

Barley and Malt Proteins and Proteinases: II. The Purification and Characterisation of Five Malt Endoproteases, Using the Highly Degradable Barley Protein Fraction (HDBPF) Substrate

Abdalla M. Osman

ABSTRACT

J. Inst. Brew. 109(2), 142–149, 2003

Five barley-malt endoproteases have been purified using the highly degradable barley protein fraction (HDBPF) as the substrate for activity detection and measurement. The five purified endoproteases were identified as the most active and, hence, we believe the most important proteolytic enzymes during barley germination and malting. This was demonstrated by showing that the component of HDBPF, degraded in test tubes during the reaction to determine their activity, cannot be recovered from malt by extraction, indicating that this component has been degraded during germination and malting. These endoproteases differ in their specificities, pH and temperature optima, thermostability and ionic-cationic behaviour. The gel filtration chromatographic-profiles of the peptide products of these enzymes *versus* parallel profiles of beer peptides exhibit very close similarities.

Key words: Barley protein substrates, characterisation, malt endoproteases, proteinases, purification.

INTRODUCTION

Barley grain at maturity contains some low proteolytic activity, which increases (up to 20-fold) during germination and commercial malting^{5,6,10}. The importance of these enzymes follows from the fact that the grain has no storage capacity for free amino acids^{7,17,20}. During initial embryo development, the grain must provide all the amino acids for the synthesis of hydrolytic enzymes and other essential nitrogenous compounds for sustaining and the continuation of germination⁷. Furthermore, the proteinases facilitate the breakdown of the cell walls and the freeing of starch granules and the release and activation of β -amylase^{8,21}. These are particularly important for the efficient processing and maximising the yield of extract in the brew-house¹⁷.

Endoproteinases are considered to be the key limiting proteolytic activity enzymes amongst the proteinases^{7,23}.

Leslie Research Centre, Farming Systems Institute, Queensland Department of Primary Industries, Agency for Food and Fibre Sciences, P O Box 2282, Toowoomba, QLD 4350, Australia.
Corresponding author. E-mail: Abdalla.Osman@dpi.qld.gov.au

Publication no. G-2003-1616-110

© 2003 The Institute & Guild of Brewing

Malt endoproteases make up a complex mixture of enzymes belonging to the four classes, namely, aspartic, cysteine, metallo- and serine endoproteases^{1,4,13,23}. Up to 42 different activities were recorded using different forms of native two-dimensional polyacrylamide gel electrophoresis²³.

Many attempts have been made to purify and study these endoproteases^{2,3,9,10,18,19}. Most agree that cysteine endoproteases are the most active at acidic pH and constitute the most effective malt endoproteases^{7,10,23}. Researchers in different laboratories have purified two malt endoproteases MA and MB and classified them as cysteine endoproteases^{9,18,19}. They were shown to hydrolyse hordein and their formation was shown to be induced by gibberellin during germination. All these purifications were based on the use of non-barley protein substrates, such as casein, haemoglobin and gelatin for the detection and assay of the purified enzymes^{2,3,9,18,19,22}.

The aim of this study was to purify malt endoproteinases using a natural barley protein substrate (HDBPF) for their detection and assay throughout the whole process. This paper describes the purification of 5 endoproteases and illustrates their true importance in degrading the stored reserve grain proteins during germination and malting.

MATERIALS AND METHODS

Malt

Joe White Maltings, Brisbane, and Barrett Burston Malting, Toowoomba, kindly supplied Grimmitt and Schooner malts respectively, which were used in the purification of endoproteases.

Chemicals

All chemicals were of the highest grades, purchased from Bio-Rad, Millipore, Pharmacia and Sigma-Aldrich, Australia.

Extraction

Malt was milled into flour using a Buhler Miag Mill (0.2 mm). It was established, in preliminary trials that the best extraction medium and the best grist/liquid ratio was 50 mM acetate buffer (pH 5.0) and 1/3 (grist/liquid) ratio. Usually, 250 g flour was extracted with 750 mL 50 mM

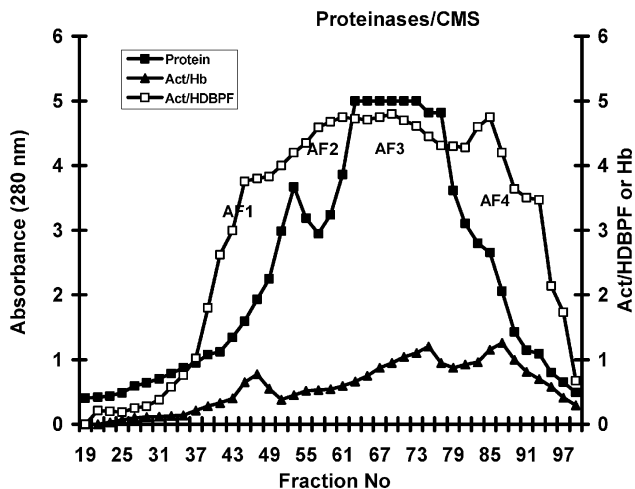


Fig. 1. Separation of malt proteinases on a carboxymethyl sepharose column. Malt extract, after $(\text{NH}_4)_2\text{SO}_4$ treatment was loaded on the column, washed with buffer to remove unadsorbed proteins and eluted with a (0–0.5 M) NaCl gradient. Details under Materials and Methods.

acetate buffer (pH 5.0) for 1 h at room temperature (RT 20–22°C) with gentle and continuous stirring. The extract was centrifuged at 3500g for 20 min and the supernatant collected.

Purification of endoproteases

All the major steps of endoproteases purification, except the dialysis (at 4°C), and precipitation steps were carried out at RT (20–22°C).

Separation by ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ precipitation

Two consecutive $(\text{NH}_4)_2\text{SO}_4$ precipitations were carried out at 30% saturation first, followed by a 2 h rest in the cold room at 4°C and centrifugation at 3500g for 30 min. In the first step, the pellet was discarded and the supernatant was up saturated to 70% by adding the extra weight of $(\text{NH}_4)_2\text{SO}_4$ powder, to achieve the 70% saturation, with gentle stirring. The solution was left in the cold room for 2 h and then centrifuged. This time the supernatant was discarded and the pellet dissolved in a minimum amount of 25 mM acetate buffer (pH 5.5) and dialysed against two changes of 10 L of the same buffer, in the cold room (4°C) for 24 h. At the end of dialysis, the content of the dialysis tube was collected, centrifuged for 10 min at 3500g and the supernatant was collected. The dialysed material was further concentrated 2 to 3-fold, using ultra-membranes 10 k molecular weight cut off, before use in the next step of purification.

Separation by liquid chromatography

Separation by Carboxymethyl Sepharose (CMS). A column (2.6 × 90 cm) was loaded with CMS and equilibrated with 25 mM acetate buffer (pH 5.5) containing 0.01% sodium azide. The partially purified endoproteases from the ammonium sulphate fractionation step were loaded to the CMS column. The column was washed with the equilibration buffer until all the unadsorbed proteases

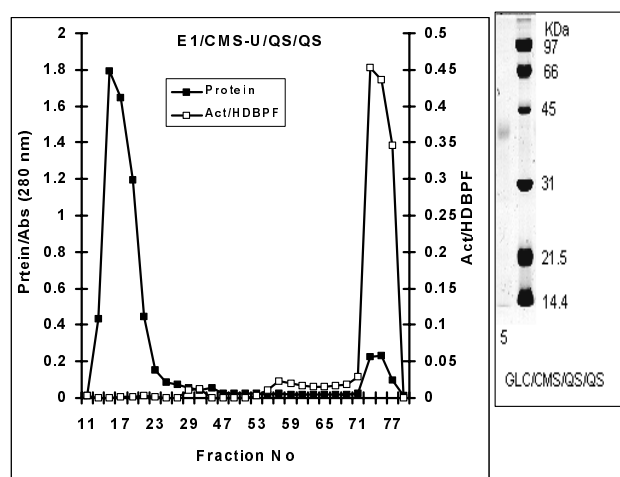


Fig. 2. The Q Sepharose chromatogram and SDS-PAGE electrophogram of the purified malt endoprotease 1 (E1). Prior to this step, it was chromatographed on a CMS column, and a larger QS column.

and proteins were washed out. Then a NaCl gradient (0 to 0.5 M) was applied to elute the adsorbed proteases. This gradient was enough to free all the endoproteases and some proteins from the column. The remaining proteins were washed out with 0.1 M NaOH before re-equilibrating the column for reuse.

The endoproteases' active fractions from the CMS column were designated e.g. CMS unadsorbed, eluted with equilibrating buffer, fraction (CMS-UF) and CMS-bound fractions 1, 2, 3 and 4 (CMS-BF1, CMS-BF2 etc.) eluted by NaCl gradient. Each fraction was combined, concentrated, filtered through DG 10 columns (Bio-Rad) to remove salt and used in further purification on QS columns.

Separation by Q Sepharose (QS) column. Two columns, a small (1.6 × 40 cm) and a large (2.6 × 40 cm), were loaded with Q Sepharose (Pharmacia) and equilibrated with 25 mM acetate buffer (pH 5.5). The choice of the use of the large or small column was made depending on the size of the fraction. The same chromatographic procedure as in the CMS column was used. The sample in 25 mM acetate buffer (pH 5.5) was loaded onto the column, washed with the same buffer to elute the unadsorbed proteins and endoproteases. This was then followed by the application of the NaCl gradient to elute the adsorbed proteins and endoproteases.

Enzyme assay

Endoproteolytic activities were measured using 1% casein and haemoglobin (Hb) solutions and 0.2 mL hordein and HDBPF extracts. The standard reaction medium, final volume 0.5 mL, contained 0.2 mL of 0.2 M acetate buffer (pH 4.0 Hb, pH 4.5 casein and hordein and pH 5.0 HDBPF), 0.2 mL substrate and 0.1 mL adequately diluted enzyme solution. The incubation was carried out at 40°C, for 10 min HDBPF and 30 min for others, unless specified otherwise. The reaction was terminated by adding 1 mL cold 10% trichloroacetic acid (TCA). The samples were kept at 4°C for at least 10 min, centrifuged at 3500g for 20 min and the absorbance measured at 280 nm. For each assay a control was treated in the same way except that

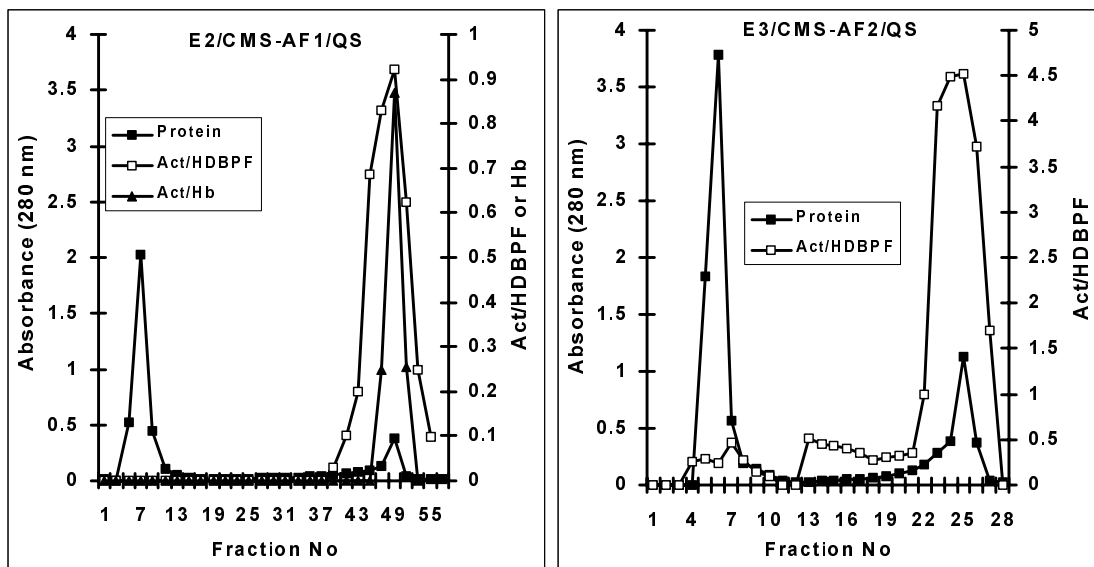


Fig. 3. The Q Sepharose chromatograms of pure malt endoproteases 2 and 3 (E2 and E3) which correspond to CMS active fractions 1 and 2 (F1 and F2).

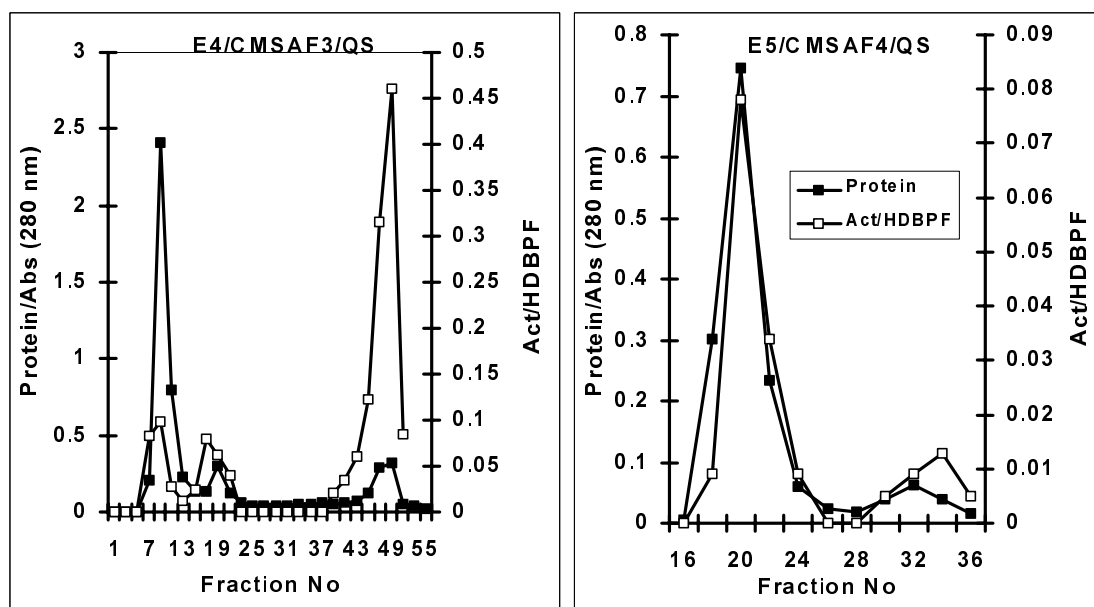


Fig. 4. The Q Sepharose chromatograms of pure malt endoprotease 4 (E4) left and endoprotease 5 (E), right. They were separately chromatographed, as described under Materials and Methods.

enzyme was added after the TCA. The control absorbance was subtracted from the test absorbance to express the enzyme activity as the difference in absorbance or converted into enzyme units¹³.

Temperature and pH optima and thermostability

These parameters were measured using haemoglobin and HDBPF as the substrates according to the previously reported procedures¹³.

Enzyme inhibition

Effects of the native barley inhibitors and the general class-specific inhibitors on the endoproteolytic activities of the purified endoproteases were investigated using the pro-

cedures reported in the literature^{6,18}. The inhibitors used were: N-ethylmaleimide (NEM), leupeptin, trans-epoxy-succinyl-L-leucylamido (4-guanidino)-butane (E-64) for cysteine endoproteases, ethylenediaminetetraacetic acid (EDTA) for metallo-, N,N'-diisopropylcarbodiimide (DIC) for serine and pepstatin A for aspartic endoproteases. In addition, endogenous barley inhibitors were prepared by extraction from barley flour and their inhibitory effect on the purified endoproteases investigated⁶. The inhibition studies were performed in test tubes by including the inhibitor in the enzyme reaction medium and incubating as normal reaction. The inhibitor was also added to the control to eliminate any absorbance effect contributed by the inhibitor or associated proteins.

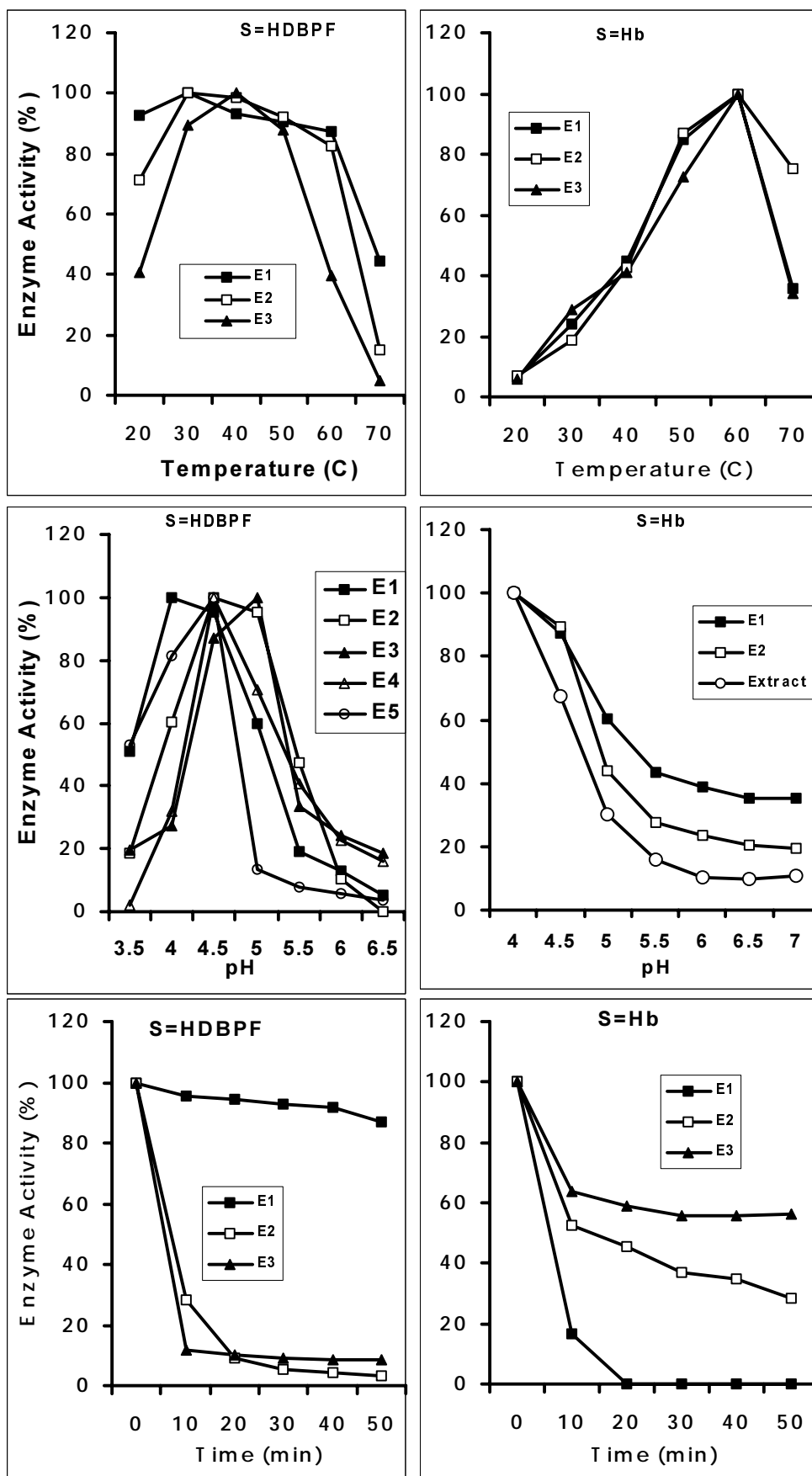


Fig. 5. The optimal temperatures (top), optimal pH (middle) and thermostability at 65°C (bottom) as a function of the natural substrate HDBPF, left and haemoglobin, right. The details as described earlier.

Extraction of HDBPF

HDBPF was extracted from barley and malt by the method and procedure previously described^{16,17}.

Protein assay

Total proteins in the malt extracts and purified endoprotease fractions were determined using Folin-Ciocalteu reagent as described in Lowry method¹¹.

RESULTS AND DISCUSSION

Purification of 5 malt endoproteases

The results summarised in Figs. 1–4 demonstrate the steps employed to purify the five enzymes. These steps were comprised of acidic (pH 5.5) and very low salt aqueous extraction. Separation and elimination of proteolytically inactive proteins by $(\text{NH}_4)_2\text{SO}_4$ precipitation and ion exchange liquid chromatography combined with the minor but essential intermediate steps of centrifugations, resuspensions, dialysis etc. The classical table, usually supplied in purification studies of one enzyme, at a time, has not been included because, here, we have purified 5 different endoproteases, from the same extract in one attempt. We believe our extraction was inclusive and contained all malt endoproteolytic activities, as manipulation to extract more from the pellet, after centrifugation, with different extraction media failed to produce any evidence to suggest otherwise. However, it is important to add that the five purified enzymes do not present the total extractable proteolytic activity. There were minor activities detected in discarded $(\text{NH}_4)_2\text{SO}_4$ separations and in peaks disregarded during ion exchange chromatography. Our guess estimate is that the right number of malt endoproteases could be around ten, plus or minus two, but perhaps not as large as 42 endoproteases²³.

Although the purity of the five endoproteases was not thoroughly investigated in this study, we assume that each enzyme was highly purified as attested by SDS-PAGE of endoprotease 1 (E1) Fig. 2. In addition, the specific activity of each enzyme was in the order of hundreds of units, well in excess of the combined specific activity of the initial extract, which was less than ten units. In fact, this suggests that each of the five endoproteases was purified a few thousand-fold, assuming the total initial extract contained ten endoproteases.

Characterisation of the five partly purified endoproteases

The five enzymes exhibited distinct characteristics, which demonstrate their differences in structure, mode of action, specificity and thermostability. Optimal temperatures and pHs as well as their thermostabilities at 65°C, as a function of two substrates, namely, haemoglobin and HDBPF are shown for E1, E2 and E3 in Fig. 5. It can be seen that the endoproteases have different optimal temperature and pH and variable tolerance to heat exposure. These effects were substrate dependent, confirming our previous reports on the study of the combined behaviour of these malt endoproteases¹².

Additionally, this justifies the doubt expressed about the applicability of using unnatural substrates, such as haemoglobin to estimate the activity of these proteinases and then project those results as a true reflection of their action on native barley proteins^{12,13}.

For example, a glance at the pH optima of these enzymes (Fig. 5) with the HDBPF substrate clearly indicates that four out of the five have their pH optima at pH 5.0 or very close to it. Moreover, pH 5.0–5.5 is the pH range in commercial mashing and can be expected to prevail during germination and malting. This is significantly different from the pH observed with haemoglobin as substrate (ie. less than 4.0). The same can be said with regard to optimal temperature and thermostability (Fig. 5). In summary, to monitor more closely and to understand the function and behaviour of these endoproteases during germination and malting and brewing, there is no alternative to the use of natural substrates^{12,14}. Only the use of natural barley protein substrates in estimating their activities will allow the correct prediction of their functional roles and behaviour during malting and mashing.

More support to this contention is provided by the findings of the investigation with the inhibitors, both the native barley and class-specific inhibitors (Fig. 6). The results with native barley inhibitors (Fig. 6, top) clearly indicate that their effects on these enzymes were substrate

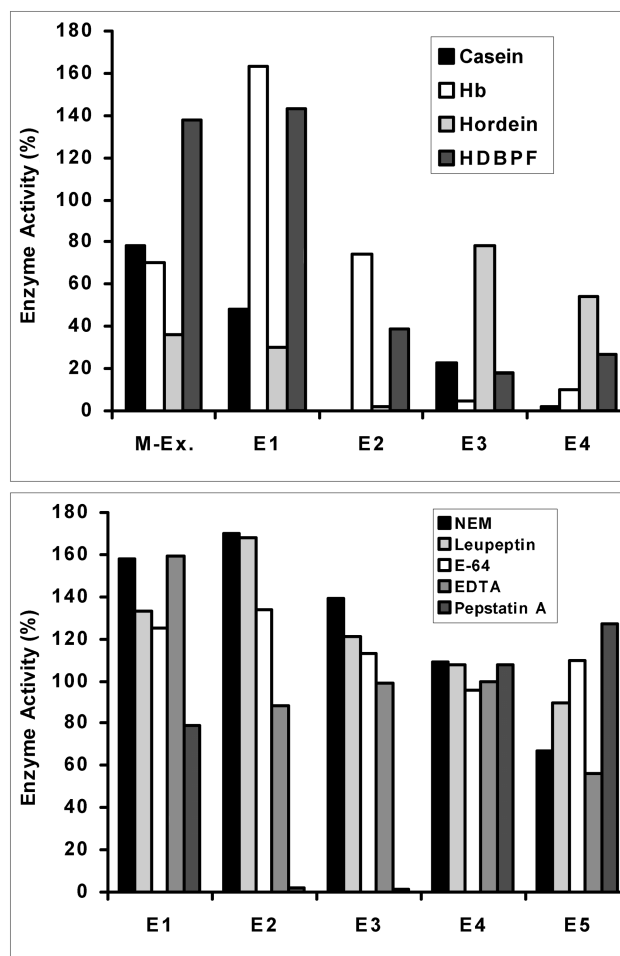


Fig. 6. (Top) The effects of native barley inhibitors on the actions of the purified and unpurified (malt extract) on casein, haemoglobin (Hb), hordein and HDBPF. (Bottom) The effects class-specific inhibitors on HDBPF (E1,2,3,4,5).

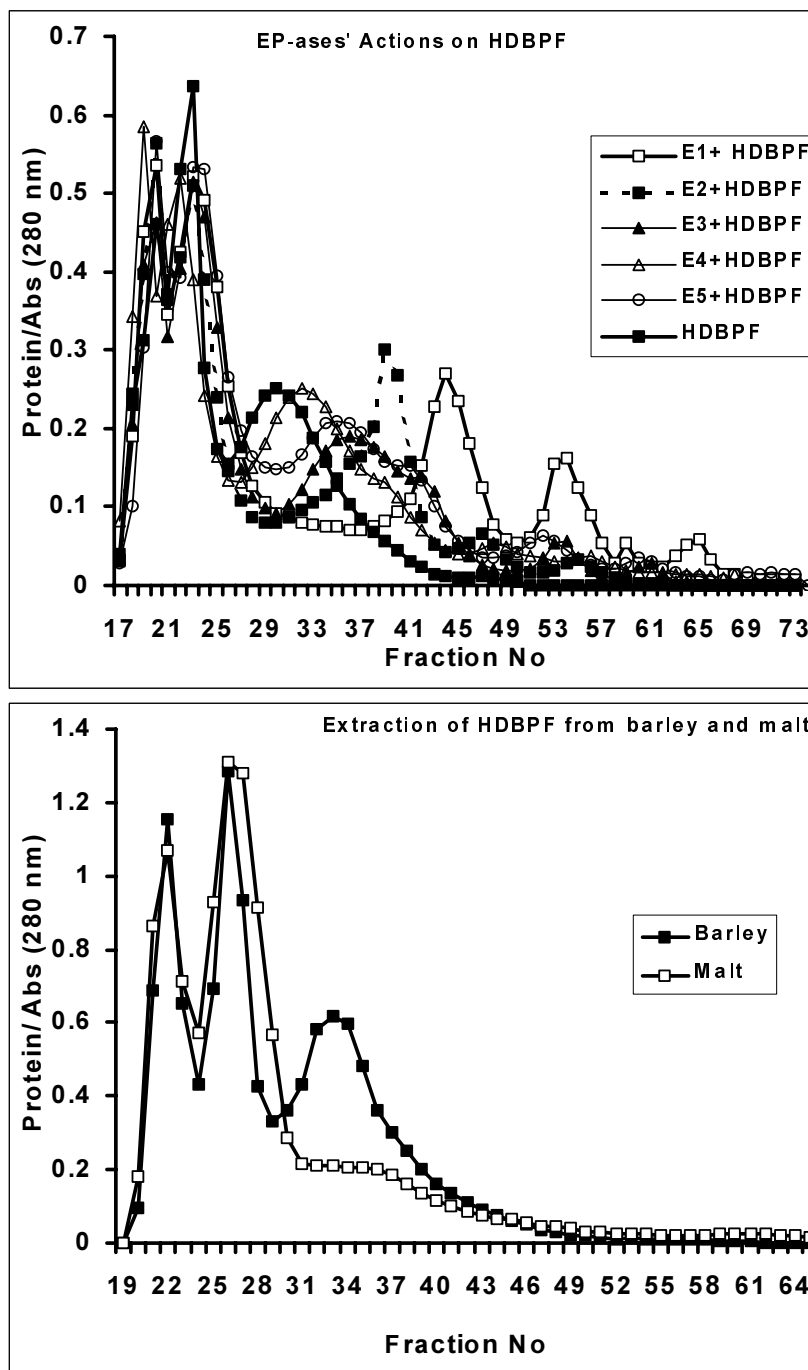


Fig. 7. The chromatograms of the profiles of HDBPF before and after degradation with the purified endoproteases (top) and HDBPF extracted from barley and malt (bottom). Details as under Materials and Methods.

dependent. All the five endoproteases were inhibited when the substrate was either casein or hordein, however, the activities with these two substrates were very low, compared to the other two, even without inhibitors. Unexpectedly, the activity of endoprotease 1 (E1) was highly enhanced by barley inhibitors measured with either haemoglobin or HDBPF. This suggests that these two substrates not only protect this endoprotease against the inhibitors but also mediate the effect of an activator(s). Hence, it appears that no general conclusion on the effects of these

inhibitors is warranted from the studies of one substrate, especially, if the substrate is not the natural substrate for the enzyme involved.

Similarly, classification of these enzymes from the results of class-specific inhibitors on one substrate should be treated with caution. Data from the study of the effects of some class-specific inhibitors (Fig. 6, bottom) on the purified endoproteases, with the barley protein HDBPF as the substrate, strongly support this view. None of the enzymes was inactivated with cysteine or metallo- class inhibitors.

Endoproteases 2 and 3 (E2 and E3) were inhibited more than 98% with pepstatin A, suggesting that they belong to the aspartic class of endoproteases. On the other hand, all the five enzymes were completely (100%) inactivated with 10 mM N,N-diisopropylcarbodiimide (DIC) indicating that they are serine class endoproteases. However, the latter could not be confirmed at lower concentrations of the inhibitor. This flies in the face of the most widely reported conclusion, which stipulates that the most active, at acidic pH, green malt endoproteases are cysteine class endoproteases^{2-7,9,10,17,21,22}.

It is very important to note that our enzymes were purified from malt rather than green malt, which is very commonly used. This was based on our previous finding that more than 90% of green malt endoproteolytic activity survives kilning¹³. It remains to be investigated whether kilning affects these endoproteases in a way that changes their protein conformation and subsequently their active sites, or is this solely the effect of native barley protein substrate? Unfortunately, we could not include other substrates in the study of class-specific inhibitors because of the limited amounts of pure enzymes at hand.

Further characterisation of the purified endoproteases was achieved through the gel filtration analysis of the peptide products of their actions on HDBPF (Fig. 7, top). These results unambiguously confirmed the different identity and specificity of the five endoproteases. Each enzyme produced different length peptide products from the same protein(s). This suggests that each enzyme specifically hydrolysed different peptide bonds formed by different amino acid combinations. It is pertinent to note that the profiles of some of these peptides are very similar to the beer peptide profiles reported previously¹⁵. The beer peptides develop mainly during malting and, presumably, as the result of the endoproteolytic action of malt endoproteases. This finding presents an opportunity to identify the important peptides with impacts on beer quality such as those peptides which form and stabilise beer foam and the endoproteases producing them. Equally the detrimental peptides involved in colloidal instability and haze formation and the endoproteases associated with them. The identification of the positively and negatively associated beer quality endoproteases may enable the breeders to incorporate that knowledge in their endeavour to improve malting barley quality.

The importance of these five purified endoproteases purified, using HDBPF as the substrate, was supported and advanced by the absence of this protein(s) in the extract of malt (Fig. 7, bottom). The evidence suggests that this protein(s) has been degraded by these endoproteases, most likely, in preference to other storage proteins and that is why it was not detected in the malt extract.

CONCLUSIONS

The five most active and important barley-malt endoproteases were purified. They differed in their specificities, pH and temperature optima, and thermostability. None was identified as a cysteine class endoprotease. The use of a barley native protein(s) HDBPF as the substrate of activity assay during purification ensured the prompt and success of purifying 5 endoproteases. Our findings

confirmed the inadequacy of using unnatural substrates and the general conclusions based on the findings of those studies.

ACKNOWLEDGEMENTS

The author acknowledges the contribution of Dr. P. A. Inkerman and collaborators. This work was supported with a grant from Australian Grains Research and Development Corporation (GRDC). The author also expresses his sincere gratitude and thanks to colleagues in LRC for support, to Ms. S.M. Coverdale for technical help and to Mr. R. Nischwitz for critical reading of the manuscript.

REFERENCES

1. Barrett, A.J., The classes of proteolytic enzymes. In: Plant proteolytic enzymes, M.J. Dalling, Ed., CRC Press: Boca Raton, 1986, Vol. 1, pp. 2-14.
2. Burger, W.C., Prentice, N., Kastenschmidt, J. and Huddle, J.D., Partial purification of proteases from germinating barley. *Cereal Chem.*, 1966, **43**, 546-554.
3. Burger, W.C., Prentice, N., Moeller, M. and Robbins, G.S., Stabilisation, partial purification and characterisation of peptidyl peptide hydrolases from germinated barley. *Phytochem.*, 1970, **9**, 49-58.
4. Burger, W.C., Multiple forms of acidic endopeptidase from germinated barley. *Plant Physiology*, 1973, **51**, 1015-1021.
5. Enari, T.-M., Pupperti, E. and Mikola, J., Fractionation of the proteolytic enzymes of barley and malt. Proceedings of the European Brewing Convention Congress, Brussels, Elsevier: Amsterdam, 1963, pp. 37-44.
6. Enari, T.-M. and Mikola, J., Characterisation of the soluble proteolytic enzymes of barley and malt. Proceedings of the of the European Brewing Convention Congress, Madrid, Elsevier: Amsterdam, 1967, pp. 9-16.
7. Enari, T.-M. and Sopanen, T., Mobilisation of endosperm reserves during germination of barley. *J. Inst. Brew.*, 1986, **92**, 25-31.
8. Guerin, J.R., Lance, R.C.M. and Wallace, W., Release and activation of barley β -amylase, by malt endopeptidases. *J. Cereal Sci.*, 1992, **15**, 5-14.
9. Koehler, S. and Ho, T.-H.D., Purification and characterisation of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. *Plant Physiol.*, 1988, **87**, 95-103.
10. Koehler, S. and Ho, T.-H.D., A major gibberellic acid-induced barley aleurone cysteine proteinase which digests hordein. *Plant Physiol.*, 1990, **94**, 251-258.
11. Lowry, O.H., Roseborough, N.J., Farr, N.J. and Randall, R.J., Protein assay with Folin-Phenol reagent. *J. Biol. Chem.*, 1951, **193**, 265-275.
12. Macleod, A.M., The physiology of malting, In: Brewing Science, J.R.A. Pollack, Ed., Academic Press: London, 1979, Vol. 1, pp. 146-224.
13. Osman, A.M., Coverdale, S.M., Cole, N., Hamilton, S.E., de Jersey, J. and Inkerman, P.A., Characterisation and assessment of the of barley malt endoproteases during malting and mashing. *J. Inst. Brew.*, 2002, **108**, 62-68.
14. Osman, A.M., The advantage of using natural substrate-based methods in assessing the roles and synergistic and competitive interactions of barley malt starch-degrading enzymes. *J. Inst. Brew.*, 2002, **108**, 204-214.
15. Osman, A.M., Coverdale, S.M., Onley-Watson, K., Bell, D. and Healy, P., The gel filtration chromatographic-profiles of proteins and peptides of wort and beer: effects of processing - malting, mashing, boiling, fermentation and filtering. *J. Inst. Brew.*, 2003, **109**, 41-50.
16. Osman, A.M., Barley and malt proteins and proteinases: I. Highly degradable barley protein fraction (HDBPF), a suitable substrate for malt endoproteases assay. *J. Inst. Brew.*, 2003, **109**, 135-141.

17. Osman, A.M., Coverdale, S.M., Ferguson, R., Onley-Watson, K., Fox, G., Hamilton, S.E. and de Jersey, J., What causes low protein modification and low wort free amino nitrogen – proteins or proteinases? Proceedings of the 10th Australian Barley Technical Symposium, Canberra, 2001, Grains Research and Development Corporation: Australia, CD-ROM, contribution 32.
18. Phillips, H.A. and Wallace, W., A cysteine endopeptidase from barley malt which degrades hordein. *Phytochem.*, 1989, **28**, 3285–3290.
19. Poulle, M. and Jones, B.L., A proteinase from germinating barley. *Plant Physiol.*, 1988, **88**, 1454–1460.
20. Shewry, P.R., Field, J.M., Kirkman, M.A., Faulks, A.J. and Mifflin, B.J., The extraction, solubility and characterisation of two groups of barley storage polypeptides. *J. Expt. Bot.*, 1980, **31**, 393–407.
21. Sopanen, T. and Lauriere, C., Release and activation of bound β -amylase in a germinating barley grain. *Plant Physiol.*, 1989, **89**, 244–249.
22. Wrobel, R. and Jones, B.L., Electrophoretic study of substrates and pH dependence of endoproteolytic enzymes in green malt. *J. Inst. Brew.*, 1992, **98**, 471–478.
23. Zhang, N. and Jones, B.L., Characterisation of germinated barley endoproteolytic enzymes by two-dimensional gel electrophoresis. *J. Cereal Sci.*, 1995, **21**, 145–153.

(Manuscript accepted for publication May 2003)