH 8.0, whereas the release of both SP and FAN during mashing was lowest at this high pH. There were some quite significant differences, however. The FAN percentage of

the unmalted barley was only about half that of its SP. It is apparent that the free amino acid and small peptide levels of barley grain are very small. The barley FAN levels increased, on average, by 4.1-fold during malting, whereas the SP level only increased by 1.9-fold. These findings agree with the report by Jones and Pierce (1963) that the contribution of a flaked ungerminated barley preparation to the total (soluble) nitrogen content of worts was considerable, but that its contribution to the FAN content was small. With both Morex and Harrington malts, no detectable FAN formation occurred during the mashing at pH 8.0. Apparently none of the malt exopeptidases were active at this high pH, and none of the malt endoproteinases that increased the wort SP by 10% during the pH 8.0 mashing was able to function in an exopeptidase mode.

It is interesting that in the presence of the inhibitor mixture, where the inhibitor concentrations were relatively high, only 22% of the wort SP was solubilized during mashing, at pH 6.0 (Table 6). Under the same conditions, but with the inhibitors present individually and at lower concentrations, the sum of the inhibition caused by these inhibitors was 35% (average, Tables 2–4). Preliminary experiments had shown that the inhibition that occurred in the presence of the individual inhibitors was the same at both the higher ('mixed inhibitor' reactions) and the lower (individual inhibitor reactions) concentrations. The only obvious explanation for this observation is that possibly the different inhibitors in the mixture inhibited some of the same enzymes. However, this is unlikely, because the inhibitors used are generally quite specific, for their target enzymes.

4.6. Overview

By mashing two- and six-rowed barley malts at varying pH values and in the presence of class-specific chemical inhibitors, the protease classes that are involved in the release of SP and FAN during malting and brewing have been determined. At pH 6.0 the cysteine and metalloproteinases were the predominant enzymes involved in releasing soluble protein, the aspartic proteinases played a significant part in the process and the serine class enzymes played no role (Tables 2–4). The release of SP during mashing correlated well with the change in the endoproteolytic activities of the mashes (Table 2 and other results not shown). The serine proteinases were an exception, indicating that although these enzymes were present and active in the mashes, they did not solubilize any of the protein. Their function in the germinating grain remains unknown. All of the enzyme classes affected the formation of FAN (Tables 2 and 3). The main contributions of the non-serine endoproteinases to FAN release were probably due to their increasing of the SP levels, and thus increasing the substrate levels for FAN formation.

Mashing at varying pH values gave expected results. At low pH values, where the cysteine and aspartic activities prevail (Zhang and Jones, 1995a), the release of SP was strongly inhibited by E-64 and pepstatin A, which had no effect at pH 8.0. At pH 8.0, where the metalloproteinases are most active (Zhang and Jones, 1995a), *o*-phen inhibited strongly. The various proteinase inhibitors caused no detectable changes in the extract or (1→3,1→4)-β-glucan levels of worts (Tables 3–5). The only effect noticed when mashes were made with different malt concentrations was that the aspartic proteinases contributed more to the SP formation in the more concentrated mashes (Table 4). Inhibitor studies showed that the metalloproteinases catalyzed most of the SP formation that occurred during barley extraction at pH 3.8, but not at pH 8.0. Little FAN was present in ungerminated barley.

About a third of the final wort SP is already present as soluble protein in the ungerminated barley, approximately half is released during the malting process and the remaining 22% is solubilized during the mashing phase of brewing (Table 6). This indicates that it should be possible to vary the final wort SP levels greatly, either by genetically altering barleys or changing the malting and mashing processing methods. It is obvious that the processes whereby the various barley proteins are solubilized and converted into SP and FAN is not nearly as simple as it was believed to be ten years ago (Enari, 1995). If industry deems it important to know how to scientifically alter the amounts and compositions of the soluble protein complements of worts, the degradation process needs to be better understood and defining this process is still going to take considerable work and ingenuity.

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