Characterisation and Assessment of the Role of Barley Malt Endoproteases During Malting and Mashing[1]

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ABSTRACT

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Barley malt endoproteases (EC.3.4.21) develop as multiple isoforms mainly during grain germination and pass through kilning almost intact. Thermostability, under simulated mashing conditions, varied from low to high depending on the substrate used in the assay. This suggests that individual enzymes respond differently to heat exposure and to protein substrates. The optimal pH with haemoglobin was pH 3.5, with hordein pH 4 and with glutelin pH 5. The optimal temperature with hordein was 40°C, with glutelin 50°C and with haemoglobin 60°C. These differences suggest that it is not possible to comprehensively characterise all malt endoproteases under one set of assay conditions. In brewing, most of the barley protein degradation (> 70 %) occurs during malting. But some proteinases remain active during mashing and contribute to wort soluble proteins and free amino nitrogen. Their contribution to all malt EBC mash total free amino nitrogen was 25 % in Schooner (Australian) and 30 % in Morex (USA). The importance of proteolytic activity during mashing and the possibility that the levels may not be adequate, at high solid adjunct ratios, are acknowledged.

Key words: Barley, endoprotease, malt, mash, thermostability.

INTRODUCTION

Malt is modified grain, produced by controlled germination and drying. Germination provides the necessary hydrolytic enzymes to modify the grain. Endoproteases are essential for grain modification. They degrade the storage proteins especially hordein and glutelin. In addition, endoproteases process and activate functionally important proteins[3,6,14] such as β-amylase. Adequate modification allows the maximum extraction of useful components during mashing. The two major components of the grain, stored in the endosperm, are starch and proteins. The latter is mainly hordein and glutelin and constitutes about 8-15 % (w/w) of the grain[13].

The degradation of endosperm-stored proteins facilitates water and enzyme movements, enhances modification, liberates starch granules and increases soluble amino nitrogen[8,10]. Morgan *et al*[8] reported that endosperm endoprotease activity was the parameter most highly correlated with malt extract.

Two aspartic endoproteases have been purified from dormant barley grain[12], and are believed to be involved in 'house-keeping' activities, and not in hordein breakdown[2]. Hordein-degrading endoproteases appear during germination[9,18] and progressively increase in number and activity by 20-fold of that over the quiescent grain [2,19]. More than 90% of the green malt endoprotease activity survives kilning[9], indicating that these enzymes would be active during mashing. However, there have been conflicting reports on the increase of free amino nitrogen in wort compared with malt: from a 100 % to no increase[1,5,16]. Proteolytic activity during mashing assumes greater importance when cereal adjuncts (which contain less soluble nitrogen) are used. Endoprotease action assures the availability, in wort, of diverse protein breakdown products for yeast nutrition, beer flavour, colour and foam.

The objective of this work was to characterise malt endoproteases and to assess their contribution to malt and beer quality. We report our results on the development of malt endoproteases during germination, their thermostability, kinetic and chemical properties and an evaluation of their contribution during malting and mashing.

MATERIALS AND METHODS

Malt

Barley samples were micro-malted in a Phoenix Micromalter using standard micromalting procedures[15]. Samples of grain (100 g) from the varieties Alexis, Franklin, Harrington, Morex, Schooner and Tallon were micromalted according to the following regime at 17°C: 6: 8: 6 h (steep: air-rest: steep). The germination was for 96 h. Kilning was in five steps of gradient air temperature as follows: (i) 6 h with 35°C start and 55°C at end, (ii) 6 h

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with 55ºC start and 65ºC at end, (iii) 3 h with 65ºC and 70ºC at end, (iv) 3 h with 70ºC and 75ºC at end and (v) 3 h with 75ºC and 82ºC at end. Barley and malt samples for determination of endoprotease activity during steeping and germination were kindly donated by Barrett Burston Malting Co. Pty. Ltd., Melbourne.

Hordeins

Hordeins were extracted from barley flour with 55% (v/v) isopropanol containing 0.5% (w/v) dithiothreitol at 60ºC for 1 h with vortexing every 10 min. The extract was centrifuged at 2800 *g* for 20 min, and collected by decanting. Adding an equal volume of ice-cold 1 M NaCl precipitated hordeins or they were precipitated by extensive dialysis against distilled water. Salt precipitated hordeins were washed with cold water and resuspended in water with vortexing.

Glutelins

Glutelins were extracted from barley flour with 0.1 M Tris-HCl buffer pH 8.8, after sequential removal of soluble proteins and hordeins[17].

Endoproteases

Endoproteases were freshly prepared from fine (milled in Bühler Universal Laboratory Disc Mill Type DLFU set at a gap of 0.2 mm) ground malt, and extracted in 50 mM sodium acetate buffer pH 5.0 (1:3 g/litre) at room temperature for 1 h with vortexing every 10 min. The extract was collected by centrifugation at 2800 *g* for 20 min.

Enzyme activity assays

Test-tube method. Endoprotease activity was measured with azocasein, haemoglobin, hordeins and glutelins as substrates. The standard reaction mixture of 1.5 mL was made up of 1 mL 0.1 M sodium acetate buffer pH 4.0 for haemoglobin and hordeins, and pH 5.0 for azocasein and glutelins, 0.4 mL substrate solution, and 0.1 mL enzyme extract. Initially, the optimum pH for each substrate was determined. The concentrations of protein substrates were 1 % haemoglobin and hordein and 0.3 % glutelin. The reaction was started by the addition of the enzyme, carried out for 30 min at 40ºC and stopped by the addition of 1 mL cold 10 % (w/v) trichloroacetic acid (TCA). Precipitated proteins were removed by centrifugation (2800 *g* for 20 min). The absorbance, at 280 nm, of TCA soluble proteins was measured, except for azocasein, which was measured at 330 nm. For each measurement a control was included, which was treated similarly except that the enzyme was added after TCA addition. A unit of endoprotease activity is defined as that amount of enzyme that will release TCA-soluble protein material equivalent to one µmole of L-tyrosine per h, under the above-specified conditions[11].

Assay with N-carbobenzoxyglycine *p*-nitrophenyl ester. Protease activity with this synthetic substrate was assayed by following, in a recording spectrophotometer, the release of free *p*-nitrophenol at 317 nm. The standard assay mixture (3 mL) was composed of 2.8 mL 0.1 M acetate buffer (pH 5.5) containing 2 mM DTT and 2 mM EDTA, 0.1 mL 5 mM substrate stock solution in acetonitrile and 0.1 mL enzyme extract.

Detection of endoprotease activities by polyacrylamide gel electrophoresis (PAGE). Non-denaturing PAGE with copolymerised protein substrates was used to separate and detect endoproteases in Tris-HCl buffer (pH 8.5)[18]. The enzyme extracts from dry, steeped and germinating barley, and malt were prepared as described above. Aliquots of extracts were mixed with equal amounts of sample preparation buffer and were frozen until used. After electrophoresis, the gels were incubated overnight (16 – 18 h) at 40ºC in 0.1 M acetate buffer (pH 4.5) containing 2 mM L-cysteine, 1 mM dithiothreitol, 1 mM $CaCl_2$ and 1 mM $MgSO_4$. The gels were stained with Amido Black and destained with an acetic acid / methanol / water mixture (1:5:4). The results were documented by scanning the gels with a Hewlett Packard HP Deskscan II.

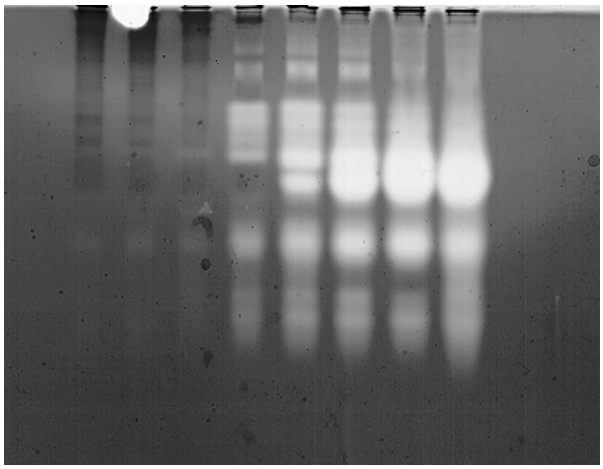

FIG. 1. Electropherogram of barley and malt endoproteases on native PAGE with incorporated gelatin substrate. Details as described under Materials and Methods. Lane 1- end of 1st immersion, 2 – end of air rest, 3 – end of steeping; d1 – day 1, d2 – day 2, d3 – day3, and d4 – day4 of germination; and m – malt. Endoprotease extracts (15 µl each) were loaded.

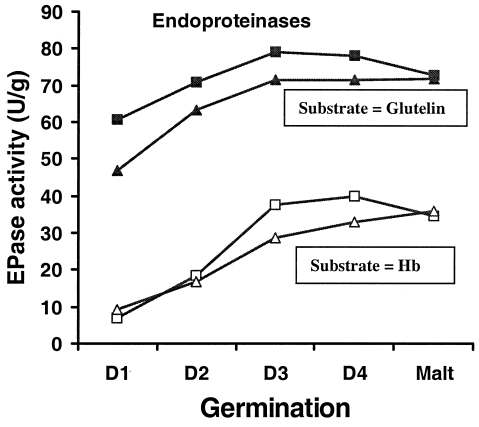

FIG. 2. Endoprotease activity in barley during germination and in malt measured in Harrington (□) and Schooner (△) varieties with haemoglobin as substrate and Harrington (■) and Schooner (▲) with glutelin as substrate. Details of enzyme extraction and assay were as described under Materials and Methods.

The finished gels were stored in a preserving solution (glycerol / methanol / water, 1:1:2).

Optimal temperature and thermostability at 65°C. The optimal reaction temperatures for malt endoproteases with haemoglobin, hordein and glutelin were determined. The assay method was the same as above except that the reaction temperature was varied and the incubation time was reduced to 10 min.

To determine thermostability of malt endoproteases at 65°C, 1 g malt samples (duplicate) were suspended in 3 mL 50 mM acetate buffer (pH 5.0) at 65°C, and incubated for different time intervals. Duplicate samples were used for each time interval at the end of which the samples were quickly removed and placed in ice water until all were through. Then the endoproteases were extracted and assayed as described earlier.

Protein assay

Soluble protein concentrations, in barley and malt extracts and wort, were measured using the Pierce BCA Protein Assay Kit, as described by the supplier (PIERCE, Rockford, USA). Samples were first filtered through Sephadex G25 to remove interfering material. The fractions of the first emerging peak absorbing at 280 nm were pooled and the protein concentration determined using bovine albumin (BSA) as the standard.

Free amino nitrogen in malt extracts and wort standard

Free amino nitrogen was determined in barley, malt extracts and wort with the ninhydrin method (8.10 Analytica-EBC, 1997). Extracts from malt at 0°C, from barley and malt at room temperature (20°C) and at 45°C from wort (Congress mashing), were prepared according to the small scale Congress mash procedure described below.

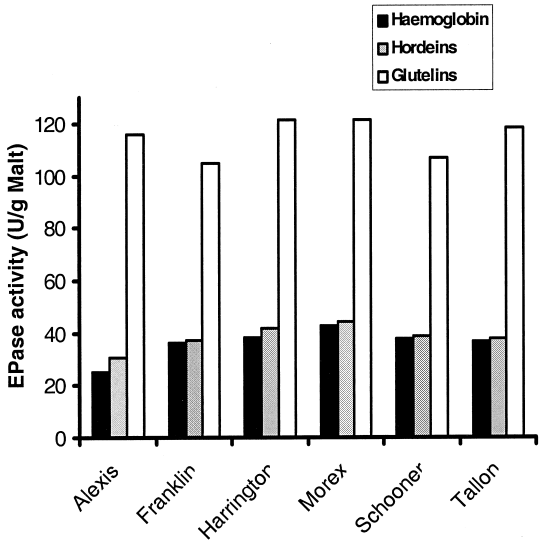

FIG. 3. Malt endoprotease activities determined with haemoglobin (filled columns), hordein (hatched columns) and glutelin (open columns) in malts of Australian and overseas barley varieties. Details as described under Materials and Methods.

Simulated EBC Congress mashing

A small scale mashing scaled down from the Congress mash (4.5.1 Analytica-EBC 1997) using 1 g malt flour instead of 50 g fine malt was adopted. Mashing was performed in test tubes in a water bath with parallel malt extraction at room temperature (RT) and on ice. All steps of the original method were followed except that magnetic stirring and filtration were replaced with vortexing at 10 min intervals and centrifugation (10 min at 2800 *g*). The rise in temperature from 45°C to 70°C was a gradual 1°C per min, and the mashing at 70°C was continued for 1 h. Soluble protein was assayed as described above, and diluted extracts and worts were used for the free amino nitrogen assay.

RESULTS

Endoprotease activity

The results of native PAGE separation of endoprotease activities in dry, steeped and germinating barley, and kilned malt are shown in Fig. 1. No activity was detected in dry or steeped barley under these conditions. Activities were detected on the first day of germination and increased as germination progressed. Both the number and intensity of the bands increased and the changes were more evident in those bands with intermediate or fast rates of migration.

The pattern of activity in kilned malt was the same as for days three and four of germination. This suggests that most endoproteases that appear during germination are heat stable and are not destroyed by kilning. Similar results were obtained when endoproteases were assayed with haemoglobin and glutelins (Fig. 2) in the same extracts used for electrophoresis (Fig. 1). The data in Fig. 2 show that more than 90% of the maximal endoprotease activity measured on day 4 of germination is retained in kilned malt.

The levels of endoprotease activities in kilned malts from a number of Australian and overseas barley varieties, measured with haemoglobin, hordeins and glutelins as substrates are shown in Fig. 3. The levels of endoprotease activity in all varieties, with each substrate, were comparable.

Optimal pH

The results of determining the pH optimum for malt endoproteases with haemoglobin, hordeins and glutelins are given in Fig. 4. The activity with haemoglobin was highest at pH 3.5, the lowest pH used in this study, which means that the optimum pH for haemoglobin could be 3.5 or lower. Hordeins were optimally hydrolysed at pH 4.0 and glutelins at pH 5.0. These results suggest that each substrate is likely to have a different pH optimum.

Thermostability and optimal temperature

The results of the thermostability studies of malt endoproteases at 65°C and their optimal temperatures of action are shown in Fig. 5. The residual activities were measured with different substrates, after heat exposure for 0 to 50 min at 65°C. Protease activity measured with the synthetic substrate N-BCZ-glycine *p*-nitrophenyl ester

was lost rapidly, more than 80 % of activity disappearing within 10 min. A similar picture was observed with the modified protein azocasein as a substrate. About 60 % of the activity was lost within the first 10 min followed by a gradual and slower loss thereafter. The activities towards haemoglobin, hordeins and glutelins followed a common pattern of slow decline of about 35 % of the original activities in 20 min. From this point on, the effect of the heating duration on the ability of the endoproteases to attack the three substrates differed. The ability to hydrolyse hordeins declined fast and was complete in another 20 min. In contrast, the decline in activity with glutelins was very slow. Hordeins and glutelins are the major components of the barley grain storage proteins. These experimental results indicate that both proteins would be hydrolysed by malt endoproteases during mashing.

The optimal temperatures of the reactions also varied with the substrate. It was 40°C with hordein and 50°C with glutelin. It appears that different protein substrates may provide varying degrees of protection against heat for malt endoproteases.

Evaluation of proteolysis during malting and mashing

The results of determining the amounts of free amino nitrogen and soluble proteins in barley, malt extracts and wort are shown in Fig. 6. The levels of free amino nitrogen in malt compared to barley were more than five and seven times in Morex and Schooner, respectively. The increase in soluble proteins was about two-fold. These results also indicate that free amino nitrogen increased during the protein rest at 45°C and during EBC Congress mashing compared to malt. Free amino nitrogen in malt was extracted at 0°C and at room temperature (20-22°C), following the Congress mashing procedure as described under the methods. There was a small difference between the levels at 0°C and at room temperature, presumably due to proteolysis at the latter temperature. The levels extracted at 0°C are considered to be the best indicator for the amounts present in malt. The increases in free amino nitrogen during the protein rest were 19 and 24 % and in wort 33 and 42 % in Schooner and Morex, respectively. The increases indicate that proteolysis was active during both the protein rest and mashing. In contrast, the levels of soluble proteins remained almost the same. This means that both endoproteases and exopeptidases were active and the same level of soluble protein was maintained by degrading malt insoluble proteins. These results also indicate that mashing contributed about 25 % and 30 % of the total free amino nitrogen in worts of Schooner and Morex, respectively.

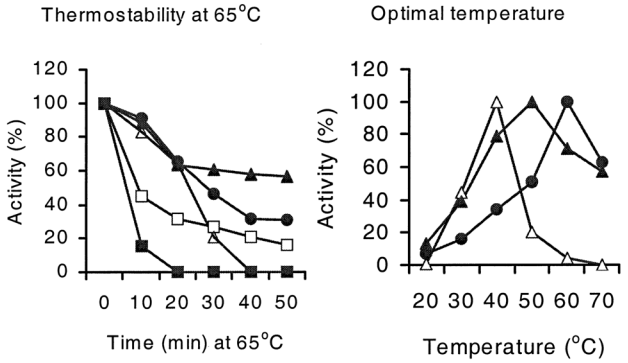

FIG. 5. Thermostability (left) and optimal temperature (right) of malt endoproteases determined with different substrates. Substrates were azocasein (□), haemoglobin (●), hordein (△), glutelin (▲) and N-BCZ-glycine *p*-nitrophenyl ester (■). Details as described under Material and Methods.

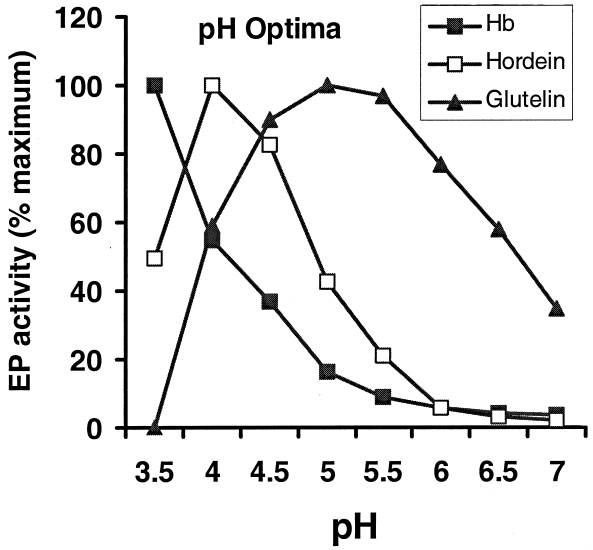

FIG. 4. The pH optima of malt endoproteases determined with haemoglobin (■), hordein (□) and glutelin (▲) as substrates. Buffers used in the assay were sodium acetate in the pH range of 3.5 - 5.5 and MOPS (3-[N-Morpholino] propane-sulfonic acid) in the pH range of 6 - 7.

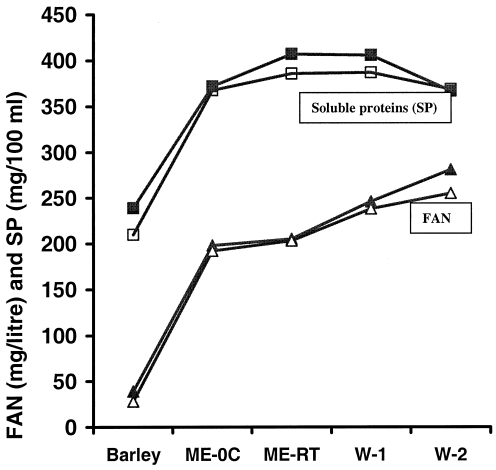

FIG. 6. Free amino nitrogen (Morex ▲, Schooner △) and soluble proteins (Morex ■, and Schooner □) in barley and malt extracts and in worts of Morex (USA) and Schooner (Australian) varieties. Details of extraction and EBC Congress mashing are described under Materials and Methods. Labelling on the X-axis: barley = barley extract at room temperature (RT); ME-0C = malt extract at 0°C ; ME-RT = malt extract at RT; similarly, W- 1 and W- 2 stand for Congress wort after protein rest and at end of mashing, respectively.

DISCUSSION

The results of this study confirm previous reports[7,9,18] that barley grain storage protein-degrading endoproteases develop during seed germination. The electrophoretic studies demonstrate that they are diverse and appear on the first day of germination , and increase subsequently in number and intensity[9,18,19]. This is consistent with the timing of their physiological role of providing the developing embryo with free amino acids.

Endoproteases are synthesised in the living cells of the grain and secreted into the endosperm where the storage proteins, hordein and glutelin, are stored. In the endosperm, endoproteases hydrolyse hordeins and glutelins to peptides. Subsequently carboxypeptidases in the endosperm and aminopeptidases in the scutellum complete the hydrolysis to free amino acids[2]. It has been reported that acidic conditions (pH 4.8) prevail in the endosperm during germination[19]. The pH optima of malt endoproteases with hordeins and glutelins as substrates are pH 4.0 and pH 5.0, respectively (Fig. 4). However, it should be noted that these pH optima were measured using a mixture of endoproteases (Fig. 1) and a mixture of protein substrates (hordeins and glutelins)[13,19]. It is possible that individual endoproteases may have a different pH optimum that is different from the combined. It is also possible that different endoproteases would have different pH optima with individual hordeins and glutelins. The case of haemoglobin, as a single protein substrate with optimum pH of 3.5, different from others, (Fig. 4) is a good example. It is important to separate these enzymes to characterise their properties. Our further study with the purified endoproteinases (unpublished data) confirms our earlier assumption that each individual enzyme might have a different pH optimum with the same substrate.

For maltsters and brewers, endoproteases and their products perform multi purpose functions during germination, kilning, mashing, and fermentation, and in beer. They provide free amino acids for the synthesis of hydrolytic enzymes, for production of colour and flavour materials and for yeast nutrition. In addition, hydrolysis of storage protein and related structures facilitates water and hydrolytic enzyme movement, liberates starch from protein bodies and ensures uniform modification. Insufficient hydrolysis of storage proteins is associated with processing problems, low extract and low fermentability. On the other hand, excessive proteolysis is associated with the development of undesirable flavours and colour and diminished levels of foam forming proteins in beer.

The results of this study, comparing the levels of endoprotease activity in green and kilned malt (Fig. 1 and Fig 2), have established that endoproteases are not destroyed during kilning. This was also supported by the similar levels of activity found in kilned malts of varieties from different countries (Fig. 3) and is in line with reports in the literature[1,5,8]. Furthermore, thermostability studies under simulated mashing conditions revealed that malt endoproteases exhibit a wide range of responses to heat stress reflecting their complexity (Fig. 1 and Fig. 5). Nevertheless, these results clearly demonstrate the ability of malt endoproteases to be active and hydrolyse hordein and glutelin during mashing.

The increases in free amino nitrogen and soluble proteins in wort compared to malt (Fig. 6) furnish further evidence in support of proteolysis during mashing. There were no substantial differences between the levels of soluble proteins in the malt and wort. In contrast, there was an increase of 25 to 30% in free amino nitrogen levels. The increase in wort free amino nitrogen without a decrease in the level of soluble proteins suggests that both endo- and exoproteases were active during mashing. As the pH of mashing is in the range pH 5.5 to pH 6, it is likely that more glutelin than hordein would be hydrolysed during mashing[5]. The portion of free amino nitrogen produced during mashing was 25% in Schooner (Australian) and 30 % in Morex (USA), reflecting small varietal differences. The 25 – 30 % increase in total wort free amino nitrogen is in good agreement with the levels reported by Morgan *et al* [8] and Palmer[10], but lower than the 50% reported by Barrett and Kirosp[1] and higher than no increase reported by van Gameron[16]. Although variation could occur due to varietal differences, the 25 – 30 % level of increase during mashing seems to be reasonable, because mashing time is shorter than malting, and endoproteases are inactivated by heat and inhibitors. Consequently, the obvious question arises whether malt would have enough endoprotease activity to hydrolyse storage proteins during mashing with solid adjuncts to meet the needs in soluble nitrogen. Cereal grains are used as solid adjuncts and they are known to be low in free amino nitrogen, for example barley grain is reported to contain about 2 % of the soluble nitrogen as free amino nitrogen[13]. Our own results show that the levels of free amino nitrogen in barley are less than 20 and 15 % in Morex and Schooner malts, respectively.

The solution to ensuring adequate levels of free amino nitrogen in worts of adjunct mashing may be higher malt endoprotease activity. This could be achieved through breeding or by adding exogenous endoproteases to the mash. Currently, we are pursuing efforts to isolate and identify the individual malt endoproteases to assess their relative importance in degrading storage proteins during malting and mashing.

CONCLUSIONS

Malt endoproteases are complex and diverse, and are expressed in multiple forms during the germination of barley. They exhibit different optimal activity levels for pH, reaction temperature and thermostability with different substrates. Malt endoproteases are active during mashing and contribute to soluble proteins and free amino nitrogen of the wort. These enzymes are particularly important during mashing with solid adjuncts to compensate for the deficiency of free amino nitrogen in the adjunct. However low activity, heat and inhibitor inactivation, and shorter duration of protein rest and mashing time, may limit their effectiveness, especially at high levels of adjunct.

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REFERENCES

1. Barrett, J. and Kirosp, B. H., *Journal of the Institute of Brewing*, 1971, **77**, 39.
2. Enari, T-M., *Journal of the Institute of Brewing*, 1995, Centennial Edition.
3. Guerin, J. R., Lance, R. C. M. and Wallace, W., *Journal of Cereal Science*, 1992, **15**, 5.
4. Heisel, S. E., Peterson, D. M. and Jones, B. L., *Cereal Chemistry*, 1986, **63**, 500.
5. Jones, B. L., Proceedings of the 9th Australian Barley Technical Symposium, Melbourne, 1999, 2.39.1.
6. Koehler, S. M. and Ho, D. T-H., *The Plant Cell*, 1990, **2**, 769.
7. Marttila, S., Porali, I. and Mikkonen, A., *Micron and Microscopica Acta*, 1992, **23**, 107.
8. Morgan, A. G., Gill, A. A. and Smith, B. D., *Journal of the Institute of Brewing*, 1983, **89**, 292.
9. Osman, A. M., Hamilton, S. E., De Jersey, J. and Inkerman, P. A., Proceedings of The 48th Australian Cereal Chemistry Conference, Cairns, 1998, 287.
10. Palmer, G. H., Cereal Science and Technology, (G. H. Palmer, ed), Aberdeen University Press, 1989, 153.
11. Poulle, M. and Jones, B. L., *Plant Physiology*, 1988, **88**, 1454.
12. Sarkkinen, P., Kalkkinen, N., Tilgmann, C., Siuro, J., Kervinen, J. and Mikola, L., *Planta*, 1992, **186**, 317.
13. Shewry, P. R., *Barley:* Chemistry and Technology, (A.W. MacGregor and R.S. Bhatty, eds), American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, 1993, 131.
14. Sopanen, T. and Lauriere, C., *Plant Physiology*, 1989, **89**, 244.
15. The Malting and Brewing Industry Barley Technical Committee, Industry Guidelines for Australian Malting Barley, 1998, **4**, Index 1.
16. van Gameron, Y. M., *Master Brewers Association of the Americas Technical Quarterly*, 1995, **32**, 238.
17. Weis, W., Postel, W. and Gorg, A., *Electrophoresis*, 1992, **13**, 787.
18. Wrobel, R. and Jones, B. L., *Plant Physiology*, 1992, **100**, 1508.
19. Zhang, N. and Jones, B. L., *Journal of Cereal Science*, 1995, **22**, 147.

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