

## Purification and Characterisation of Barley Malt Endoproteases

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### Abstract

The levels of activity and the composition of endoproteases in germinating grains of Australian barley varieties were investigated using native gel electrophoresis. The results indicated that both the levels and composition of the malt endoproteases were similar to those reported for overseas varieties. The malt endoproteases that are most active at acidic pH, are largely cysteine endoproteases and have a moderate to high thermostability. The optimal temperature(s) for activity was substrate dependent, with hordeins at 40°C and with glutelins at 50°C. Among the native barley protein substrates, glutelins were hydrolysed most effectively, particularly by those endoproteases that have higher thermostability. Consequently, glutelins are likely to be degraded more extensively than other protein fractions during malting and mashing, and contribute more to the soluble nitrogen pool of the wort. However, it is not clear if the degradation of glutelins has any special impact on the production or quality of malt and beer.

### Introduction

Endoproteases play an indispensable role in the mobilisation of the reserve proteins, from the commencement and during grain germination. The adequate supply of free amino acids is vital for both plant propagation and commercial malting. Cereal grain has only a very limited capacity (if any) for storage of free amino acids (Enari and Sopanen 1986). Therefore, the reserve proteins have to be degraded by proteases to ensure the supply of amino acids for the synthesis of new proteins and other essential nitrogenous compounds. The proteases are composed of endo- and exoproteases with the endoproteases being the key and rate limiting enzymes. Firstly, their action on proteins is a prerequisite for the action of exoproteases, which act on peptides to produce free amino acids. Secondly, endoproteases are heavily involved in metabolic regulation by activating (maturing) and inactivating metabolically important proteins such as enzymes and hormones (Thum, 1993).

Commercial malting is essentially controlled germination to produce hydrolytic enzymes to degrade endosperm cell walls, hydrolyse proteins and free starch granules from protein bodies and protein matrix. These processes are effected directly or indirectly by endoproteases and have consequences for the processing and the quality of the end product. For instance, insufficient endoprotease action results in undermodification with the associated problems of beer and wort filtration, haze formation and low extract. In contrast, excessive endoprotease action diminishes foam-forming proteins and leads to excessive colour and undesirable flavour. Therefore, production of high quality malt that yields maximum useful extract with a desirable flavour profile and without processing and production problems in the brewhouse, requires an adequate control of the endoproteases. To date, endoproteases are regulated by treatment of barley with gibberellin and potassium bromate to stimulate or inhibit their development during germination (Baxter *et al* 1978). However, these are nonspecific regulators and there are more than 40 different malt endoproteases (Zhang and Jones 1995) that may act and impact differently. Therefore, there is a need to identify and characterise those endoproteases that are involved in specific functions during malting and mashing that

impact on the quality of malt or beer. Consequently we have opted to purify and characterise some of the major malt endoproteases. In this paper, we report the results on the collective characterisation of malt endoproteases, with the purification and characterisation of the most active and important enzymes.

## Material and Methods

### *Malt*

Commercial Grimmatt malt for purification of endoproteases was obtained from Joe White Maltings Ltd, Redbank, Queensland. Other samples were micromalted in the Barley Quality Laboratory, at Leslie Research Centre, Toowoomba.

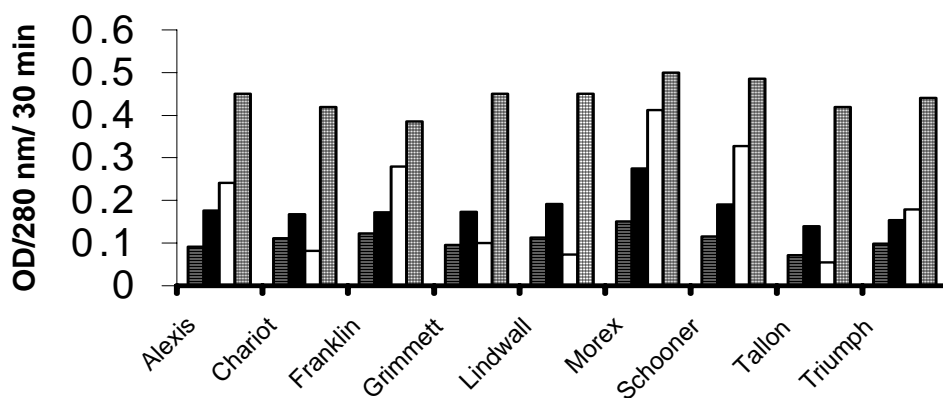
### *Enzyme Extraction for Assay and Purification*

Malt endoproteases were extracted from ground malt flour in (1:3 g/l) 20 mM acetate buffer (pH 5) for 1h at room temperature. After centrifugation (2860g, 20 min), extracted endoproteases were precipitated using 25 - 65 % ammonium sulfate, centrifuged, dissolved and dialysed. Enzymes in the dialysate were purified by a combination of anion (CM) and cation (Q Sepharose) exchange chromatographies and gel filtration. Activities were assayed using a variety of substrates including azocasein, haemoglobin, the synthetic compound N-BCZ-glycine *p*-nitrophenyl ester and native barley proteins, hordeins and glutelins. The latter two proteins were sequentially extracted from barley by a modified procedure of Weis et al 1992.

## Results and Discussion

### *Endoprotease Activity in Australian and Overseas Malt Varieties*

In Figure 1, the activities of unfractionated malt endoproteases in Australian and elite overseas varieties are compared using four protein substrates, azocasein and haemoglobin, hordeins and glutelins. The levels of activity, measured with azocasein, haemoglobin and glutelins were similar in all varieties. The widest variation was observed with hordeins and the least variation with glutelins.



**Figure 1.** Malt endoprotease activities in Australian and overseas barley varieties, measured with azocasein (1 from left), haemoglobin (2), hordeins (3) and glutelins (4 from left).

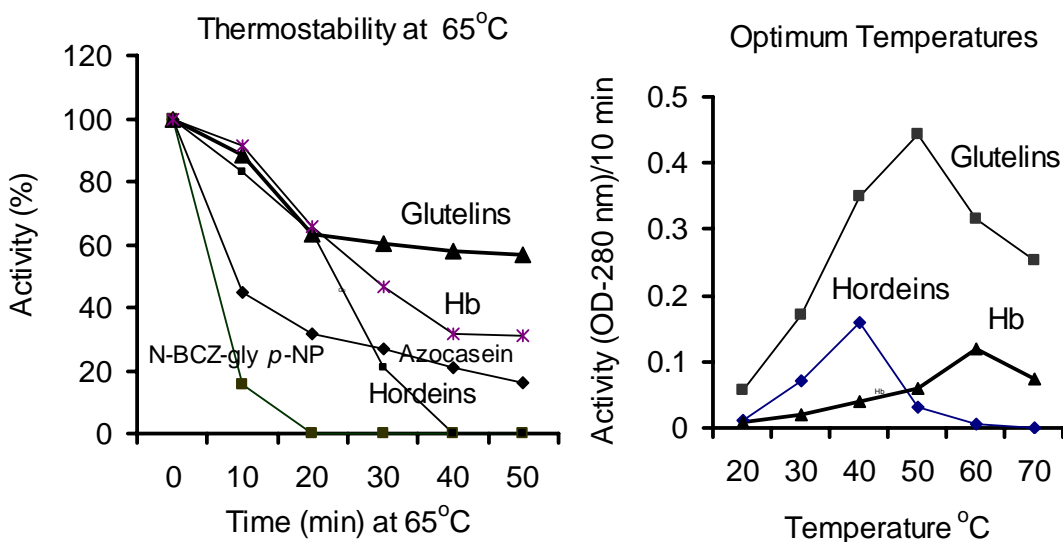
To our knowledge, this is the first report of glutelins being used as substrate in a direct enzyme assay of barley malt endoproteases. The method seems very promising, as the rate of

degradation of glutelins to form products soluble in trichloroacetic acid is much higher than from haemoglobin or hordeins (Figure 1 and Figure 2, right). Glutelins are the second largest group of barley reserve proteins, constituting about 30 % of the total level (MacLeod 1995). Their degradation during malting and mashing could contribute towards desirable levels of grain modification (Palmer 1989). Moreover, the use of glutelins as a substrate for malt endoproteases provides a better assessment of their action on barley storage proteins.

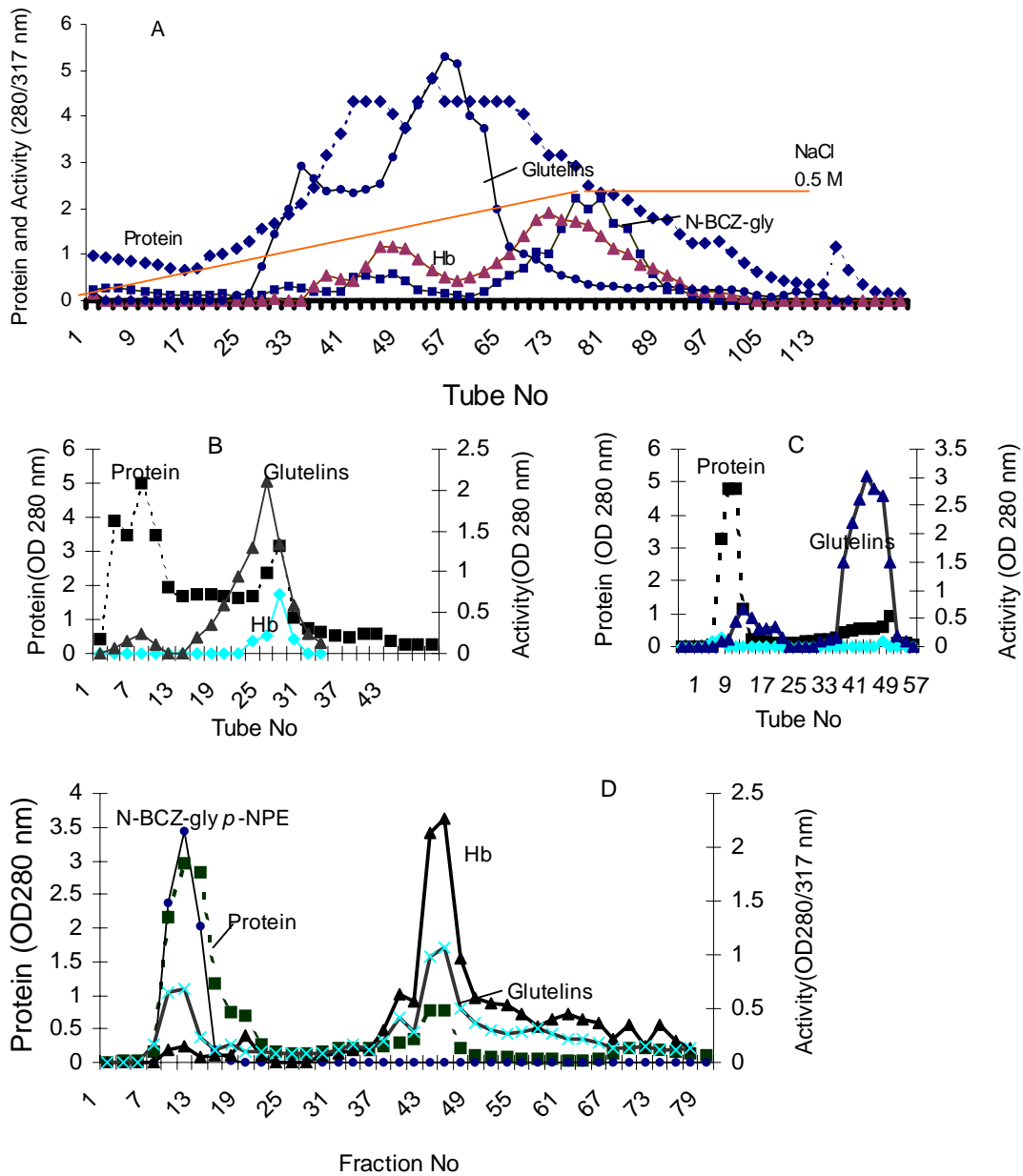
#### Characterisation of Malt Endoproteases

It is generally accepted that barley proteins are degraded during malting while their degradation during mashing is minimal due to heat inactivation (Gameron 1995). We have examined the thermostability and optimal temperature of the action of endoproteases using a number of substrates (Figure 2). Overall, the results indicate that both thermostability and optimal temperature are dependent on the substrate used in the assay. This suggests that different substrates may be hydrolysed by different endoproteases. Also it appears that some endoproteases may be heat sensitive while others are relatively thermostable.

Thermostable endoproteases were more effective in degrading glutelins than the other substrates tested. This property combined with the higher optimal temperature suggests that, during mashing, the glutelins are more likely to be degraded than hordeins. It is plausible to assume that the degradation of glutelins, which are structural and storage proteins, would enhance modification. The latter will be due to the removal of the subcellular structural impediment to the movement of water and the hydrolytic enzymes including the proteases that hydrolyse hordeins. Further, the degradation of the storage proteins would facilitate the release and activation of functionally important proteins such as  $\beta$ -amylase and liberate starch granules from protein bodies and protein matrix resulting in high extracts. The thermostable enzymes will be more valuable during mashing with cereal adjuncts.



**Figure 2.** Thermostability and optimum temperatures of malt endoproteases with different substrates. For thermostability determination, aliquots of enzyme extracts were incubated at 65°C for the indicated periods, cooled and assayed for the residual activities with the substrates shown in the graph. Optimal temperatures were determined by incubating the enzyme extracts with the substrates shown at the indicated temperatures.



**Figure 3.** Elution profiles of endoproteases from CM (graph A) and Q (graphs B, C and D) Sepharose columns. B endoproteases unadsorbed on CM Sepharose, C adsorbed (tubes 52-63) and D adsorbed (tubes 72-90). Protein is indicated by dotted lines and enzyme activities measured with haemoglobin (Hb), glutelins and N-BCZ-gly *p*-nitrophenyl ester are indicated by solid lines and labelled.

#### *Purification and Characterisation of Malt Endoproteases*

A combination of ammonium sulphate precipitation, and CM and Q Sepharose ion exchange chromatography was employed to purify malt endoproteases. The results are summarised in Figure 3.

CM Sepharose separation of malt endoproteases resulted in two distinct groups, adsorbed and unadsorbed. The adsorbed enzymes were then eluted with a NaCl gradient (0 - 0.5 M). Three different activity peaks (Fig.3, A) emerged close to each other but were distinguishable by their differential action on haemoglobin, glutelins and the synthetic substrate. All the fractions were concentrated by ammonium sulphate precipitation, dissolved, dialysed and rechromatographed on Q Sepharose column Figure 3 (graphs B, C and D). Graph B represents the CM Sepharose unadsorbed fraction rechromatographed on the Q Sepharose column. Only a small amount of the activity was detected in the fraction with the bulk of the protein that eluted at the void volume, with the highest endoprotease activity corresponding to the second peak of protein. Graph C represents the CMS adsorbed fraction (tubes 52-63) and the outcome from Q Sepharose was similar to the unadsorbed fraction, the major activity being confined to the second protein peak. Endoproteases most tightly adsorbed to CM Sepharose (Graph A, tubes 72-90) also displayed a similar picture, namely the major protein fraction emerged at the void volume and the smaller fraction eluted with NaCl (D, Fig.3). All the activity measured with synthetic substrate N-BCZ-glycine *p*-nitrophenyl ester emerged with the first protein peak while the second peak was more active with haemoglobin than with glutelins.

In summary, the chromatography of malt endoproteases on CM and Q Sepharose columns resulted in four different endoproteases with different structural properties and substrate specificities. The presence of endoproteases with specificities to different substrates partly explains the differences in thermostability and optimal temperature observed with unfractionated enzymes on different substrates (Figure 2). The fractionated endoproteases are being further investigated to determine their properties and their involvement in the production of high quality malt and beer.

## Conclusions

Barley malt endoproteases are a mixture of enzymes with different substrate specificities, thermostabilities, optimal temperatures and ionic properties. The thermostable endoproteases would be active during mashing contributing more soluble nitrogen to wort, particularly when cereal adjuncts are used. Endoproteases exhibiting different substrate specificities are most likely to have different impacts on malt and beer qualities.

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