

# Barley and Malt Proteins and Proteinases: I. Highly Degradable Barley Protein Fraction (HDBPF), a Suitable Substrate for Malt Endoprotease Assay

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## ABSTRACT

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Barley grain proteins were extracted and fractionated, based on their solubility, to investigate their proteolytic digestibility and suitability to be used as a substrate for the assay of malt proteinases. The fraction extracted with alkaline buffer (Tris-HCl or Tris-glycine pH 8.8–9.5), at the end of the sequence, exhibited remarkably high degradability by malt proteases compared to other fractions or any known protein substrate. Gel filtration chromatographic analysis of this fraction revealed that it is composed of three different molecular weight components. Further investigation, after proteolytic treatment, demonstrated that the third and the low molecular weight component is the highly degradable protein(s) (HDP). We designated the whole fraction, the mixture of the three components, as the highly degradable barley protein fraction (HDBPF) and used it (and recommend it) as the substrate for the assay of malt endoproteases activity.

**Key words:** Barley, endoproteases, highly degradable, malt, proteinases, proteins.

## INTRODUCTION

The importance of barley grain protein component as an essential determinant of barley quality for malting, brewing and feeding is widely recognised<sup>9,11,16</sup>. Proteins are important components of the grain. Both the quantitative and qualitative aspects of the grain protein are highly considered by the end users. Maltsters and brewers demand a limit range of 9–12% grain protein content that is easily modifiable while food and feed industries desire high lysine containing proteins that can be readily digested.

For brewing, barley is initially transformed into a range of malts. Malt has two remarkable technological features that make it different from barley. These are the possession of the acquired hydrolytic enzymes synthesized during germination and the friability of the internal grain structure. The latter is mainly due to the breakdown of the

cell walls and proteins<sup>1,3,12</sup>. Hence, the capability of a barley variety to synthesize the hydrolytic enzymes, during grain germination, is of utmost importance for malt quality and for the classification of barley as being fit for malting<sup>1,12</sup>. Amongst these enzymes, proteinases are instrumental in the initiation of the grain modification because they supply the amino acids for the synthesis of other hydrolytic enzymes. They hydrolyse storage proteins such as hordeins and glutelins and enhance the actions of others such as  $\beta$ -glucanases that break down cell walls<sup>1</sup>. Furthermore, they free starch granules and  $\beta$ -amylase to ensure the maximum desirable diastatic power and extraction from the grain.

Therefore, the estimation of the levels of activity of these enzymes in malt is a potential means for determining an important aspect of malt quality and its performance in the brewhouse. Consequently, breeders and maltsters, routinely, measure the activity of enzymes such as  $\alpha$ - and  $\beta$ -amylases, and diastase to evaluate the capability of starch degradation, including limit dextrinase contribution, and  $\beta$ -glucanases for cell wall breakdown. However, and despite that protein-degradation is required right from the start of germination to supply amino acids for the synthesis of hydrolytic enzymes and others, estimation of the levels of proteinases is not yet a common practice. Lack of suitable and true natural substrates is given as the reason why proteinases are not routinely assayed for assessing malt quality<sup>2,9,10</sup>.

Traditionally, the convention has been to use animal protein substrates such as casein, haemoglobin and gelatin for endoproteases and synthetic substrates for exoproteinases and peptidases<sup>4,8,22</sup>. Although these substrates are acceptable for evaluation of proteinases' activity in malt *per se*, extrapolation to reflect their action during malting and mashing is highly doubtful<sup>9,15,17,19</sup>. Many attempts have been made to use extracted barley proteins such as the hordeins used as a substrate by Baxter<sup>2,12,17,22</sup>. None of the methods have gained popularity. This is because of the difficulty of extracting the barley protein fractions and ensuring a homogeneous suspension in proteinase reaction medium and is made more difficult by the fact that any suitable preparation should also be free from proteinases' inhibitors<sup>5,6</sup>.

The aim of this study was to extract and fractionate barley proteins, in a way to remove the inhibitors, and inves-

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tigate proteolytic degradability. A fraction that is highly degradable by malt proteinases has been recognized and identified.

## MATERIALS AND METHODS

Ten malting and feed barley (MB and FB) varieties of varying qualities and three varieties of wheat were tested. These were barley varieties: Harrington (MB, Canada), Morex (MB, USA), Valier (FB, USA), Franklin (MB), Gairdner (MB), Grimmett (MB), Lindwall (MB), Schooner (MB), Tallon (MB) and Tantangara (FB), Australia, and Australian wheat varieties: Banks, Janz and Sunco. Barley grain samples were obtained from the breeders and ground to flour using a Buhler Miag Mill (0.2-mm), while Wheat Quality Laboratory, LRC, kindly supplied wheat flour samples. Malt samples for the extraction of malt proteinases were obtained from the Barley Quality Laboratory or from Barrett Burston Malting House, Toowoomba.

All chemicals were of the highest grades and supplied by Sigma-Aldrich and Bio-Rad Australia.

### Sequential extractions of the proteins

Most of the preliminary extraction trials were performed with the flour of the four barley varieties: Franklin, Harrington, Morex and Tallon. The procedures described by Weiss et al.<sup>20</sup> were followed for sequential extraction and investigation of the degradability of the protein fractions. Modifications were made to simplify the procedure and to ensure extraction of the highest quantity and degradability of the proteinase substrate fraction. The 4% SDS solution was in 50 mM acetate buffer (pH 5.0) and the final extraction of the last fraction of proteins was in 50 mM Tris-HCl or glycine buffer pH 8.8–9.5. The extraction was carried out at room temperature (20–22°C) while dialysis was performed at 4°C. The extraction process is outlined in Fig. 1.

Initially, albumins and globulins were extracted with distilled water and dilute salt solutions, collected and investigated for their digestibility with malt proteinases. Next, the hordeins were extracted with 70% ethanol alone for hordein 1 (H1), followed by extraction in 70% ethanol containing 0.1–0.5% dithiothreitol (DTT) and 1–2% sodium dodecyl sulfate (SDS) in acetate buffer (pH 5.0) for hordein 2 (H2).

The remaining hordeins (H3) were extracted with 4% SDS solution. The hordein fractions were extensively dialysed against distilled water containing 0.05% sodium azide and investigated for their digestibility with malt proteinases.

Finally, and after washing the pellet twice with 50 mM acetate buffer (pH 5.0) and distilled water, glutelins were extracted with 50 mM Tris-HCl or Tris-glycine buffer (pH 8.8–9.5). The fraction was extensively dialysed against distilled water containing 0.05% sodium azide and investigated with malt proteinases for digestibility.

In all steps the suspension, resuspension and extraction were facilitated by mixing with glass rods every 5 min.

### Proteinases: extraction and assay

Proteinases were extracted from malt flour 1 g in 3.0 mL 50 mM acetate buffer (pH 5.0) for 30 min at RT with

vortexing, centrifuged and filtered. Enzyme activities were assayed with all the fractions, including water and salt soluble proteins, extracted from barley prepared as above, as well as with haemoglobin using the procedures reported previously<sup>10</sup>. The method entailed the proteolytic degradation of the substrate proteins by proteinases, rendering them TCA soluble to be measured spectrophotometrically at 280 nm. The proteolytic activities of malt extract were estimated using the substrates: 1% casein and haemoglobin (Hb) and 0.2 mL extract of hordein and HDBPF prepared as above. The standard reaction medium, final volume 0.5 mL, contained 0.2 mL of 0.2 M acetate buffer (pH 4.0 Hb, 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) solution. The samples were kept at 4°C for at least 10 min, centrifuged at 3500 g for 20 min and the absorbance measured at 280 nm. For each assay a control was treated exactly the same way except that the 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 units<sup>12</sup>. Though it is possible that this assay will detect most proteases, the short duration of the reaction and the extraction conditions, used here, would be more in favour of endoproteases.

The reaction conditions, described above, were determined empirically after trying different variations such as variable substrate (extract) volumes and different concentrations and volumes of TCA. The latter seemed to be critical and dependent on the substrate volume and concentration.

### Gel chromatography

Gel filtration chromatography of the HDBPF and its peptide products after the endoproteolytic actions of purified endoproteases (see the accompanying paper 2 in this issue) were performed on a (2.6 × 90 cm) column packed with Marex Cellufine GCL-300 (Millipore). The column was equilibrated and eluted with 0.1 M NaOH, details as described previously<sup>13</sup>.

### Amino acid analysis

The amino acid composition of HDBPF and some of its peptide products were determined with the Water's PICO-TAG method.

## RESULTS AND DISCUSSION

### Extraction and degradability

The results of the proteolytic action of malt proteases on the 70% ethanol, hordein-1 and 2 (H1, H2), 4% SDS-solution and Tris buffer extractions in comparison to haemoglobin degradability are shown in Fig. 2. The barley proteins including albumin and globulins were sequentially extracted from the barley varieties Harrington, Morex and Tallon. The data from the albumins and globulins were not included in this graph. This is because they were negative and in line with the widely accepted view that

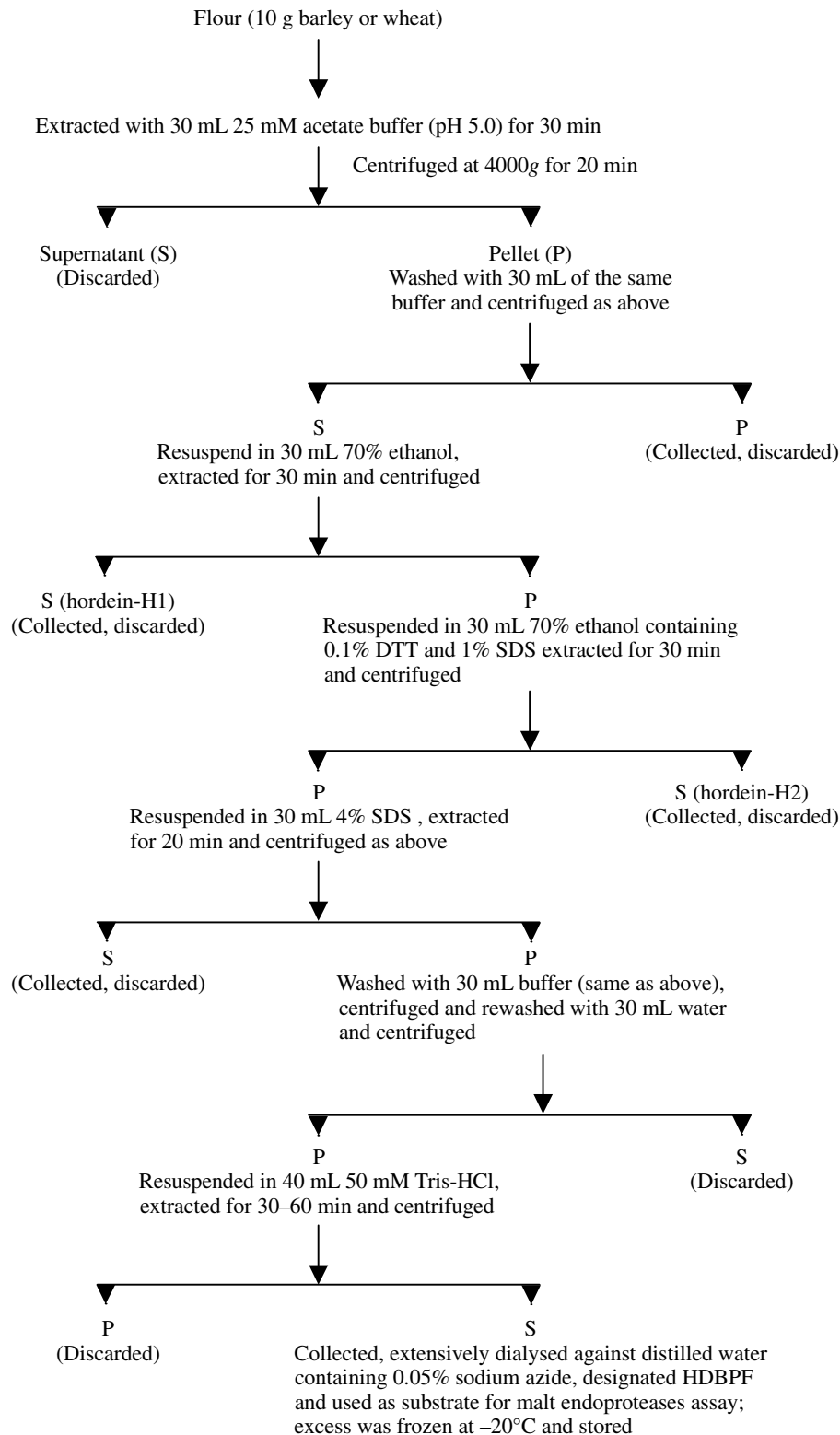


Fig. 1. Sequential extraction of barley and wheat proteins.

they are unsuitable proteolytic substrates, perhaps due to the co-extraction of the inhibitors<sup>5-7,10</sup>. Both hordein-1 (H1) and the Tris buffer extract (HDBPF) fractions from the three barley varieties were degraded much faster than haemoglobin by malt proteinases. In contrast, hordein-2 (H2) interaction with proteinases exhibited an opposite

effect. Whereby, it appeared that the proteinases instead of hydrolysing the TCA insoluble proteins to increase, as expected, TCA soluble fraction, they formed larger polypeptides insoluble in TCA from smaller and already soluble ones. It is pertinent to add that one of the differences between the H1 and H2 extraction is the presence of the

reducing agent DTT in H2 extraction. It is possible that DTT reduction might have produced more TCA soluble proteins in this extract. Subsequently, some of these reduced proteins were re-oxidised in the proteinase reaction medium but not in the control, creating the negative effect. The SDS extracted fraction was deemed unsuitable for use as proteinase substrate because of the mixed effects (negative and positive) and very low levels of degradability, if any. This could be another project to be investigated further in the future – such experiences are one of the reasons why these proteins are not successfully used in the assay of proteinases.

Reproducibility of extraction of H1 with positive proteolytic degradability proved to be difficult to achieve. Some extractions would be degraded, whereas others could not be degraded by the same malt extract proteases. In contrast, it was easy to reproduce the Tris-buffer extraction of HDBPF, although the degree of degradability also

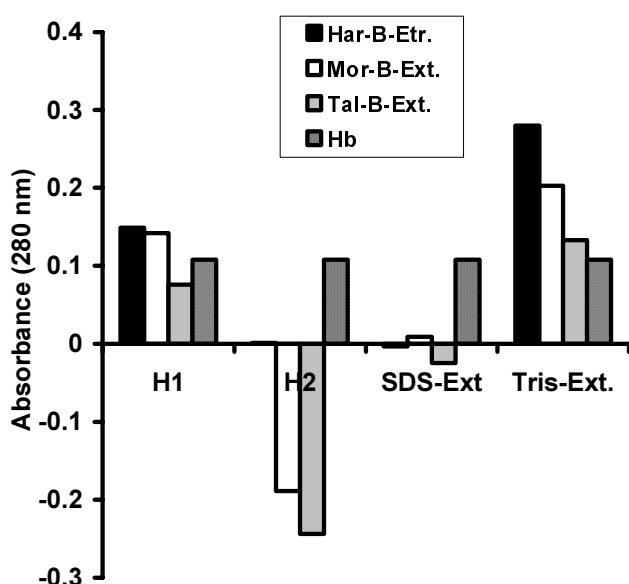


Fig. 2. The malt proteinase degradability of the sequentially extracted barley protein fractions: hordein-1 (H1), hordein-2 (H2), SDS extracted fraction (SDS-Ext.) and Tris buffer (pH 9.0) extraction (Tris-Ext. – designated HDBPF), compared with haemoglobin (Hb). Details of extraction procedure and proteinases assay method are given under ‘Materials and Methods’.

Table I. The extraction and degradability with malt proteinases of the HDBPF from 8 malt (MB) and 2 feed (FB) barley and 3 (W) wheat varieties.

Variety	OD (280 nm)
Franklin (MB)	0.267
Gairdner (MB)	0.168
Grimmett (MB)	0.165
Harrington (MB)	0.285
Lindwall (MB)	0.135
Morex (MB)	0.293
Schooner (MB)	0.199
Tallon (MB)	0.23
Tantangara (FB)	0.24
Valier (FB)	0.11
Banks (W)	0.102
Janz (W)	0.118
Sunco (W)	0.091

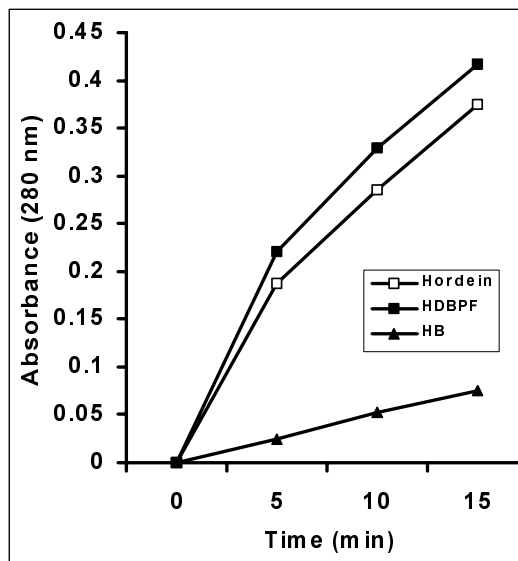
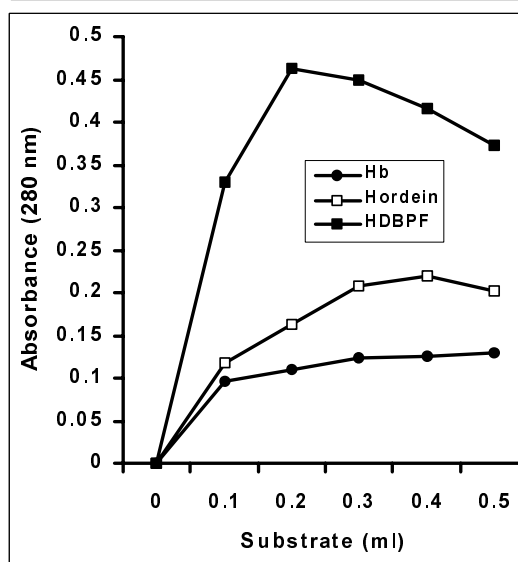
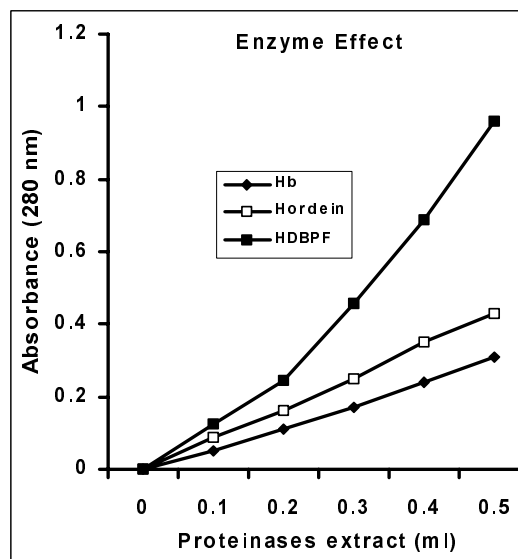


Fig. 3. The comparative kinetics of malt proteinases action on hordein-1 (H1), haemoglobin (Hb) and HDBPF as a function of enzyme and substrate concentrations, top and middle, respectively, and duration of the reaction, bottom.

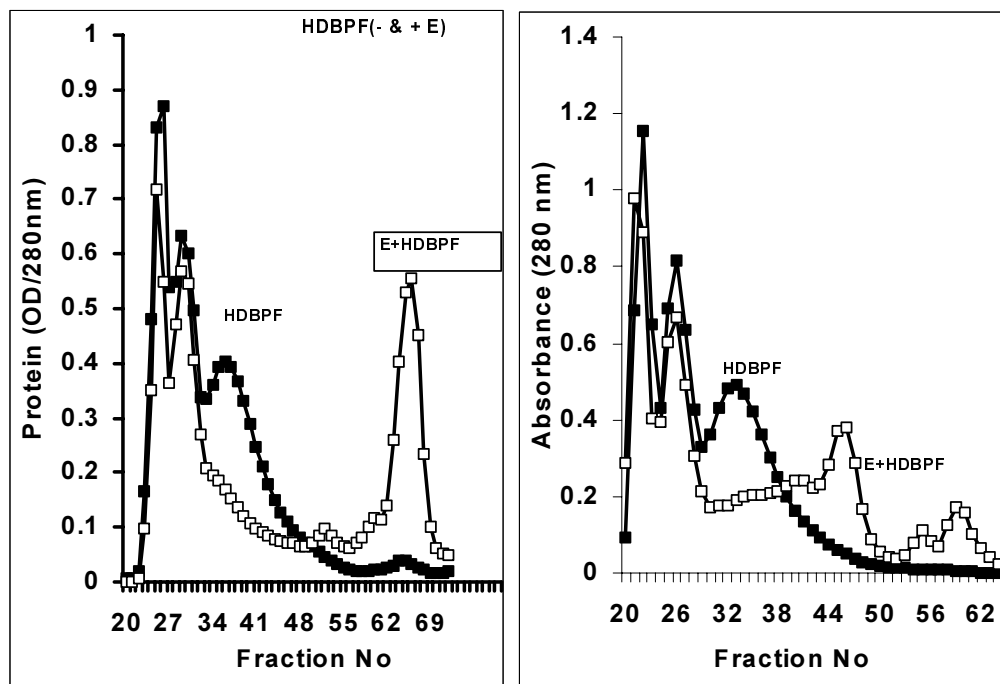


Fig. 4. The gel filtration chromatograms of the HDBPF with or without treatment with two purified malt endoproteases.

varied between extractions from the same barley variety and between varieties as evident from the results in Table I. It seems that HDBPF can be extracted with hordeins depending on the extraction conditions. For instance, when hordeins were extracted in 70% ethanol at 60°C, HDBPF was co-extracted with hordeins (data not included). We found, the best criterion for assessing the success of the extraction is the gel filtration profile of HDBPF, where the presence and the proportion of the third peak determine the rate of success. The higher and larger this peak the higher is the rate of degradability and hence the success of extraction. This means that the extraction procedure should be followed scrupulously, especially, regarding mixing and the use of slightly acidic media (pH 5.0–5.5) all the way through to the SDS extraction step. The varietal differences in degradability are interesting and could be used as a quality parameter and measured as suggested in the third paper of this series in this issue of the Journal.

#### The kinetics of proteinases with HDBPF

The kinetics of malt proteinases action on HDBPF are shown in Fig. 3 (effect of enzyme concentration, top, substrate concentration, middle and reaction time, bottom). The proteinase actions on the H1 fraction and haemoglobin are also included for comparison. The first impression of these results, particularly, with HDBPF indicates that they do not follow the expected classical patterns, at least as represented by haemoglobin. However, it is reasonable to expect different patterns from haemoglobin as these fractions, HDBPF and H1 are protein-mixtures and not pure proteins like haemoglobin.

While the increases in proteinases activities with haemoglobin and hordeins were linear with the amounts of extract added to the medium, the increase with HDBPF was different (Fig. 3, top). It exhibited biphasic increases,

where the increase in the second phase was more than in the first one. There are two possible explanations for this. Firstly, malt proteinases being a mixture of exo- and endoproteinases act in sequence and synergistically. Meaning that endoproteinases act first to hydrolyse proteins into peptides thus creating substrates for exoproteinases and their congruent action. As more product-substrate is produced more enzymes join in and the reaction rate accelerates more than what would be expected from the behaviour of one enzyme. A second and alternative explanation arises from the effect of substrate concentration as evident from the middle graph (Fig. 3). It is clear from this graph that proteinase action is stimulated by an increase in the HDBPF substrate concentration to a certain limit beyond which the rate decreases. Therefore, if we start with high substrate to enzyme ratio the reaction rate will be slow. But as we increase the enzyme concentration we reverse the ratio and increase the reaction rate.

The action of malt proteinase on HDBPF and H1 in comparison to haemoglobin is non-linear, bottom graph (Fig. 3). Perhaps this is due to the mixture nature of HDBPF, which is evident from the results of the chromatographic separation seen in Fig. 4. Additionally, the products may also have feed back inhibition on some of the proteinases. In situations like this where both the enzymes and substrate are a mixture it is most likely that the kinetics will not follow the classical rules.

#### Partial characterisation of HDBPF and its proteolytic products

The composition of HDBPF and the degradability of its components by purified malt endoproteases have been investigated by gel filtration chromatography (Fig. 4). It is clear that this fraction is composed of, at least, three components of different molecular weights. The third and the

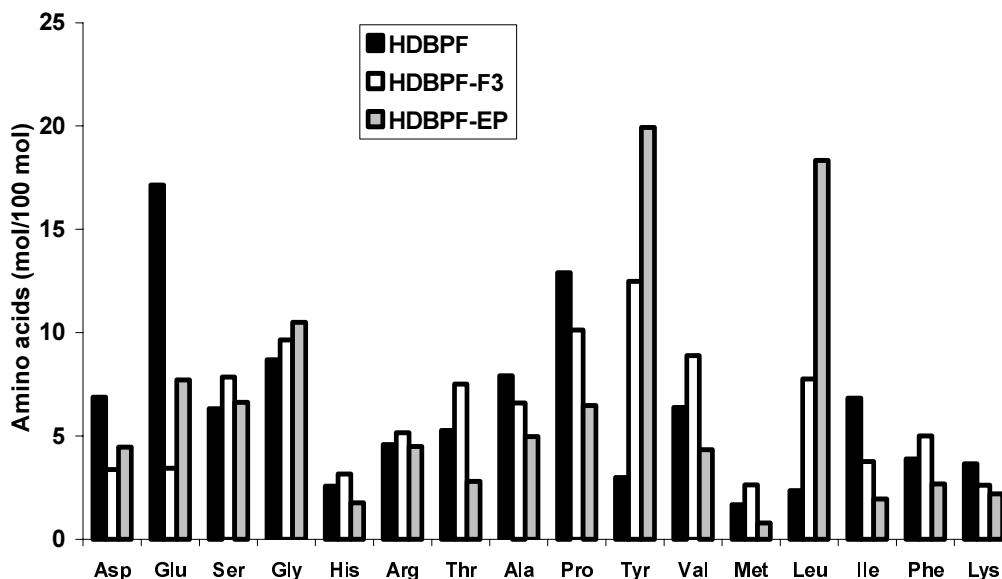


Fig. 5. The amino acid compositions of HDBPF, the third fraction of HDBPF on gel filtration chromatography (HDBPF-F3) and a product of endoprotease action (HDBPF-EP).

lower molecular weight component is the most degradable fraction of the three. The two purified barley malt endoproteinases (details in paper 2 of this series) hydrolysed this component differently to yield different peptide products. The other two components were only slightly hydrolysed by the two endoproteases used. Although there are plenty of endoproteases in malt<sup>2,19</sup>, the evidence presented in the second paper of this sequence indicates that only the third fraction is missing in the malt extract of HDBPF. The latter suggests that only the third component of HDBPF is actually highly degradable.

Attempts to purify this third component of HDBPF and use it as a pure protein substrate for endoproteinases have not yet been successful. During its separation from the other composites, and most likely during its concentration, it polymerised to form the other two higher molecular weight components and was no longer readily degradable (data not presented). This suggests that the relationships between the three components are much more complex than it seems and could be also different in the grain.

The amino acid compositions were analysed for HDBPF, its third component and one of the peptide products of endoprotease action (Fig. 5). The two amino acids cysteine and tryptophan were not measured as the method used did not include them. All the other amino acids are present in reasonably balanced comparable quantities. It is assumed that this has something to do with the fact that this fraction is preferably degraded by endoproteases. For plant propagation the embryo depends on the degradation of the storage proteins for the supply of all amino acids until it can assimilate nitrogen. Therefore, degradation of such protein will provide all amino acids compared to hordeins, which are skewed towards proline and glutamine. Similarly, the yeast in the brewhouse would prefer the supply of all amino acids it needs in wort rather than spend energy and other resources to synthesize them. Additionally, this fraction would be most nutritious in human food and animal feed. Hence breeding for its higher content would be advantageous.

### General discussion and remarks

A barley protein fraction with balanced amino acid composition (all the 18 amino acids determined present in comparable quantities) and high degradability with malt proteinases has been discovered. It is composed of at least three fractions of varying molecular weights. The third fraction is the one preferably degraded and its presence and relative quantity determines its suitability as a substrate for proteinases assay. Therefore, the extraction should aim at maximizing the quantity of the third component, which is the preferable substrate of proteinases.

It has been demonstrated in the accompanying paper (paper 2 of this series) that HDBPF is suitable to use as the substrate for malt endoproteases. Moreover, it has been shown that most of the activities measured in the malt extract, by this method, belong to endoproteases (Fig. 5 and paper 2 of this series).

Another important factor associated with the success of using HDBPF, as substrate for proteinases is the quantity and final concentration of TCA. This has to be worked out for each extract to avoid opaqueness in the reaction medium. Often in the literature the protein fraction degraded during malting or natural germination is described as the hordein fraction<sup>6,12,14,15</sup>. The amino acid composition of HDBPF indicates that it is not a typical hordein with the usual high proline (>50%) or glutamic acid plus glutamine content<sup>11</sup>. However, these amino acids were among the highest in this fraction and therefore it can be classified as having the properties of both glutelin and hordein<sup>18</sup>.

### CONCLUSIONS

A barley fraction extracted by Tris-buffer (pH 8.8–9.5) after the removal of other fractions was identified as the most suitable substrate for the assay of malt proteinase, especially, the endoproteinases. The lowest molecular weight component of this fraction is the most important component because it is the fraction preferentially de-

graded in test tubes and during malting. It contains 18 amino acids measured, and possibly all, which makes it a valuable nutritional asset, the increase of which should be pursued in future breeding improvements.

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