ENDOPEPTIDASES IN BARLEY SEED AND THEIR ACTION DURING GERMINATION

by

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CHAPTER 5: THE EFFECTS OF ENDOPEPTIDASE ACTIVITY AND BARLEY STORAGE PROTEINS ON MALTING QUALITY

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any University. To the best of my knowledge and belief, no material described herein has been previously published or written by any other person except when due reference is made in the text.

If accepted for the award of Doctor of Philosophy, this thesis will be available for loan or photocopy.

Jennifer Ross Guerin

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LIST OF PUBLICATIONS

Part of the work described in this thesis has been presented at scientific meetings and published in journals.

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LIST OF ABBREVIATIONS.

ABA ..... absisic acid
ABTS ..... 2, 2'-azinobis(3-ethylbenzthiazolinesulfonic acid)
BANA ..... sodium benzoyl-DL-arginine-β-naphthylamide
BAPNA ..... sodium benzoyl-DL-arginine-p-nitroanilide
BCIP ..... 5-bromo-4-chloro-3-indolyl phosphate
BSA ..... bovine serum albumin
CBB ..... Coomassie brilliant blue
CBZ-phe-ala ..... *N* -carbobenzoxy-*L* -phenylalynyl-*L* -alanine
CM-Sephadex ..... carboxymethyl Sephadex
CPI ..... Commonwealth Plant Introduction number
cv ..... cultivar
d ..... day
df ..... degrees of freedom
DTNB ..... 5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA ..... ethylenediamine~~trichloroa~~tetra-cetic acid
et al. ..... and others
FPLC ..... fast protein liquid chromatography
g ..... gravitational force
GA ..... gibberellic acid
h ..... hour
HPLC ..... high performance liquid chromatography
IEF ..... isoelectric focussing
kDa ..... kilodaltans
2-ME 2-mercaptoethanol
M ..... molar
mM ..... millimolar
μM ..... micromolar
MDR ..... menadione reductase
MEP-1 ..... malt endopeptidase 1
min ..... minute
Mr ..... relative molecular mass
NBT ..... nitro blue tetrazolium
nm ..... nanometre
ns ..... not significant
osc/min ..... oscillations per minute

PA.....phaseic acid
PAGE .....polyacrylamide gel electrophoresis
PBS.....phosphate buffered saline
pI .....isoelectric point
pmole.....picomole
PMSF .....phenylmethylsulphonyl fluoride
ppm.....parts per million
PVPP .....polyvinylpolypyrrolidone (insoluble)
RO .....reverse osmosis
RP-HPLC .....reversed phase high performance liquid chromatography
SDS .....sodium dodecyl sulphate
SE-HPLC .....size exclusion high performance liquid chromatography
TCA.....trichloroacetic acid
Temed.....N,N,N', N' - tetramethylethylenediamine
Tris .....Tris(hydroxymethyl)-aminomethane
TST.....20 mM Tris-HCl pH 7.5; 0.5 M NaCl; 0.1% Tween 20
v/v.....volume by volume
w/v.....weight by volume

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ABSTRACT

Barley endopeptidases have a primary role during germination (or malting) in the catabolism of storage proteins to smaller peptides and amino acids. Insufficient or excessive protein degradation during malting often leads to problems in the brewing process. More information on the action of endopeptidases is required to establish what level of endopeptidase activity is desirable in a malting barley. The general aim of this project was to investigate the malt endopeptidases and their action during germination.

The main malt endopeptidase enzyme (MEP-1), a cysteine endopeptidase of approximately 30,000 Mr, had previously been isolated in three different laboratories (Poulle and Jones, 1988; Phillips and Wallace, 1989; Koehler and Ho, 1990a). In the present study MEP-1 and other endopeptidases found in malt were characterised further. The N-terminal amino acid sequence for twenty residues was determined for MEP-1 and found to be identical to the sequence published by Koehler and Ho (1990a).

Utilising ditelosomic and ditelocentric wheat/barley addition lines, the gene encoding MEP-1 was located on the long arm of barley chromosome 3. The gene has been given the symbol *CepB* (von Wettstein-Knowles, 1992). A variant allele of *CepB* was found in a survey of accessions of wild barley (*Hordeum vulgare* ssp *spontaneum*) from Israel, Turkey and Iran. This rare variant has an isoelectric point of 4.1 compared with 4.3 for the form commonly found in cultivated barley and most accessions of wild barley. Analysis of the F_2 progeny of the cross between a Turkish accession having the variant *CepB* allele and barley cultivar Clipper segregated for MEP-1 isozyme type in a simple co-dominant Mendelian fashion. Linkage analysis between endopeptidase, esterase (*Est2*)and NADH diaphorase (*Ndh2*) enzymes in the F_2 progeny was used to map both the *CepB* and *Ndh2* gene loci in relation to *Est2*.

Malt endopeptidases have been postulated to contribute to endosperm modification by increasing the activity of β-amylase through a conversion of the relatively inactive bound form to the more active soluble form. Bound β-amylase was released after incubation with purified MEP-1. A range of free β-amylase isoforms were released which had identical pI values to the β-amylases found in malt. Thiol treatment also caused some activation of β-amylase but did not generate the same isoforms detected either in malt or after treatment with endopeptidase. It was concluded that the action of MEP-1 is the main mechanism by which β-amylase is released from the bound to the free form during malting.

The effect of hordein on malting quality was assessed, using near isogenic lines of Pallas barley which differ in hordein SDS-PAGE banding patterns. The lines were examined for differences in hordein content using SDS-PAGE and size exclusion HPLC. Malt extract, a major malting quality parameter, and endopeptidase activity were also measured. Genetic differences between the isolines affected all of the parameters measured. Peak areas relating to the different hordein groups estimated by size exclusion HPLC had no relationship with malt extract, demonstrating that the amount of any hordein group could not be used to predict malting quality. However, isolines P03 and P08A had the most strikingly different hordein banding patterns on SDS-PAGE from the parent and the malt extract values for these lines were significantly lower than all the other isolines. These differences may have been due to genetic carry-over from the donor barleys or to the different types of hordein donated.

Endopeptidase activity may be important in malting quality through its involvement in the modification of the endosperm. Fourteen different barley varieties, grown at three different sites, were malted and assayed for endopeptidase activity as well as other parameters routinely screened by the Barley Quality Evaluation Laboratory; α- and β-amylase, free amino nitrogen, diastatic power, % grain protein and % EBC extract. Analysis of variance revealed that endopeptidase activity was significantly correlated with % EBC extract, free amino nitrogen and % grain protein if the effects of site and variety were ignored. However, when site and variety were included in the analysis, endopeptidase activity had

no significant effect on any of the parameters. The environmental and genetic differences between the barleys that were surveyed obscured the effects of endopeptidase in this analysis.

CHAPTER 1.

GENERAL LITERATURE REVIEW

1.1 INTRODUCTION

This thesis focuses on the endopeptidases which catabolise barley storage proteins during malting. Most of the endopeptidase activity in malt is due to the cysteine endopeptidases (Enari and Sopanen, 1986). A 37,000 Mr cysteine endopeptidase named EP-A was isolated by Koehler and Ho (1988) and a 30,000 Mr species, named MEP-1 and EP-B, has been isolated by three groups (Poulle and Jones, 1988; Phillips and Wallace, 1989; Koehler and Ho, 1990a). The isolation and characterisation of the endopeptidases has been recent in comparison to other hydrolytic enzymes such as the α- and β-amylases and (1-3, 1-4) β-D-glucanases. The activities of the starch and cell wall degrading enzymes during germination are recognised as important factors in the production of quality malt and are routinely screened in barley breeding programs around the world. The roles of the endopeptidases in malting are also important but as yet have not been fully assessed. The degradation of the storage proteins and the generation of amino acids for enzyme and protein synthesis are important functions of the endopeptidases but they also have a crucial role in the regulation of other hydrolytic enzymes.

The processes of malting barley and brewing beer involve a series of complex physiological and biochemical steps and the constituents of malt influence many stages of the brewing process. Proteolysis affects the fermentability of wort, colloidal stability, flavour and colour of beer (Enari, 1981). Insufficient proteolysis can cause poor brew house yields and filtration problems. Commercial proteases such as papain are used in brewing to assist the breakdown of proteins which cause hazes in beer (Rastogi *et al.*, 1988). Potassium bromate is sometimes added during malting to control the level of proteolysis, especially when gibberellic acid is used. The use of additives during malting and brewing is undesirable

considering the increasing demand for natural products. If proteins are optimally modified during malting the use of such additives would not be necessary.

1.2 GERMINATION OF THE BARLEY SEED

Germination begins after the seed imbibes water and is followed by many metabolic processes. A mature barley grain will germinate, given the right conditions, unless it is dead or dormant (Briggs, 1978). Physical and chemical barriers may retard germination. The seed coat (or pericarp) can impede the entry of water and solutes whilst the growth regulator abscisic acid (ABA) chemically inhibits seed germination (Webb and Waering, 1972). Mechanical damage to the pericarp enhances water and solute entry, thus increasing the rate of germination (Freeman and Palmer, 1984). 'Mechanical abrasion' of barley seeds is a commercial practice which is sometimes used to promote germination. Grains germinated under different environmental conditions (such as temperature and moisture content) undergo chemical changes at different rates but the chemical compositions and enzyme components of the seedlings also differ (Briggs, 1978). Seed germination is an important area in barley research as the production of a good malt relies on a high and uniform germination rate.

As germination continues, the seed reserves support the growing seedling until it can obtain nutrients from the environment. The endosperm contains reserves of starch, protein and cell wall β-glucans which are hydrolysed to smaller molecules by specific enzymes. Barley storage proteins are broken down by several peptidases to small peptides and amino acids which are transported to the developing embryo. This process depends on the co-ordination of physiological events between the embryo, aleurone layer, endosperm and scutellum. Growth regulators are synthesised in the embryo and secreted into the aleurone layer and scutellum. They influence the synthesis of hydrolytic enzymes and their release into the endosperm.

1.2.1 Secretion of the hydrolases.

The relative contributions of hydrolases from the aleurone and the scutellum to the mobilisation of seed reserves is a matter of conjecture. Mundy *et al.* (1985) isolated germ and aleurone layers from germinating barley and prepared mRNA from these tissues which was translated *in vitro*. The products of translation were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation with antibodies toward carboxypeptidase, α-amylase and endo-(1-3, 1-4)-β-D-glucanase. The results show that α-amylase and endo-(1-3, 1-4)-β-D-glucanase are synthesised primarily in the aleurone layer. Carboxypeptidase is predominantly synthesised in the scutellum and to a lesser extent in the aleurone. However some enzymes such as the endo-(1-3, 1-4)-β-D-glucanase isozymes appear to be secreted from both scutella and aleurone tissues. Isoenzyme I is secreted from isolated scutella and is also found in aleurone tissue whereas isozyme II is secreted from isolated aleurone layers (Stuart *et al.*, 1986).

The expression of the endopeptidase EP-B has recently been studied using *in situ* hybridisation and immunomicroscopy (Marttila *et al.*, 1993). EP-B mRNA was observed in the scutellar epithelium and the aleurone cells adjacent to the embryo after one day of germination. As germination progressed the mRNA levels were highest in the aleurone layer and proceeded to the distal end of the grain. On the first day of germination the EP-B protein was secreted into the endosperm from the scutellar epithelium and surrounding aleurone cells. EP-B secretion followed the expression of mRNA along the aleurone layer to the distal end of the seed in later stages of germination. Apparently both the scutellum and aleurone layer are able to synthesise and secrete EP-B. The secretion pattern for EP-A was examined, using similar methods, and appears to be the same as EP-B except that it is not secreted until day three of germination (Marttila *et al.*, 1992b). It appears that EP-B is the first to hydrolyse insoluble storage proteins in the endosperm and that EP-A is involved in further processing and production of smaller peptides.

The secretion and processing of the endopeptidases EP-A and EP-B were also studied using cDNA clones (Koehler and Ho, 1990b). The proteins encoded by clones of EP-B isozymes

contained large preprosequences preceding the sequences of the mature protein. The prosequences are processed after translation in a multistep fashion. The intermediate forms of EP-B are detected in aleurone layers and are secreted into the surrounding medium whereas EP-A is only secreted as a mature protein. Thus the endopeptidases appear to be differentially processed after translation.

An aspartic acid endopeptidase was detected in both the scutellum and proximal aleurone layer of resting seed. During germination it was found in all living tissues of the seedling, including the coleoptile, but was not secreted into the starchy endosperm (Marttila *et al.*, 1992a). It was concluded that the endopeptidase functions in the vacuoles of living tissue, unlike the cysteine endopeptidases which are secreted into the endosperm. However, an aspartic acid endopeptidase found in wheat was shown to hydrolyse the storage protein gliadin in the endosperm (Sarbakanova *et al.*, 1988).

1.2.2 Effects of growth regulators on the hydrolases.

Gibberellic acid (GA) influences the synthesis and secretion of several hydrolase enzymes, including endopeptidases, from the barley aleurone (Jacobsen and Varner, 1967). Paleg (1960) showed that α-amylase was produced by distal half seeds and isolated barley aleurone layers in response to exogenous gibberellic acid. Nicholls *et al.* (1986) noted that α-amylase production, in the absence of exogenously applied GA, differs between barley seeds of the same genetic stock that have been grown in different environments. They concluded that the environmental conditions in which the parent barley is grown influences the amount of GA released during germination and consequently the hydrolytic activity of the germinating seed.

Although many of the effects of GA have been studied, there is still debate over how GA actually influences enzyme activity. Events of hormone perception, signal propagation, regulation of transcription and other processes influencing gene expression are subjects which are currently under investigation (Jones and Jacobsen, 1991). Studies on the GA induction of α-amylase have indicated that expression is controlled at the transcriptional

level. GA increases transcription resulting in rapid production of mRNA in the aleurone for α-amylase (Higgins *et al.*, 1976), (1-3, 1-4)-β-D-glucanase (Mundy and Fincher, 1986) and the barley peptidase aleurain (Rogers *et al.*, 1985).

The presence of GA stimulates the activity of barley endopeptidases during germination (Jacobsen and Varner, 1967). GA is required for the synthesis of peptidases in the aleurone layer and their secretion into the endosperm (Hammerton and Ho, 1986). Koehler and Ho (1990b) examined the hormonal regulation of EP-A and EP-B. Time course experiments monitoring the *in vivo* synthesis of pro-EP-A and pro-EP-B in response to GA_3 showed that the two enzymes are differentially regulated. This has also been shown at the mRNA level with the α-amylase enzymes (Nolan and Ho, 1988a; Nolan and Ho, 1988b).

GA_3 is not required for the synthesis of carboxypeptidases but is necessary for their secretion into the endosperm (Hammerton and Ho, 1986). Thus GA influences endosperm hydrolysis by directing the transcription and translation of hydrolases and/or their secretion into the endosperm. Although GA_3 has been used for many of these studies it has been suggested that the main endogenous gibberellin in germinating barley is GA_1 (Radley, 1967).

Abscisic acid (ABA) opposes all GA responses in aleurone layers of barley (Higgins *et al.*, 1982). It retards germination and the release of hydrolytic enzymes. The first stable product of ABA catabolism is phaseic acid (PA). The conversion of ABA to PA is enhanced by ABA and it is proposed that ABA stimulates its own metabolism (Uknes and Ho, 1984). PA has inhibitory effects similar to ABA and is thought to contribute to ABA action (Nolan and Ho, 1988b).

The mode of action of ABA is still unclear. It was proposed that ABA directly prevented either the translation or transcription of α-amylase. Nolan and Ho (1988b) suggested that GA suppresses the synthesis of a protein which destabilises the mRNA of α-amylase. The removal of GA or the addition of ABA or PA prevents the suppression of the destabilising protein and consequently α-amylase mRNA is destabilised. Mundy and Fincher (1986)

studied the affects of GA and ABA on the translatable mRNA for (1-3, 1-4)-β-D-glucanase in barley aleurone layers. They concluded that (1-3, 1-4)-β-D-glucanase mRNA levels increased with the addition of GA but this effect was blocked by the addition of ABA.

1.3 PROTEOLYSIS IN THE BARLEY SEED

The breakdown of protein reserves in germinating barley occurs in three main stages (Mikola, 1983a). Initial hydrolysis of specific proteins (mainly globulins) is catalysed *in situ* by endopeptidases present in the embryo. This phase of proteolysis is essential for the production of amino acids which are consequently used for protein synthesis. The second stage of protein breakdown occurs as a rapid phase of proteolytic activity which is due to the synthesis of endopeptidases. These enzymes are secreted into the endosperm where they degrade the main storage proteins (the prolamins and glutelins) to peptides and amino acids. The peptides are degraded further to amino acids by exo-peptidases during the third stage of proteolysis.

1.3.1 Proteolytic enzymes.

There has been confusion as to the nomenclature used to describe the proteolytic enzymes in plants, in particular with the term peptidase. Barrett and McDonald (1986) proposed that endopeptidase and exopeptidase were more rational and less confusing than the terms endo- or exo-proteinase and endo- or exo-protease. The terminology and classification systems recommended for describing plant proteases have been outlined in a review by Storey and Wagner (1986). Endopeptidases are further classified into four sets according to the mechanism of the active site. These are serine, cysteine, aspartic acid and metallo-endopeptidases. Exopeptidases cleave terminal peptide bonds and are classified according to substrate specificity. The groups most commonly found in plants are: carboxypeptidases which cleave amino acids units from the α-COOH terminus of a peptide and amino-peptidases which remove amino acid units from the α-NH_2 terminus.

The rate of proteolysis of the protein reserves appears to be limited by endopeptidase activity (Burger and Schroeder, 1976). Endopeptidases catalyse the initial breakdown of the insoluble storage proteins to soluble peptides by cleaving internal peptide bonds. Exopeptidases are thus dependent on endopeptidase activity for the release of available substrate.

1.3.1.1 Endopeptidases.

Most resting seeds appear to contain pepstatin-sensitive endopeptidase activity (Sarkkinen *et al.*, 1992). An aspartic acid endopeptidase purified from resting wheat seeds hydrolysed ω- and γ-gliadins, suggesting that the enzyme may initiate proteolysis of storage proteins (Belozersky *et al.*, 1989). Doi *et al.* (1980) described an aspartic acid endopeptidase present in rice endosperms. Morris *et al.* (1985) detected high amounts of a pepstatin-sensitive endopeptidase in the embryo and endosperm of resting barley grains. Two heterodimeric aspartic acid endopeptidases were purified from resting barley grains by affinity chromatography on a pepstatin-Sepharose column, followed by ion exchange chromatography on a Mono S column (Sarkkinen *et al.*, 1992). A 48 kDa enzyme and a smaller 40 kDa enzyme were isolated. Amino acid sequence analysis revealed that the 40 kDa enzyme was formed by proteolytic processing of the 48 kDa enzyme. The enzymes did not hydrolyse globulins isolated from the barley embryo or denatured α-hordothionin which suggests that they are substrate specific. These enzymes are evidently not responsible for the hydrolysis of globulins in the embryo and aleurone layer described by Mikola (1983a). An aspartic acid endopeptidase has been detected in barley leaves (Kervinen *et al.*, 1990) which shows that this type of enzyme is not tissue specific. Sarkkinen *et al.* (1992) speculated that the enzymes may have a regulatory role in the processing of other specific regulatory proteins.

Rastogi and Oaks (1986) studied the action of endopeptidases in germinating barely seeds by observing the hydrolysis patterns of a hordein substrate. They concluded that the initial release of large polypeptide fragments was due to the action of an enzyme(s) that was not

dependent on gibberellic acid and that further hydrolysis was mediated by enzymes which were induced by the presence of exogenously applied gibberellic acid.

Several endopeptidases are synthesised in the germinating barley grain. Inhibitor studies have shown that most of the proteolytic activity is due to cysteine endopeptidases which are active in the endosperm which has a slightly acid pH of about 5 (Enari and Mikola, 1967). Early studies with inhibitors indicated the presence of at least two cysteine endopeptidases as well as a metal activated endopeptidase component (Enari and Mikola, 1967; Burger *et al.*, 1970; Sundblom and Mikola, 1972). A recent study by Wrobel and Jones (1992) utilising electrophoresis and 'in-gel' substrates estimated a minimum of seven endopeptidases to be present in five-day germinated grains. Four cysteine endopeptidases and an aspartic acid endopeptidase (also present in resting grains) were active at acidic pH. The other two enzyme fractions were active at neutral pH, one was partially inhibited by serine endopeptidase inhibitors and the other could not be identified by inhibitor studies. It was suggested that these two fractions were composed of multiple enzymes which would obfuscate the inhibition study results.

Cysteine endopeptidases of similar size and function have been isolated from germinating barley seeds by three main groups. Poulle and Jones (1988) isolated a green malt endopeptidase from barley cv. Morex which had an apparent Mr of 30,000 and a pH optimum of 3.8 for the hydrolysis of haemoglobin. It was inhibited by leupeptin indicating that it was a cysteine endopeptidase. They estimated that it was one of the predominant cysteine proteinases secreted into the endosperm during barley germination. They also studied its action on barley proteins. The hydrolysis of hordeins, incubated in the presence of the endopeptidase, was observed by utilising SDS-PAGE. Hordeins extracted with 2% (v/v) 2-ME were rapidly hydrolysed into small fragments of less than 14,000 Mr (Jones and Poulle, 1987). Hydrolysis of polypeptides with known amino acid sequences (α- and β-hordothionins) by the enzyme demonstrated that cleavage sites occured where leucine, and to a lesser extent tyrosine and valine, were positioned one residue away from the hydrolysed bond (Jones and Poulle, 1990).

Phillips and Wallace (1989) isolated a cysteine endopeptidase from germinating seeds of cv. Schooner with a Mr of 29,000 which separated into two main bands during IEF with pI values of 4.2 and 4.3. The endopeptidase (MEP-1) had pH optima for the hydrolysis of haemoglobin, hordein and azocasein of 4.0, 4.5 and 5.5 respectively. They also pointed out that the presence of 2-ME increased the activity of MEP-1 against hordein suggesting that the reduction of sulfhydryl groups renders the hordeins more susceptible to proteolytic attack.

Koehler and Ho (1990a) purified a 30,000 Mr endopeptidase from Himalaya barley which was named EP-B. The enzyme was strongly inhibited by leupeptin and *p*-hydroxy-mercuribenzoate indicating that EP-B was a cysteine endopeptidase. The N-terminal amino acid sequence of 22 residues showed 81 % and 90 % similarity to papain and a cysteine endopeptidase isolated from *Vigna mungo* respectively. EP-B hydrolysed haemoglobin and azocasein optimally at pH 4.6 and 4.5 respectively and the enzyme was sensitive to high pH. Degradation of hordein, enriched in the B and D fractions, was monitored on SDS-PAGE gradient gels (10-20 %) and products of hydrolysis were found ranging from 2,000 to 25,000 Mr.

Koehler and Ho (1988) isolated a gibberellic acid induced, 37,000 Mr cysteine endopeptidase named EP-A from embryoless half seeds of Himalaya barley. This enzyme was more stable than EP-B at high pH and had a pH optimum of 5.0 against haemoglobin. Three isozymes detected on a native PAGE haemoglobin gel were closely related, having overlapping N-terminal amino acid sequences, and could be products of a single gene. Koehler and Ho (1990a) established a 90% similarity between the N-terminal amino acid sequences of EP-A and EP-B and obtained cDNA clones for both enzymes. Genomic DNA gel blot analysis found that there were four or five genes for cysteine endopeptidases (Koehler and Ho, 1990b).

The presence of endogenous endopeptidase inhibitors has been reported in resting barley seeds (Mikola and Enari, 1970). Two barley fractions which inhibit malt endopeptidases have been partially purified, both were heat stable and inhibited at least 75% of the

endopeptidase activity present in a crude malt extract, measured against haemoglobin (Jones, 1991). The concentration of the inhibitors declines during germination and are not thought to restrict endopeptidase activity during malting (Mikola and Enari, 1970). However, proteolysis is reduced during mashing when unmalted barley is added to the wort as a brewing adjunct (Enari *et al.*, 1964).

1.3.1.2 Carboxypeptidases.

Carboxypeptidases hydrolyse terminal peptide bonds cleaving off one amino acid at a time. Five carboxypeptidases, extracted from the endosperm of germinating barley seeds at pH 5, have been separated by ion-exchange chromatography (Mikola, 1983b). The enzymes (I to V) are all serine dependent carboxypeptidases. Carboxypeptidases I, II and III have been isolated and characterised (Breddam *et al.*, 1983; 1985; Breddam and Sørensen, 1987). Enzymes I and II are dimers of 100,000 and 120,000 Mr respectively. Each monomer consists of two peptide chains which are linked by disulphide bonds (Breddam *et al.*, 1985). cDNA analysis has shown that both chains of enzyme I are translated from a single mRNA and that the mature proteins are formed after endoproteolytic excision from a precursor polypeptide chain (Doan and Fincher, 1988). Enzyme III is a monomer of 48,000 Mr while enzymes IV and V have estimated Mrs of 170,000 and 95,000 respectively (Mikola 1983b). The action of each enzyme appears to complement the others; carboxypeptidases I, II and III cleave uncharged aliphatic, positively charged and aromatic amino acid residues respectively from the C terminal end (Breddam and Sørensen, 1987) while enzymes IV and V hydrolyse peptides containing a penultimate proline residue (Mikola, 1983b). This suggests that the five enzymes can hydrolyse peptides only by sequential co-operation.

1.3.1.3 Aminopeptidases and dipeptidases.

The third stage of protein breakdown occurs in the scutellum. Peptides which are transported from the endosperm are rapidly hydrolysed by aminopeptidases and dipeptidases to amino acids (Mikola and Mikola, 1980). These enzymes have optimal activities at the neutral to alkaline pH of the scutellum.

Aleurain is another barley cysteine endopeptidase which was first recognised as a cDNA derived from gibberellic acid treated-aleurone cell mRNA (Rogers *et al.*, 1985). The N-terminal amino acid sequence of 22 residues has 59 % similarity to MEP-1. It has since been purified to homogeneity from barley leaves and characterised (Holwerda and Rogers, 1992) and the gene has been reported to be located on barley chromosome 1 (Li, *et al.*, 1991). The enzyme has now been described as an aminopeptidase according to its substrate specificity. It has Mr of 32,000 and separates into two charged forms, on two dimensional electrophoretic gels, with pI values of 6.0 and 6.1 (Holwerda and Rogers, 1992). Inhibition studies indicate that a cysteine residue is present in the active site. In the short term it is most active at pH 6.8 but after 16 h incubation it is more stable at pH 4.8. Aleurain is expressed constitutively in all barley tissues yet its function in the plant is obscure.

Aminopeptidases and dipeptidases have been detected in the embryo and aleurone layers of resting barley grains (Sopanen and Mikola, 1975). The transport of peptides from the endosperm to the embryo appears to be an efficient pathway for nitrogen transfer. The amino and dipeptidases break down peptides which were transported via peptide carriers into the scutellum (Salmenkallio and Sopanen, 1989). The amino acids released are converted to glutamine and transported to the developing seedling.

1.3.2 Uptake of peptides by the scutellum.

The products of hydrolysis from the endosperm and scutella epithelial cells are transported to the developing seedling (Sopanen, 1984). Amino acids and peptides are taken up by separate active transport systems (Higgins and Payne, 1978). The peptide carrier has a broad specificity and can transport di-peptides and oligo-peptides containing up to 5 amino acid residues (Higgins and Payne, 1978; Sopanen 1984). The uptake of one peptide is inhibited competitively by other peptides but not by amino acids (Sopanen and Väisänen, 1985). The rapid increase of peptide transport at the beginning of germination suggests that the uptake of peptides by the scutellum is a very important process in the mobilisation of seed reserves.

Sopanen and Väisänen (1985; 1986) studied the transport systems of glutamine and proline in the scutella of germinating barley seeds. Glutamine is taken up by two or more rather unspecific amino acid carrier systems. The transport of glutamine is of particular interest since it is the most abundant amino acid in hordein (Shewry and Miflin, 1985). Glutamine also regulates the uptake of other amino acids in the scutellum by repressing the synthesis of the amino acid carrier protein (Nyman *et al.*, 1983). Proline transport is mediated by a specific proline uptake system presumably because it has an unusual imino group. The transport of proline is important as it is the second most abundant amino acid in hordein (Shewry and Miflin, 1985).

1.4 BARLEY PROTEINS

1.4.1 Albumins and globulins.

Barley proteins are classified on the basis of their solubility in certain solvents as defined by Osborne (1924). Proteins extracted in water and salt solutions are termed albumins and globulins respectively. These proteins primarily have a metabolic function. Some, however, are present in large amounts indicating that they have secondary roles as storage proteins. Examples of these are β-amylase and protein Z, the former comprising up to 2 % of the total protein (Nishimura *et al.*, 1987).

1.4.2 Prolamins and glutelins.

Proteins extracted in aqueous alcohol solutions, with or without a reducing agent, are the prolamins, so named because they are rich in proline and glutamine. These have been classified further into three groups; high molecular weight (HMW), sulphur rich (S-rich) and sulphur poor (S-poor) prolamins (Shewry and Miflin, 1985). The remaining proteins, extracted in dilute acid or alkali, are termed glutelins. This group is composed mainly of structural proteins such as cell wall glycoproteins, ribosomal and endoplasmic reticulum proteins.

Complications arise with this classification system as the conditions of preparation influence the type of protein that is extracted. Many factors such as the fineness of milling, physiological state of the seed, temperature, type of alcohol and the presence of reducing agents affect the quantity and type of protein extracted from the meal. This problem is highlighted when prolamins and glutelins are extracted from cereals. Many proteins in cereals are present as disulphide-linked aggregates which are difficult to extract in aqueous alcohol solutions. The reduction of disulphide bonds disaggregates these sulphur rich proteins and, once reduced, the proteins are more soluble in alcohol solutions. Thus it becomes difficult to distinguish between prolamins and glutelins purely by their solubility in aqueous alcohol solutions.

The prolamins in barley are storage proteins and have the specific name of hordeins. The HMW, S-rich and S-poor prolamins correspond to the D, B and C hordeins respectively. Hordeins are synthesised in the endosperm and the amount of each group produced is regulated by the nutritional status of the plant (Rahman *et al.*, 1983). Hordeins account for 30-50 % of the total protein in the grain. Of the total hordeins the B, C and D components are 70-90 %, 10-20 % and 2-4 % respectively (Shewry and Miflin, 1985). Hordeins are synthesised relatively late in the development of the seed, when the endosperm enters the rapid growth phase, and continue to accumulate until maturity. During development they appear as discrete protein bodies which are thought to be derived from the rough endoplasmic reticulum (Miflin *et al.*, 1983).

1.4.2.1 D hordein.

The HMW D hordeins have been estimated (by SDS-PAGE) to have Mr > 90,000 (Miflin *et al.*, 1983). They are rich in lysine and low in proline residues relative to the other hordein groups. The presence of cysteine enables these proteins to form disulphide-linked aggregates. Shewry *et al.* (1988) have shown a high degree of homology between D hordein of barley, HMW secalins of rye and HMW glutelins of wheat. This indicates that D hordein is in fact a glutelin rather than a prolamin. Determining the homology of proteins among cereals will greatly aid the classification of protein groups.

1.4.2.2 B hordein.

Cysteine residues account for the presence of disulphide aggregates in B hordeins. Disulphide links may occur as intermolecular and intramolecular disulphide bonds. The Mr of B hordeins range from 36,000 - 44,000 as estimated by SDS-PAGE (Miflin *et al.*, 1983). These hordeins have been separated by two dimensional electrophoresis into three structurally distinct groups (BI, BII and BIII) (Faulks *et al.*, 1981). Groups I and III are 80% homologous and are composed of two domains. The amino-terminal domain is proline rich and contains a series of hepta, hexa and heptapeptide repeats whereas the carboxy-terminal domain is poor in proline and non repetitive (Shewry *et al.*, 1988). The secondary protein structure of BI hordein was shown to be 28-30% α-helical form, 8-10% β-sheets and the remainder of aperiodical structure (Shewry, 1988). It is noteworthy that the positions of the cysteine residues in the B hordeins are highly conserved (Forde *et al.*, 1985).

1.4.2.3 C hordein.

The amino acid composition of C hordein is poor in lysine, rich in proline and lacks charged amino acids. The Mr estimated by SDS-PAGE ranges from 40,000 - 80,000 (Miflin *et al.*, 1983). It contains no cysteine and only a small amount of methionine so it does not participate in disulphide aggregates. C hordein is composed primarily of tandem repeats of an octapeptide and the secondary structure is a loose spiral composed of β-turns.

The function of storage proteins is to provide a source of carbon, nitrogen and sulphur to the germinating seed. The nutritional status of the plant will affect the proportion of hordein components deposited during grain maturation. An environmental study by Marchylo and Kruger (1984) showed that the interaction of climate and site significantly affected the proportion of B to C hordeins. With ample available nitrogen the amount of C hordein is likely to increase whereas B hordein increase depends on the amount of available sulphur (Rahman *et al.*, 1983).

1.4.3 Hordein degradation.

Reversed phase-HPLC (RP-HPLC) has been used to assess hordein degradation during germination. Marchylo and Kruger (1985) analysed malt samples and found that hordein content decreased during germination. In some cases the chromatograms of malt and grain differed in the proportion of various components which indicates that certain hordeins may be preferentially broken down during malting or denatured during kilning. About 30 % of total hordein is degraded during malting (Smith, 1990). There appeared to be no major intermediate hordein products in malt which suggests that hordein is degraded to soluble amino acids or peptides. The degradation products may be able to be detected on amino acid or peptide reversed phase columns.

Marchylo *et al.* (1986) studied the hordeins of two contrasting malting quality barleys and recorded differences between the amounts of B, C and D hordeins in the different cultivars. The decrease in the total hordein during malting differed between cultivars as did the rate of break down of each hordein group. Hordein was modified more rapidly in the better malting cultivar. D hordein was degraded most rapidly followed by B and then C in samples extracted in 50 % (v/v) propanol, 1 % (w/v) DTT. However greater amounts of D hordein were detected in 8 M urea, 1 % (w/v) DTT extractions. This D hordein fraction was detected throughout malting, suggesting that it is firmly bound in the endosperm and less susceptible to proteolytic degradation than the more easily extractable fraction. About 80% of D hordein was present in the tightly bound fraction (Marchylo *et al.*, 1986).

Skerritt and Henry (1988) quantified the hydrolysis of hordeins using enzyme- and radio-immunoassays. Carbohydrate modification and malt extract were significantly correlated with antibody binding throughout malting in the varieties tested. Skerritt (1988) showed that hordein groups were degraded at different rates during malting of barley varieties which differed in malting quality. In general hordeins were degraded faster in the better malting varieties.

1.4.4 Gel protein.

Another barley protein fraction which is thought to be related to quality is the 'gel protein' fraction. Gel protein is a sodium dodecyl sulphate (SDS) insoluble protein which has been isolated from wheat (Graveland *et al.*, 1982) and barley (Smith and Simpson, 1983 ; Smith and Lister, 1983). It is extracted in SDS (15 g/l) and, after centrifugation, forms a gel layer on top of the pellet. Gel protein isolated from wheat flour was found to have the same chemical structure as glutenin (Graveland *et al.*, 1982). As glutenin is a name for wheat proteins, gel protein is generally used for other cereals.

Marchylo *et al.* (1986) examined gel protein by RP-HPLC and determined that it consisted of both B and D hordeins. It is a high molecular weight protein comprised mainly of B and D hordeins (Smith and Simpson, 1983). The presence of cysteine in these hordeins results in the formation of disulphide aggregates. Smith and Lister (1983) suggested that the amount of aggregated proteins in the gel was determined by the concentration of D hordein. Moonen *et al.* (1987), under the conditions they describe, determined that the barley gel protein HMW subunit generally had 6 cysteine residues. Of these 4-5 are located at the termini and 1-2 toward the centre of the subunit. They have proposed a model of gel protein in which the HMW subunits are linked by inter and intra chain disulphide bonds in a head to tail formation. LMW subunits are linked to each HMW subunit by disulphide bonds.

The amount of gel protein present in wheat flour is correlated with bread-making quality (Graveland *et al.*, 1982) and the disulphide bonding characteristics of gel protein are thought to contribute to the viscoelastic properties of dough. Smith and Lister (1983) reported that the amount of gel protein isolated from barley flour compared to total protein was inversely correlated with malting quality (as determined by % malt extract). They proposed that the malting quality of barley varieties could be predicted by the amount of gel protein extracted from the flour. More recently, Skerritt and Janes (1992) studied the relationship between the content and composition of gel protein and malting quality, using protein assays, electrophoresis and HPLC. They confirmed that greater amounts of gel

protein, specifically B hordein subunits, could be extracted from poor malting samples. A relationship between amount of disulphide and sulphhydryl groups in gel protein and malting quality was not observed. They concluded that the number and orientation of disulphide bonds between subunits in barley protein complexes may influence malting quality.

It has been demonstrated that disulphide linked aggregates inversely affect the rate of lauter-tun filtration (Van den Berg *et al.*, 1981). The reducing conditions during mashing also influence the rate of filtration indicating the importance of the oxidation state of disulphide bonds (Van den Berg and Van Eerde, 1982). Moonen *et al.* (1987) concluded that negative brewing properties are caused by a disulphide bonded network of gel protein that prevents water from extracting material from the modified endosperm. Glutelins and gel protein appeared to aggregate which contributing to this impenetrable protein layer which also causes problems during filtration.

1.5 ASPECTS OF PROTEIN AND PROTEOLYTIC ACTION RELATING TO PROCESSING QUALITY

Malting is the process of germinating and kilning barley. Barley grains are initially steeped in water for a period of approximately two and a half days. Germination proceeds under conditions of controlled temperature, humidity and aeration and is halted after 4-5 days by kilning. Kilning involves heating the grains to temperatures around 80°C for a period of about 24 - 48 hours. During this time many of the components affecting the aroma, colour and flavour of malt are produced from the modified carbohydrate and nitrogenous compounds. Metabolic activity is arrested as the seed dries out.

Mashing involves a hot water extraction of soluble material from milled malt or grist. The extract is filtered through a bed of insoluble residue (mainly husk) and allowed to settle after mashing. The filtered product is termed the wort which is further processed into beer. The specific gravity of the wort is used to estimate the malt extract value which is one of the parameters used to estimate malting quality. A well modified endosperm is achieved

through adequate hydrolysis of cell wall components. Starch and protein and the action of proteolytic enzymes may be involved in each of these steps.

The action of endopeptidases appears to be a determinant of malting quality. Morgan *et al* . (1983) correlated malt extract with endopeptidase activity and found that endopeptidase activity accounted for 20% of the variability of the HWE. Some of the many ways in which protein and proteolytic activity effect the composition of malt and wort are discussed below.

The degradation of cell walls is necessary for starch granules and storage proteins to be exposed to attack by hydrolytic enzymes. Incomplete degradation results in the retention of high molecular weight β-glucans in the beer, which can cause clarity and filtration problems (Bamforth, 1985). The main constituents of the cell wall, the β-glucans, are degraded by (1-3, 1-4)-β-D-glucanases. It has been proposed that proteinaceous material is covalently bound to the β-glucan in the barley endosperm cell wall (Forrest, 1977) although the precise nature of the linkage has not been determined. It has been proposed that proteolysis is a pre-requisite for cell wall degradation. A putative acidic carboxypeptidase termed "β-glucan solubilase" has been reported to catalyse the first step of cell wall degradation (Bamforth and Martin, 1981). Baxter (1978) found that the presence of purified carboxypeptidase fractions reduced the viscosity of β-glucan extracted from endosperm walls. However, this has been a controversial area as it is difficult to conceive how a carboxypeptidase could cleave a bond between protein and β-glucan. Brunswick *et al.* (1988) investigated the degradation of isolated barley endosperm call walls by purified endo-(1-3, 1-4)-β-D-glucanases and malt extracts. They showed that degradation by isolated endo-β-glucanases was limited but was increased by the presence of crude malt extract. However, cell wall degradation by the β-glucanases was not altered after pretreatment with a general protease (proteinase K) and α-amylase. They concluded that proteolysis may not be a pre-requisite for β-glucan degradation.

The hydrolysis of starch to soluble carbohydrates is important to produce the requisite amount of fermentable sugars in the wort and endopeptidase action is indirectly involved in this process. Starch granules are largely embedded in a protein matrix (Palmer, 1972)

which is postulated to limit the action of α-amylase. Slack *et al.* (1979) showed that high Mr B and C hordeins are closely associated with starch granules and that the protein surrounding starch granules inhibited amylolysis. Furthermore the hordeins were modified by cysteine or malt endopeptidases. Smith and Gill (1986) concluded that about 15 % of the original starch granules remain in the spent grains and are associated with protein. β-Amylase is a protein which is deposited in a bound form on the surface of starch granules (Hara-Nishimura *et al.*, 1986). The action of malt endopeptidases converts the enzyme from the bound to a free, more active state (Guerin *et al.*, 1992a; Sopanen and Laurière, 1989). Consequently endopeptidases may be responsible for removing protein surrounding starch granules and rendering them open to attack by the amylases as well as increasing the activity of β-amylase.

The action of malt endopeptidases affects stages of processing beyond malting and kilning. The products of proteolysis are a matter for concern throughout the brewing process and are present in beer. Foam stability, or head retention, is an important property of beer. Hydrophobic proteins derived from the repetitive domains of the hordeins which are resistant to proteolytic attack are thought to contribute to foam and lacing properties in beer (Smith, 1990). Yokoi and Tsugita (1988) isolated three of the major foam proteins of 10,000, 8,000 and 40,000 Mr by SDS-PAGE and HPLC. The 40,000 Mr protein is identical to protein Z of barley (Sørensen and Ottesen, 1978). Asano and Hashimoto (1980) characterised proteins of 40,000 and 15,000 Mr from beer, as well as some higher Mr proteins. A positive correlation between the amount of beer proteins and head retention was established. Immunological studies showed that the proteins were mainly produced during germination. The amount of 'foam proteins' increased as endopeptidase activity increased which indicates that endopeptidases are instrumental in releasing these proteins during germination. However, excessive endopeptidase activity results in degradation of the foam proteins (Palmer and Bathgate, 1976).

Chill hazes are formed from soluble materials which precipitate during storage and shorten the 'shelf-life' of beer. Asano *et al* (1982) characterised four proteinaceous fractions from

beer that were responsible for chill haze. The fractions ranged from 1,000 Mr to 40,000 Mr. Each of the fractions readily combined with polyphenols to form hazes. A positive correlation between proline content and affinities for polyphenols was established. Immunological studies confirmed that the haze forming proteins were derived from hordein (Asano *et al.*, 1982). The proline content of hordein appears to be important in the formation of hazes as the free oxygen atom in the pyrrolidine ring forms hydrogen bonds with hydroxyl groups of polyphenols.

1.6 SUMMARY

An assembly of endopeptidases is present in the germinating barley seed and although several have already been isolated and characterised our knowledge of these enzymes is by no means complete. More specific detail is required about the endopeptidases from the point of basic research as well as a means of benefiting the malting and brewing industries. It is important to determine both the number and biochemical class of the endopeptidases in germinating barley. Characterisation of the barley endopeptidases requires a reconciliation of the results obtained at both the protein and DNA level. Information is also required regarding the sites of synthesis and secretion, mechanisms of control and the substrate specificities of these enzymes. The biochemical and genetic characterisation of the endopeptidases, analysis of their action during germination and the estimation of their contribution to the quality of malt and beer are important research goals which need to be addressed.

CHAPTER 2.

MATERIALS AND GENERAL METHODS

2.1 Materials.

2.1.1 Plant material.

Barley (*Hordeum vulgare* ssp *vulgare*) seeds were supplied by the Waite Barley Quality Evaluation Laboratory, Department of Plant Science, Waite Institute.

Wild barley seeds (*Hordeum vulgare* ssp *spontaneum*) from Israel, Turkey and Iran were supplied by Dr A. H. D. Brown, Division of Plant Industry, C. S. I. R. O., Canberra. The seeds of the F_2 generation of a cross between an accession of wild barley, *Hordeum vulgare* ssp *spontaneum* and barley, *Hordeum vulgare* ssp *vulgare*, cultivar Clipper were also supplied by Dr A. H. D. Brown. The wild barley accession (Commonwealth Plant Introduction number 109861) originated 9 km east of Siverek, Turkey.

Betzes barley and Chinese Spring disomic and ditelosomic addition lines were supplied by Drs A. K. M. R. Islam and K. W. Shepherd, Department of Plant Science, Waite Institute.

2.2 Methods.

2.2.1 Germination of barley seeds.

2.2.1.1 Preparation of green malt.

Seeds were surface sterilised with sodium hypochlorite solution (5% (v/v)) for 5 min and rinsed five times in distilled water. The third rinse included soaking the seeds for 30 min. The seeds were placed on absorbent, wetted cardboard in a sealed petri dish and left in a dark humid environment at 15°C for four days.

2.2.1.2 Preparation of malt.

Malt samples were prepared essentially by the method of Sparrow *et al.* (1987). Malting was performed in a micromalter (Phoenix Systems) in the Waite Barley Quality Evaluation Laboratory under the following schedule.

Step	Cycle time (h)	Temperature (°C)	Time on rollers (%)
Wash	0.17	15	100
Rinse	0.25	15	100
Pre-condition	0.25	15	100
Steep 1	8	15	25
Air rest 1	8	15	20
Steep 2	8	15	25
Air rest 2	94	15	22
Kiln 1	6	50	100
Kiln 2	8	55	100
Kiln 3	10	63	100
Kiln 4	4	70	100
Tank drain	0.25	20	100

2.2.2 Electrophoretic techniques.

2.2.2.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Discontinuous SDS-PAGE, based on the method of Laemmli (1970), was performed at room temperature in a Bio Rad Mini-PROTEAN II system. Separating gels were prepared by combining 2.25 ml acrylamide solution [acrylamide (30% (w/v)): bisacrylamide (0.8 % (w/v))], 3.13 ml Tris-HCl pH 8.8 (0.75 M); SDS (0.2% (w/v)); 0.88 ml H_2O; 8.75 µl Temed and 23.5 µl ammonium persulphate (10% (w/v)). The solution was mixed and quickly poured between two glass plates supported by the BioRad casting stand. The separating gel was overlayed with H_2O (approximately 1 ml) and left to polymerise for at

least 30 min.

The stacking gel was prepared by combining 0.25 ml acrylamide solution (as above), 1.25 ml Tris-HCl pH 6.8 (0.25 M); SDS (0.2% (w/v)); 0.98 ml H_2O and 2.75 µl Temed. This solution was de-aerated for 10 min before 11 µl ammonium persulphate (10% (w/v)) was added and gently mixed. The stacking gel was poured on top of the separating gel with the comb set in place and left to polymerise for at least 45 min. Stacking gels were always poured to be twice the length of the comb teeth. After polymerisation the comb was removed and the wells were washed at least twice to remove any unpolymerised acrylamide.

Gels were covered with running buffer [Tris-HCl pH 8.3 (0.1 M); SDS (0.1 % (w/v); glycine (0.2 M)] and electrophoresed under constant voltage, stacking at 50 V and running at 100 V. The average running time was 1.25 h.

2.2.2.2 Horizontal IEF.

Horizontal IEF, polyacrylamide gels (0.5 mm thick) were cast onto the hydrophilic side of gel bond PAG film (FMC BioProducts, USA) using the LKB gel ultrathin casting unit (Pharmacia, Sweden) according to the manufacturers instructions. The gel solution was composed of 10 ml of distilled water; 3.6 ml glycerol (50 % (v/v)); 3.3 ml acrylamide solution [acrylamide (30% (w/v)): bisacrylamide (0.8 % (w/v))]; 1.0 ml of ampholytes and 25 µl of Temed. The solution was de-aerated for 10 min before the addition of 50 µl of ammonium persulphate (10 % (w/v)) and quickly poured onto the gel bond in the casting unit.

The pH gradients were achieved by mixing different ampholyte ranges with the gel solution and the choice of anode and cathode solutions. These details are specified in the methods section concerned with each experiment.

Horizontal IEF was performed at 4°C, gels were prefocussed at 5 W, 1000 V for 30 min before samples were applied and then run at 20 W, 2000 V for 2 h. The distance between electrodes was approximately 9.5 cm. Crude samples were applied in 10 µl aliquots to 0.5

cm plastic loading wells, which were removed after 1000 Volt hours. IEF standards (broad range (4.6 - 9.6) supplied by BioRad, Australia) and purified MEP-1 were applied in 5 µl aliquots.

2.2.2.3 Vertical IEF.

Gels were prepared by mixing 10 ml of distilled water; 3.6 ml glycerol (50 % (v/v)); 3.3 ml acrylamide solution [acrylamide (30% (w/v)): bisacrylamide (0.8 % (w/v))]; 1.0 ml of ampholytes and 25 µl of Temed. The pH range of the gels was achieved by mixing LKB ampholytes pH 3.5-10, pH 4-6 and pH 3-3.5 in a ratio of 1: 2: 3, respectively. The solution was de-aerated for 10 min before 50 µl of ammonium persulphate (10 % (w/v)) was mixed in. The gels (0.75 mm) were poured in a BioRad casting stand between a glass plate and an alumina plate which was part of a Hoeffer Mighty Small II SE 250 kit. The gel was left to set for at least an hour. Vertical IEF was performed at 4°C using the Hoeffer Mighty Small II SE 250 unit, according to the manufacturer's instructions. The anode solution was sodium hydroxide (0.02 M) and the cathode solution was acetic acid (0.02 M). The gels were prefocussed at 5 W, 500 V for 30 min. Samples were applied to the sample wells with a microsyringe in 5 µl aliquots and run at 20 W, 700 V for 4 h.

2.2.3 Immunological detection of MEP-1 and immunologically related proteins.

Proteins were transferred from IEF or SDS-PAGE gels to 'Immobilon' PVDF membrane (supplied by Millipore, USA) as described in the methods section relating to each experiment. After transfer membranes were blocked for 2 h or overnight in a solution of skimmed milk (5% (w/v)) in a Tris-saline-tween solution (TST) composed of Tris-HCl (20 mM) pH 7.5; NaCl (0.5 M); Tween 20 (0.1 % (v/v)). The membranes were then incubated for 1 h with polyclonal antibodies, raised against MEP-1 in a rabbit (Phillips and Wallace, 1989), diluted 1:1000 with TST. After two 5 min washes with TST, membranes were incubated for another hour with goat anti-rabbit antibodies conjugated with alkaline phosphatase (BioRad, Australia) diluted 1:1000 with TST. The membranes were sequentially washed 2 x 5 min in

TST, 5 min in Tris-HCl (20 mM); NaCl (0.5 M) and 5 min in substrate buffer [Tris-HCl (0.1 M); NaCl (0.1 M); $MgCl_2$ (5 mM) pH 9.5]. The colour reaction was produced by incubation for 5 min in nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution which consisted of 330 µl of NBT (50 mg/ml in dimethylformamide) and 165 µl of BCIP (50 mg/ml in 70% dimethylformamide) in 50 ml substrate buffer. The membranes were then rinsed with EDTA (0.2 M) and finally with distilled water before air drying.

2.2.4. Protein assay.

Protein concentrations were estimated using the dye binding method described by Bradford (1976). Bovine Serum Albumin (BSA) was used as a standard.

CHAPTER 3.

MAPPING THE MEP-1 GENE.

3.1 INTRODUCTION

There is considerable interest in mapping the barley genome, the location of genetic markers and linkages with genes of interest being valuable tools for breeders in the development of improved barley varieties. The development of the disomic addition lines containing the complete complement of wheat chromosomes (cv. Chinese Spring) and a pair of barley (cv. Betzes) chromosomes 1, 2, 3, 4, 6 or 7 provides the opportunity to investigate the gene content of most of the barley chromosomes (Islam *et al.*, 1981; Soliman and Allard, 1989). In this study IEF and immunoblotting were employed to identify the barley chromosome containing the gene for the malt endopeptidase (MEP-1) isolated by Phillips and Wallace (1989). The ditelosomic addition lines (Islam, 1983) have also been examined to assign the gene to a barley chromosome arm.

An endopeptidase similar to MEP-1 has been isolated and cloned by Koehler and Ho (1990a, 1990b) who named it EP-B. The symbol for the gene encoding EP-B has been named *CepB* (Von Wettstein-Knowles, 1992). The endopeptidase (EP-B), isolated from germinating seeds of the barley cultivar Himalaya (Koehler and Ho, 1990a) has almost identical properties to MEP-1 with the exception of the reported pI values. Both proteins appear to resolve into two bands after IEF; MEP-1 at 4.2 and 4.3 and EP-B at 4.6 and 4.7. It was also shown that MEP-1 is a glycoprotein whereas EP-B is not glycosylated.

The genetic variation of enzymes between cereal varieties (both in a qualitative and quantitative sense) has many uses in plant breeding and related disciplines. Variation in enzyme activity, stability or time of expression can be valuable assets for crop improvement. The genetic diversity of wild barley (*Hordeum spontaneum*) is a valuable source of such

variation which can be integrated into new barley varieties (Frey, 1976). Variations of morphological, biochemical or molecular traits are also useful as markers for gene mapping and inheritance studies.

In this study accessions collected from Iran, Turkey, Israel and Australia were screened for variation of the MEP-1 banding pattern on IEF gels. These accessions are genetically diverse and many enzyme variants have previously been found within this collection (Brown *et al.*, 1978). One of these lines had been backcrossed to cv. Clipper and the resulting progeny was available for studying the linkage between endopeptidase (*CepB*), esterase (*Est2*) and a previously undescribed NADH diaphorase gene (*Ndh2*) loci on chromosome 3. A putative endopeptidase first detected by Hart *et al.* (1980) and given the gene symbol *Enp1* (Brown, 1983) was also examined.

The main aims of the work presented in this chapter are as follows:

i) To examine the relationship between EP-B and MEP-1, in particular their pI values and N-terminal amino acid sequences.

ii) To assign the MEP-1 gene to a barley chromosome (and chromosome arm) using disomic and ditelosomic wheat-barley addition lines.

iii) To investigate the variation of MEP-1 in wild barley (*H. spontaneum*).

iv) To estimate linkage relationships between the MEP-1 gene and other genes located on the same barley chromosome.

3.2 EXPERIMENTAL PROCEDURE

3.2.1 Plant material.

Betzes barley and Chinese Spring wheat-barley addition lines and ditelosomic addition lines were supplied by R. A. K. M. R. Islam and K. W. Shepherd, Department of Plant Science, Waite Institute.

Wild barley seeds (*Hordeum vulgare* ssp *spontaneum*) from Israel, Turkey and Iran were supplied by Dr A. H. D. Brown (C. S. I. R. O. Division of Plant Industry, Canberra). Linkage analysis was conducted on the F_2 generation of a cross between an accession of wild barley, *Hordeum vulgare* ssp *spontaneum* and barley, *Hordeum vulgare* (ssp *vulgare*), cultivar Clipper, supplied by Dr A. H. D. Brown (as above). The wild barley accession (Common-wealth Plant Introduction number (CPI) 109861) originated from a collection made by E. Nevo and D. Zohary, 9 km east of Siverek, Turkey.

3.2.2 Germination and extraction of barley seeds.

Seeds were germinated as in Section 2.2.1.1 except that the wheat-barley addition lines were germinated in the presence of gibberellic acid (7 ppm). Single seeds were mashed, on ice, with a tissue grinder in 1.5 ml Eppendorf tubes with sodium acetate (50 mM); 2-ME (1 mM) pH 4.5 at a concentration of 3 ml/g fresh weight. The tubes were then subjected to centrifugation for 10 min at 12,000 *g*. The supernatants were decanted into fresh tubes and kept on ice or at 4°C before use.

The F_2 generation seeds from the cross between wild barley (*Hordeum vulgare* ssp *spontaneum,* CPI 109861) and barley (*Hordeum vulgare* ssp vulgare, cultivar Clipper) were germinated and bisected before extraction. The half seeds containing the embryo were labelled and sent to A. H. D. Brown, Canberra, for isozyme analysis while the corresponding half seeds were extracted as above.

3.2.3 Analysis of MEP-1 in barley cvs. Schooner and Himalaya by horizontal IEF and immunoblotting.

Extracts of 5 day green malt of barley cvs. Schooner and Himalaya (prepared as in Section 3.2.2) and a sample of purified MEP-1 were loaded on to an horizontal IEF gel (Section 2.2.2.2). The pH gradient of the gel was achieved with 1.0 ml of Pharmalyte pH 2.5 - 5.0 (Pharmacia, Sweden). The anode solution was sulphuric acid (0.1 M) and the cathode solution was sodium hydroxide (0.1 M).

3.2.4 Transfer of horizontal IEF gels.

After separation by horizontal IEF, proteins were transferred to a PVDF membrane by passive diffusion. The gel was rinsed with acetic acid (0.7 % (v/v)) to remove any traces of sample from the surface. A piece of 'Immobilon' PVDF membrane (supplied by Millipore, USA), was equilibrated in acetic acid (0.7 % (v/v)) and placed on top of the gel followed by 3 pieces of Whatman 1 paper soaked in acetic acid (0.7% (v/v)), 5 pieces of dry paper and a glass plate. The gel was left to transfer for 30 min before the membrane was removed and probed with antibodies raised against MEP-1 as described in Section 2.2.3.

3.2.5 Chromosome location of *CepB* using the disomic and ditelosomic wheat-barley addition lines.

Extracts of the wheat-barley disomic addition lines and the wheat-barley ditelosomic addition lines were run on vertical IEF gels as described in Section 2.2.2.3. The proteins in the gel were electro-transferred to PVDF membrane in acetic acid (0.7 % (v/v)) for 1 h at 100 V in a mini transblot unit (supplied by BioRad, Australia) according to the manufacturer's instructions. The initial buffer temperature was 4°C. After transfer the membrane was probed with antibodies raised against MEP-1 as described in Section 2.2.3.

3.2.6 Horizontal IEF analyses of wild barley and the F_2 generation of the wild barley cross for *CepB* genotype.

The horizontal IEF procedure used for the analyses of the wild barley populations from Israel, Turkey and Iran is described in Section 2.2.2.2. The pH range was achieved by mixing LKB ampholytes (supplied by Pharmacia, Sweden) pH 3.5 - 10.0, pH 4.0 - 6.0 and pH 3.0 - 3.5 in a ratio of 1: 2: 3, respectively. The anode solution was NaOH (0.02 M) and the cathode solution was acetic acid (0.02 M).

The pH gradient of the IEF gels used for the analysis of the F_2 seedlings was achieved by using 0.5 ml of Pharmalyte pH 2.5 - 5.0 and 0.5 ml of the narrow range Pharmalyte pH 4.2 - 4.9 (supplied by Pharmacia, Sweden). The anode and cathode solutions are described above.

The proteins separated on IEF gels were transferred to PVDF membranes (described in Section 3.2.4) and the immunological detection system described in Section 2.2.3 was used to identify the MEP-1 bands.

3.2.7 Determination of pI values of proteins separated by IEF.

All pI values given are apparent and were estimated with reference to the pI markers. The pI markers (broad range IEF standards supplied by BioRad, Australia) were loaded onto the IEF gels and transferred to PVDF membranes. The coloured markers are visible at pI 4.6 on the membrane which resists shrinking and stretching and this section was cut away before the immunodetection procedure. The bands visualised by immunodetection were compared with the markers by reassembling the main part of the membrane with the strip of coloured standards.

3.2.8 N-terminal amino acid sequence.

The N-terminal sequence of MEP-1 (200 pmoles) was determined by Edman degradation. The amino acids were separated by HPLC on an Applied Biosystems 475A protein

sequencer. The analysis was undertaken in the Department of Biochemistry, University of Adelaide.

3.2.9 Isozyme analyses for esterase, endopeptidase and NADH diaphorase genotypes.

The distal halves of 78 germinating seeds were scored for *CepB* genotype (Section 3.2.6) and the remaining half seeds, containing the embryo, were transported to the Canberra laboratory where they were planted out in pots. Isozyme analyses were carried out in the laboratory of Dr A. H. D. Brown C. S. I. R. O., Division of Plant Industry, Canberra, ACT, Australia. The analyses of the esterase (*Est2* and *Est5*), endopeptidase (*Enp1*) and NADH diaphorase (*Ndh2*) genotypes was undertaken in Dr Brown's laboratory (Division of Plant Industry, Canberra) and are described by Guerin *et al.* (1993).

3.3 RESULTS

3.3.1 Comparison of MEP-1 and EP-B by IEF.

An immunoblot of crude extracts from barley cvs. Schooner and Himalaya separated by horizontal IEF is shown in Figure 3.1. A band with a pI value 4.3 is detected in both the Schooner and Himalaya extracts and coincides with the purified MEP-1 band from Schooner. In each sample the 4.3 band is the most prominent. This immunoblot shows that there is no varietal difference in the position of the main endopeptidase band between the cvs. Schooner and Himalaya.

3.3.2 N-terminal amino acid sequence of MEP-1.

The N-terminal amino acid sequence of a purified MEP-1 fraction from Schooner barley was determined by Edman degradation. Twenty amino acid residues were identified as NH_2-asp-leu-pro-pro-ser-val-asp-trp-arg-gln-lys-gly-ala-val-thr-gly-val-lys-asp-gln. The sequence was identical to the N-terminal sequence for EP-B determined by Koehler and Ho (1990a).

3.3.3 Chromosomal location of the MEP-1 gene.

3.3.3.1 IEF and immunoblotting of the wheat-barley disomic addition lines.

Crude extracts of each line were analysed by IEF and immunoblotting (Figure 3.2). The endopeptidase band is present at pI 4.3 in germinated seeds of Betzes barley (lane g) but is not detected in Chinese Spring (lane h). The band is seen in the addition line containing Betzes chromosome 3 (lane d) but is not present in the other addition lines. It is therefore concluded that the gene controlling the expression of MEP-1 is located on barley chromosome 3.

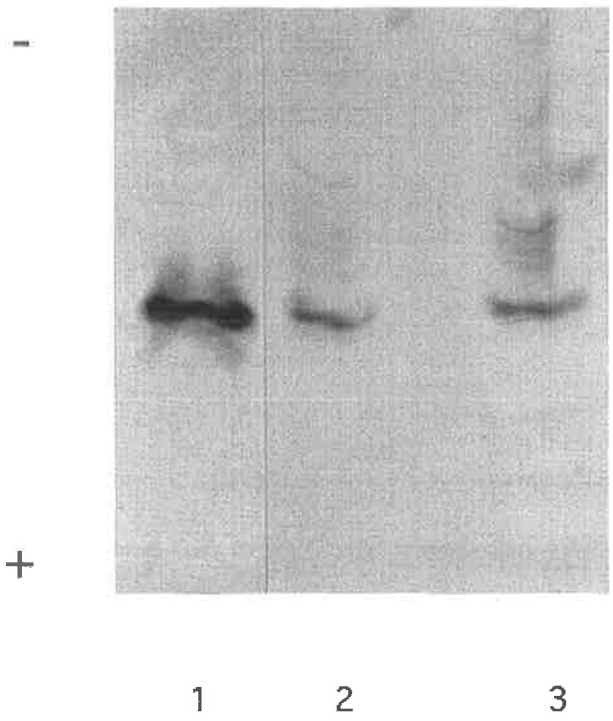

Figure 3.1 Immunoblot of Himalaya and Schooner malt extracts after separation by IEF.

Crude extracts from barley cvs. Schooner and Himalaya were run on a horizontal IEF gel (pH range 2.5 - 5.0). The main endopeptidase bands from each barley cultivar were detected using immunoblotting.

Lane 1) MEP-1 (5µl); 2) Schooner extract (10 µl); 3) Himalaya extract (10 µl)

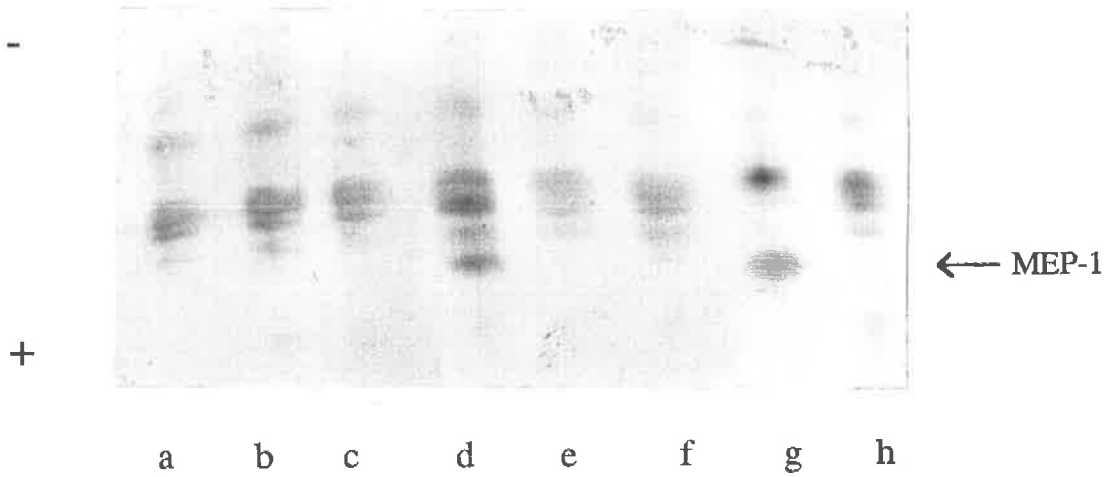

Figure 3.2 Immunoblot of crude extracts of the wheat-barley disomic addition lines.

Crude extracts were separated by vertical IEF (pH range 3.5 - 10 enriched in the 3 - 6 zone). The main endopeptidase bands in each sample were detected using immunoblotting. The extracts shown are 5 µl of addition lines containing Betzes chromosome 7 (a), 6 (b), 4 (c), 3 (d), 2 (e) and 1 (f), Betzes (g) and Chinese Spring (h).

3.3.3.2 IEF and immunoblotting of the wheat-barley ditelosomic addition lines.

The ditelosomic addition lines containing the Betzes chromosome 3 long arm (3L) and short arm (3S) were also analysed by IEF and immunoblotting (Figure 3.3). The barley endopeptidase band appears in addition line 3L (lane b) but not in 3S (lane c). Thus the gene controlling the expression of MEP-1 has been assigned to the long arm of barley chromosome 3.

3.3.4 Genetic variation of MEP-1 in wild barley populations.

3.3.4.1 Analysis of endopeptidase banding patterns in wild barley populations.

MEP-1 banding patterns on isoelectric focussing gels did not vary between *H. vulgare* cv. Schooner and the *H. spontaneum* lines that were tested from Israel (Figure 3.4). Five of the cultivars tested from Turkey and Iran, however, showed banding patterns which appeared to be lower than the other varieties (Figure 3.5 samples 13, 14, 17, 19 and 21). The CPI numbers and collection sites for each cultivar are shown in Appendix 3.1. The endopeptidase variant in these five cultivars appears to have a slightly lower pI value than MEP-1.

Endopeptidase bands were not observed in sample numbers 3, 4, 11, and 20. These seeds had not reached a level of germination equal to that of the others. Rootlet development was minimal and the coleoptile had not emerged whereas the other seedlings had an average rootlet length of 2-3 cm and coleoptiles of 1-2 cm. The lack of endopeptidase bands in the underdeveloped seeds would be due to the immature physiological stage of the seed rather than the absence of a malt endopeptidase.

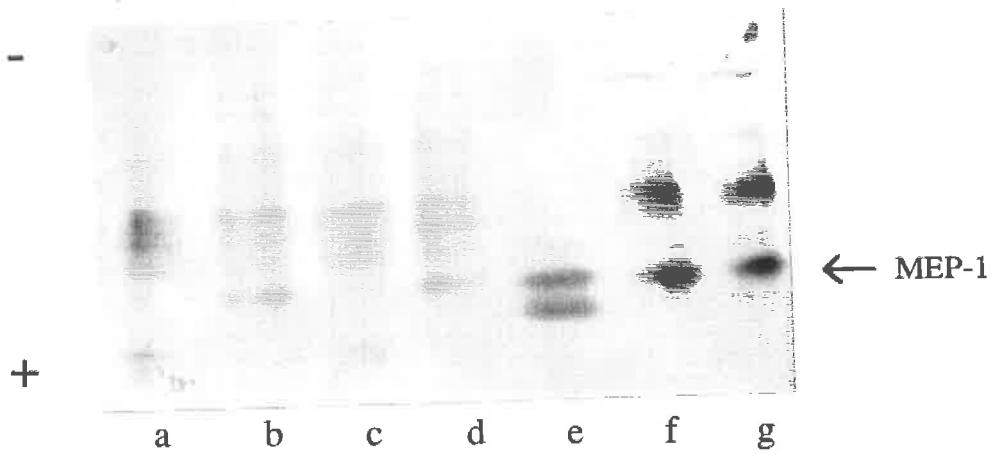

Figure 3.3 Immunoblot of crude extracts of the wheat-barley ditelosomic addition lines.

Crude extracts were separated by vertical IEF (pH range 3.5 - 10 enriched in the 3 - 6 zone). The main endopeptidase bands in each sample were detected with immunoblotting. The extracts shown are 5 µl aliquots of Chinese Spring (a), ditelosomic addition lines 3L (b), and 3S (c), disomic addition line 3 (d), MEP-1 (e), Schooner (f), and Betzes (g).

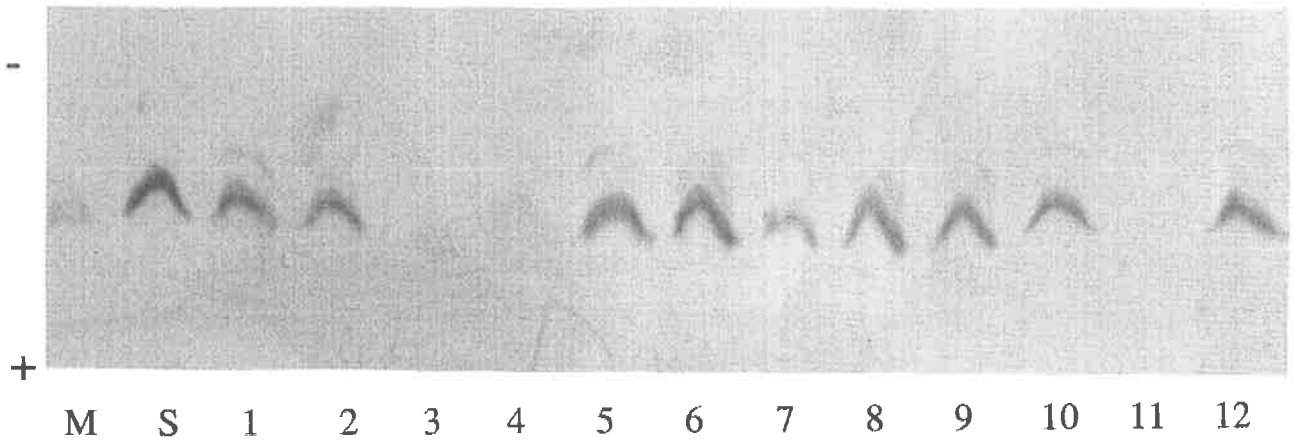

Figure 3.4 Immunoblot of extracts of *H. spontaneum* lines collected from Israel.

Crude extracts were separated by horizontal IEF (pH range 3.5 - 10.0 enriched in the 3.0 - 6.0 region). The main endopeptidase bands in each sample were detected by immunoblotting. Lane M) 5 µl purified MEP-1 from Schooner; lane S) 10 µl of Schooner extract; lanes 1-12) 10 µl of extract from *H. spontaneum* lines collected from Israel.

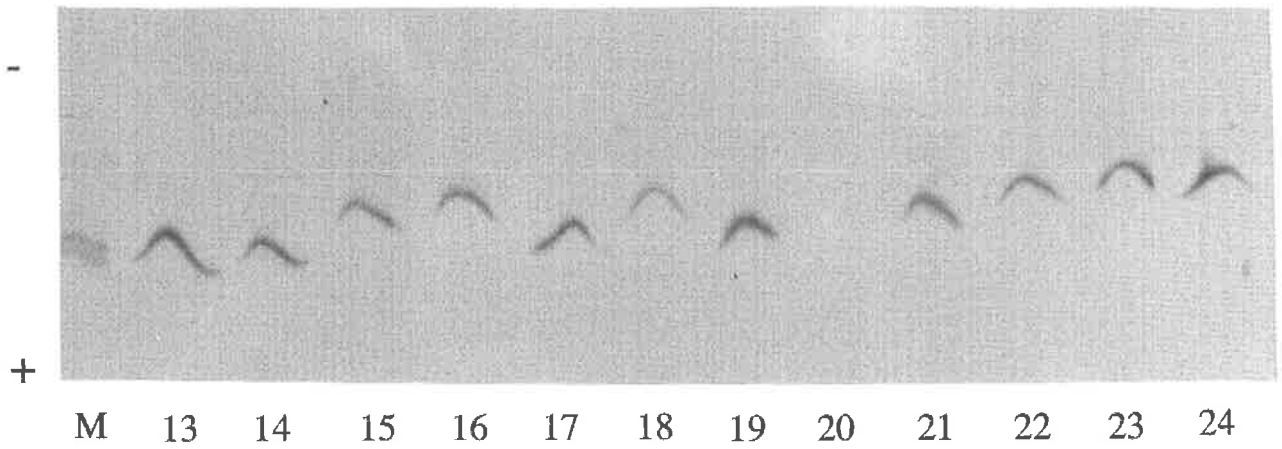

Figure 3.5 Immunoblot of extracts of *H. spontaneum* lines collected from Iran and Turkey.

Crude extracts were separated by horizontal IEF (pH range 3.5 - 10.0 enriched in the 3.0 - 6.0 region). The main endopeptidase bands were detected by immunoblotting.
Lane M) 5 µl purified MEP-1 from Schooner; lanes 13-18) 10 µl of extract from *H. spontaneum* varieties collected from Iran; lanes 19-24) 10 µl of extract from *H. spontaneum* lines collected from Turkey.

3.3.4.2 Verification of the MEP-1 variants.

The variant endopeptidase bands were difficult to identify as their pI value is close to the that of the normal MEP-1 band. IEF gels are subject to small disturbances in the pH gradient caused by many factors such as salts present in the sample. To ascertain whether the putative variant bands actually differed from MEP-1 in Schooner several *H. spontaneum* samples were 'spiked' with 5 µl of crude extract prepared from 5 day germinated seedlings of Schooner (as in Section 3.2.2). It was expected that two bands would be observed if a variant were present, one band due to Schooner and the other a variant. This is shown in Figure 3.6, and the five accessions with putative variant bands (sample numbers 13, 12, 17, 19 and 21) are shown to be true variants as the Schooner band in each case is seen above that of the variant form of the endopeptidase. The pI value of the variant endopeptidase is ~ 4.1 compared to MEP-1 which has a pI value of 4.3 (Phillips and Wallace, 1989).

3.3.5 Chromosomal mapping of the MEP-1 gene.

3.3.5.1 Analysis of endopeptidase genotypes in the F_2 generation of the wild barley cross.

The F_2 progeny of the cross between the Turkish accession line (CPI 109861) and barley cv. Clipper was examined for endopeptidase banding type. The different mix of ampholytes in these gels allowed the 4.1 and 4.3 bands to be clearly distinguished (Figure 3.7). Three banding patterns were detected in the F_2 population, the two parental homozygous and the heterozygous IEF banding patterns are shown in Figure 3.7. The F_2 seeds segregated for endopeptidase type in a simple, co-dominant Mendelian fashion. Out of a total of 78 seeds tested for endopeptidase genotype; 42, were heterozygous, 21 were homozygous for the 4.3 band, and 14 were homozygous for the 4.1 band (χ^2 (2 df) = 2.10 ns).

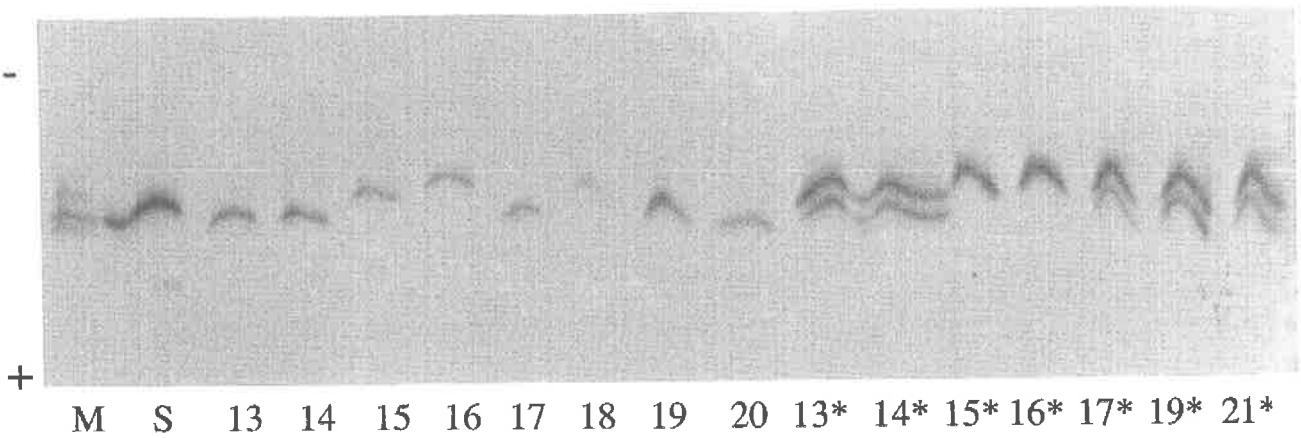

Figure 3.6 Immunoblot of crude extracts of *H. spontaneum* lines verifying the presence of an endopetidase allele.

Crude extracts were separated by horizontal IEF (pH range 3.5 - 10.0 enriched in the 3.0 - 6.0 region) and the main endopeptidase band was detected by immunoblotting. Lane M) 5 µl purified MEP-1 from Schooner; lane S) 10 µl of crude Schooner extract; lanes 13-20) 10 µl of extract of the designated *H. spontaneum* lines. * = 10 µl of extract from *H. spontaneum* lines 'spiked' with 5µl Schooner extract.

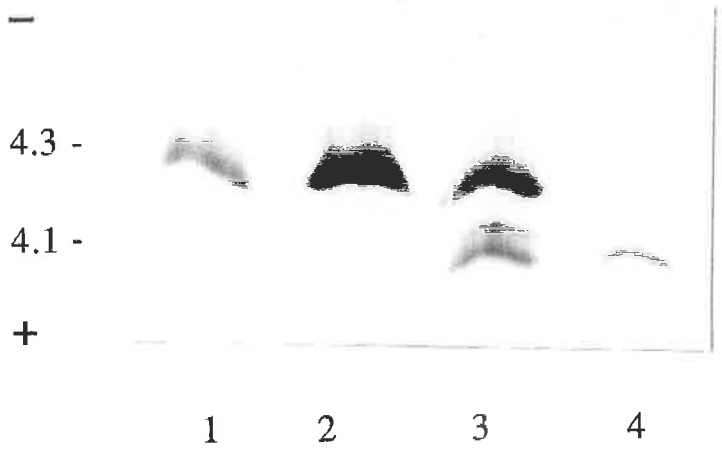

Figure 3.7. Immunoblot showing the endopeptidase genotypes present in the F_2 generation of the barley and wild barley cross.

Crude extracts were separated by horizontal IEF (pH range 2.5 - 5.0 enriched in the 4.2 - 4.9 zone) and the main endopeptidase bands were detected by immunoblotting.
Lane 1) Clipper extract (10 µl). Lanes 2, 3 and 4) samples of the F_2 population extracts (10 µl) showing the three different genotypes in the population : homozygous for the Clipper endopeptidase, heterozygous and homozygous for the wild barley endopeptidase respectively. The apparent pI values for each of the bands are marked on the left.

3.3.5.2 Endopeptidase activity of *H. spontaneum* extracts.

The endopeptidase activities of several individual F_2 seedlings were measured using the azocasein assay. There was no relationship between endopeptidase activity against azocasein (Section 5.2.2) and the endopeptidase genotype. The average activity for the heterozygous genotypes was 11.3 ± 2.0; 10.1 ± 2.3 for homozygous genotypes for the Schooner type endopeptidase and 10.3 ± 1.8 for the homozygous, variant genotype. Analysis of variance showed that there was no significant difference in the activities between these groups.

3.3.5.3 Isozyme segregation.

Table 3.1 summarises the specifications of the isozyme loci that were segregating in the F_2 family and the enzymes coded by those loci. The chromosomal location of each locus is shown with reference to published sources (Brown, 1983; Guerin *et al.*, 1992b and 1993). The table lists the source of tissue found to be optimal for enzyme activity and the gel buffer system(s) that were preferable for each locus. The anodal mobility of the parental isozymes was measured as the distance (mm) from the sample insert in the gel, or as apparent pI value in the case of MEP-1.

The simplest zymogram was that which detected the endopeptidase (Figure 3.7). The esterase zymogram for barley is well known (Brown, 1983) and segregation at the *Est* -2 locus occurred in this cross.

The diaphorase zymogram proved to be more useful than esterase with respect to mapping the new endopeptidase locus (*CepB*). Table 3.2 presents the maximum likelihood estimates of the recombination fraction between the loci listed in Table 3.1. As expected from the different chromosomal locations, the endopeptidase locus (*CepB*) segregated independently of that detected with a histochemical stain (*Enp1*). However the *CepB* locus showed statistically significant linkage with the *Ndh2* locus which, in turn, was linked to the *Est2* complex. This result confirmed the location of the *CepB* locus on chromosome 3. Figure 3.8 shows the

most likely order of these enzymes and their relative positions on chromosome 3L. The values for recombination fractions were converted to centimorgans using the Kosambi function (Kosambi, 1944).

Table 3.1 Enzyme loci segregating in the F_2 progeny.

Locus	Chr[1]	Tissue[2]	Gel[3]	Mobility[4] (mm)	
				Cl	Sp
Enp1	1 (R1)	L, R	M	60	57
Est5	1 (R1)	L	T, L	67	70
Est2	3 (R1)	L, R	T, L	28	22
Ndh2	3 (R2)	L, R	C, L	45	32
CepB	3 (R3)	E	IEF	(4.3)	(4.1)

[1]Authorities for barley chromosome locations: R1 = see citations in Brown (1983), R2 = Guerin *et al.* (1993), R3 = Guerin *et al.* (1992b).

[2]Tissue : L = seedling first leaf, R = seedling root tip, E = germinating endosperm.

[3]Gel : Buffer systems T, L, M and C are as in Brown (1983), the IEF procedure is described in Section 3.2.6.

[4]Mobility (mm) : distance from the anode, or pI value in parenthesis.

Table 3.2. Maximum likelihood estimates of recombination fraction in the F_2 of *H. spontaneum* CPI109861 x *H. vulgare* cv. Clipper.

	Enp1	*Est5*	*CepB*	*Ndh2*	*Est2*
Chromosome 1					
Enp1					
Est5	0.49				
Chromosome 3					
CepB	0.48	0.53			
Ndh2	0.55	0.54	0.34**		
Est2	0.60	0.61	0.42	0.20**	

Standard error for these estimates ≈ 0.05

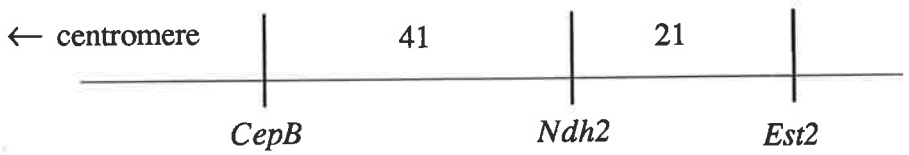

Figure 3.8 Linkage map of the long arm of barley chromosome 3.

The estimated positions (in cM) of malt endopeptidase (*CepB*), NADH diaphorase (*Ndh2*) and esterase (*Est2*) gene loci.

3.4 DISCUSSION

There are several reasons for the discrepancy in the reported pI values of the purified enzymes EP-B (Koehler and Ho, 1990a) and MEP-1 (Phillips and Wallace, 1989). Both enzymes are resolved into two bands after IEF; MEP-1 at 4.2 and 4.3 and EP-B at 4.6 and 4.7. These small differences could be due to the different procedures used to measure the pI values, a difference between the two barley varieties or that EP-B and MEP-1 are different enzymes. Also the enzymes have been purified in different states of glycosylation which may account for the differences in their pI values.

Koehler and Ho (1990a) estimated the pI values using tube gel electrophoresis. The pH profile was determined by cutting the gel into 5 mm sections, which were equilibrated in 3 ml of water, and measuring the pH of the solution. A similar gel, containing the endopeptidase, was sliced and incubated in Na succinate (20 mM); 2-ME (10 mM) pH 5.0 and activity of each slice was determined against azocasein. Phillips and Wallace (1989) performed flat bed isoelectric focussing with 0.4 mm ultrathin gels poured onto a gel bond backing. The pH profile was measured in a similar fashion to Koehler and Ho and compared with the enzyme band on the gel visualised by silver staining. These methods are subject to error as the pH profile of an IEF gel may vary between the segment of gel used to measure pH and that used to detect the enzyme. Therefore it would be inappropriate to make the assumption that the two enzymes differ using the reported pI values from the two different laboratories as a base.

When both varieties were examined together using IEF, the endopeptidase bands ran to the same position, thereby ruling out varietal differences. The polyclonal antibodies react with two bands in the MEP-1 sample purified from the barley cultivar Schooner, yet only the upper band of these is detected in crude extracts. The lower band appears in crude extracts stored at 0°C for several days (data not shown) and it is assumed that the second band which appears after storage and in purified MEP-1 samples is a product of autolysis rather than another gene product. The identical sequences of the first twenty amino acids indicates that the enzymes

are the same, although a full amino acid sequence is required to verify this. Furthermore both enzymes account for the major portion of endopeptidase activity in the germinating seed.

Several variations of the IEF technique have been used to analyse MEP-1 in this study. The two MEP-1 bands were not clearly separated in Figure 3.1 using the pH range 2.5 - 5.0. The two MEP-1 bands are distinct in Figures 3.2 to 3.6 where a different ampholyte mix was used (pH range 3.5 - 10 enriched in the 3 - 6 zone). Further separation in the MEP-1 banding region was achieved using a pH range of 2.5 - 5.0 enriched in the 4.2 - 4.9 zone (Figure 3.7). Using this pH range the endopeptidase genotypes in the F_2 generation of the barley and wild barley cross were clearly distinguished without the need for 'spiking' with Schooner extract as was necessary to identify the variant endopeptidase bands in Figure 3.6.

Vertical IEF was used for Figures 3.2 and 3.3 as these gels tend to run more evenly than the horizontal IEF gels which are prone to wavy lines and disturbances caused by factors such as localised heating caused by salt concentration in the sample. The regularity of the vertical gels may be due to more effective cooling in this system. The increased thickness of the gel also reduces the errors of uneven casting which can occur with ultrathin gels, particularly if a support film is used. The absence of a support film allowed the vertical gels to be transferred by the electrotransfer method (described in Section 3.2.5) which uses a voltage gradient to assist the transfer of the proteins from the gel to the membrane. However, the passive method of transferring the horizontal ultrathin gels (described in Section 3.2.4) was equally if not more effective.

The wheat-barley addition line seeds were germinated in the presence of gibberellic acid (7 ppm) as it enhances the level of endopeptidase (Jacobsen and Varner, 1967). IEF was chosen over SDS-PAGE as it allowed the MEP-1 band to be differentiated from a wheat protein band of similar size which cross reacted with the polyclonal antibodies. The antibodies cross-react with two bands, other than MEP-1, in Betzes with pI values of 4.7 and 5.4 and several bands in the Chinese Spring extracts. It is not known if these bands have a similar function to the endopeptidase or share an immunological identity only.

A structural gene designated *Ep-H1* encoding an endopeptidase was located in Betzes chromosome 1 (Hart *et al.*, 1980) which is synonymous with the gene *Enp1* described by Brown (1983). Extracts from 7 d germinated coleoptiles were electrophoresed in horizontal starch gels and the endopeptidase was detected with sodium benzoyl-DL-arginine-p-nitroanilide (BAPNA) and Black K stains. The endopeptidases Ep (Hart *et al.*, 1980) and MEP-1 are not related as the latter does not degrade BAPNA (Phillips and Wallace, personal communication). Indeed there is no substantiated evidence to suggest that enzymes which degrade BAPNA or sodium benzoyl-DL-arginine-β-naphthylamide (BANA) are true endopeptidases (Wallace and Lance, 1988).

The lack of variation in the endopeptidase banding patterns in the Israeli lines shows that the enzyme structure has been conserved in this region. The variants detected in the Turkish and Iranian samples 14, 18, 20, 23 and 24 all have identical pI values (4.1). The reaction with the polyclonal antibodies raised against MEP-1 indicates a structural similarity between MEP-1 and the wild barley endopeptidases. Overall the enzyme structure appears to be conserved from the ancestral barleys to the commercial varieties used today which may suggest that significant changes in the endopeptidase structure has a deleterious effect on the plant.

The lack of correlation between the endopeptidase genotype and endopeptidase activity in the half seeds of the F_2 population is not surprising. The level of seed maturity, the size of the seed and individual seed to seed variation may have more effect on activity than the type of enzyme present. As the endopeptidase activity was measured in extracts made from the distal half of the germinating seed, the position at which the seed was cut is bound to have a significant effect on the amount of enzyme present in the extract. Endopeptidase activity is secreted from the aleurone tissue at the embryo end of the seed through to the distal end during germination (Okamoto *et al.*, 1980; Marttila *et al.*, 1993), therefore an undeveloped seedling may have very little endopeptidase activity in the distal end of the seed.

The linkages determined in this study will assist the construction of barley chromosome maps. The main malt endopeptidase; EP-B has been cloned (Koehler and Ho, 1990b)

therefore DNA probes could be used in conjunction with established markers to substantiate the exact position of the gene on chromosome 3. In this study, the *CepB* locus was found to be linked to one of the NADH dehydrogenase (*Ndh2*). The discovery of the *Ndh2* locus was pivotal as a "bridging locus" in establishing the gene location of *CepB* on the long arm of chromosome 3, especially in relation to the seedling esterase gene complex. The investigation into new isozyme markers plays an important role in the mapping of barley genes.

The identification of allelic variation and mapping of the enzyme loci from wild barley creates a valuable store of information regarding genetic variation in barley which is beneficial to plant breeders. An IEF and immunoblotting technique, similar to that employed in this study, has been used to identify an endo-β-glucanase E I variant (MacLeod et al., 1991). Backcross progeny involving a cross between Clipper and the *H. spontaneum* line carrying the variant was used to map the *Glb1* locus. This is the first report of a variant endopeptidase allele (Guerin *et al.*, 1993) although wide variation in the level of endopeptidase activity has been demonstrated in a *H. spontaneum* collection from Israel (Ahokas and Naskali, 1990). The comparison of the normal (Clipper) and variant *CepB* endopeptidase allele isolated from the *H. spontaneum* line may aid the understanding of these enzymes, if it were demonstrated that the variant allele was associated with an altered level of activity, by examining how a change in structure affects the behaviour of the enzyme. The discovery and breeding of barleys with mutant genes have been valuable tools for assessing enzyme roles in ammonia assimilation, photorespiration and amino acid biosynthesis (Lea *et al.*, 1992). Further studies of variant endopeptidases backcrossed into a common genetic background such as Clipper, will be invaluable for assessing the functions and roles of individual endopeptidases during germination and ultimately their importance in aspects of malting quality. Other barley endopeptidases which have already been isolated (Koehler and Ho, 1988: Sarkkinen *et al.*, 1992) are primary candidates for this type of analysis.

CHAPTER 4.

THE ROLE OF ENDOPEPTIDASES AND THIOL COMPOUNDS IN THE RELEASE AND ACTIVATION OF β-AMYLASE.

4.1 INTRODUCTION

Starch is one of the main seed reserves in barley grain and represents about 65 % of the total dry seed weight. Amylose and amylopectin are the two main components of starch; amylose consists of long unbranched chains of α(1-4) linked glucose units and amylopectin is composed of branched glucose α(1-4) chains, cross linked via α(1-6) linkage. Starch is degraded during germination by a number of hydrolytic enzymes. α-Amylase and β-amylase hydrolyse α(1-4) linkages whereas the α(1-6) linkages are hydrolysed by limit dextrinase. β-Amylase cleaves maltose units from the nonreducing end of starch molecules.

Unlike most other hydrolytic enzymes β-amylase is not synthesised during germination (Hardie, 1975). The physiological function of β-amylase is obscure. It is an albumin which is synthesised in large amounts and accumulates in the endosperm concurrently with starch and storage proteins (MacGregor *et al.*, 1971). It has been suggested that the enzyme may function as a storage protein. However, it is synthesised as a cytosolic protein in the endosperm and is not deposited in protein bodies with the major storage proteins (Nishimura *et al.*, 1987). A large amount is located at the perifery of starch granules in the mature grain (Laurière *et al.*, 1986). The amount of β-amylase in the mature seed appears to be greatly in excess of requirement. Judging by i*n vitro* activity, the free β-amylase in one grain could theoretically degrade all the starch present (if in the form of amylose) within about 18 min, yet germination takes around 6 days (Sopannen and Laurière, 1989).

β-Amylase exists both as a free, soluble protein and as an insoluble, bound complex. The complex may be formed between β-amylase molecules or with an albumin called protein Z (Hejgaard, 1976). β-Amylase is converted from the bound to the free form by treatment with disulphide reducing agents or papain (Sandegren and Klang, 1950). Proteolytic release of β-amylases has also been described in wheat (Kruger, 1979; Bureau *et al.*, 1989), rye (Daussant *et al.*, 1991) and rice (Fursov and Khakimzhanov, 1991).

Protein Z is present in large amounts in barley grain yet it is not known to have a physiological function. It appears to be very resistant to degradation during malting and brewing. It is a major barley protein constituent of beer and may contribute to foam stabilisation and/or haze formation (Hejgaard and Kaersgaard, 1983). Homology between protein Z and protein inhibitors of the α1-antitrypsin family has been established. However protein Z had no inhibitory effect on a number of microbial and pancreatic serine proteases (Hejgaard *et al.*, 1985).

The major grain forms of β-amylase are controlled by a locus on chromosome 4 in barley (Powling *et al.*, 1981) and Southern hybridisation analysis has indicated that the β-amylase locus consists of a small gene family of at least two copies per haploid genome (Kreis *et al.*, 1987). Chromatofocusing and isoelectric focusing of β-amylase in dormant and germinating seeds demonstrate the heterogeneity of β-amylase. At least eight forms of β-amylase, extracted in the presence of thiol reducing agents, exist in barley grain and have isoelectric points ranging from 6.46 to 4.82 (Laberge and Marchylo, 1983). Treatment with papain resulted in the release of two β-amylase components with the apparent pI values of 6.89 and 6.86 (Laberge and Marchylo, 1987) which correspond with the two major malt enzymes found in barley cv. Bonanza (Laberge and Marchylo, 1986).

Lundgard and Svensson (1987) isolated four β-amylase species from barley flour in the presence of 0.1 M thiol. The β-amylases (1 to 4) were single polypeptide chains of approximate Mr 59,700, 58,000, 56,000 and 54,000, with corresponding pI values of 5.2, 5.3, 5.5 and 5.7. β-Amylase 1 was degraded to β-amylase 4 in the presence of proteolytic

agents. Papain (cysteine-endopeptidase), subtilisin and trypsin (serine-endopeptidases), collagenase (metallo-endopeptidase) and malt extract proteinases were all active on β-amylase 1 yet acid-proteinases and carboxypeptidase II had no effect. The activity of the malt extract endopeptidase was inhibited only by leupeptin and EDTA in combination, suggesting that cysteine-endopeptidases and metallo-endopeptidases are involved in cleaving β-amylase 1.

Sopanen and Laurière (1989) demonstrated that β-amylase released by papain or endopeptidases in a malt extract had a lower Mr (by ~5,000) than β-amylase released by a reducing agent. The presence of leupeptin in the malt extract inhibited the release of the smaller species suggesting the involvement of a cysteine-endopeptidase.

It appears that malt endopeptidases are involved in both the release of bound β-amylase and in modification of the free enzyme. The recent isolation of malt endopeptidases (Poulle and Jones, 1988; Phillips and Wallace, 1989; Koehler and Ho, 1990a) allows the study of their action on β-amylase.

The aims of the studies presented in this chapter are as follows:

i) To assess the effects of thiol, and the malt endopeptidase MEP-1, on the activity of bound and free β-amylase fractions from resting barley grains.

ii) To compare the profile of β-amylases in malt with the β-amylases released by MEP-1.

iii) To assess the effect of barley endopeptidases other than MEP-1 on bound β-amylase.

iv) To estimate the concentration of thiol in resting barley grains and malt.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Preparation of bound β-amylase.

Clipper barley flour (20 g) was extracted with 200 ml NaCl (0.1 M) for 20 min at 20°C on a horizontal shaker at 50 osc/min. The suspension was then centrifuged for 15 min at 10,000 *g*. This extraction was repeated five times and the final pellet was resuspended in 100 ml distilled water and used as the bound β-amylase sample. β-Amylase activity was assayed in each supernatant; the final wash had < 0.1 % of the activity in the initial wash. The bound β-amylase preparation was stored at -15°C.

4.2.2 Extraction of free β-amylase.

Clipper barley flour (5 g) was extracted with 50 ml NaCl (0.1 M) for 5 h at 20°C before centrifuging at 10,000 *g* for 15 min. The supernatant was used as a source of free β-amylase.

4.2.3 Extraction of malt amylases and preparation for IEF gels.

Seedlings were germinated (as described in Section 2.2.1.1) for 5 d then ground in a mortar and pestle with NaCl (0.1 M) at a concentration of 3 ml/g fresh weight. The mixture was centrifuged for 15 min at 12,000 *g* and the supernatant used as malt sample containing both α- and β-amylases. The samples were partially desalted before application to IEF gels in a Centricon® TM 10 device, containing 1 ml of distilled water, for 1 h at 5,000 *g* .

4.2.4 Bound β-amylase release and sample preparation for IEF gels.

Bound β-amylase samples (1.0 ml) were incubated for 3 d at 20°C with 0.6 ml sodium acetate buffer (0.2 M), pH 4.5 ± 2-ME (3 mM) and ± 0.2 ml (2.4 μg) endopeptidase sample in the presence of azide (0.02 % (w/v)). After incubation the suspensions were centrifuged at 12,000 *g* for 10 min and the activity in the supernatant was taken as that of the free enzyme.

Distilled water (1.0 ml) was added to the supernatant and the samples were partially desalted in a Centricon® TM 10 device for 1 h at 5,000 *g* before application to IEF gels.

4.2.5 Free β-amylase release and preparation for IEF gels.

Free β-amylase (0.5 ml) was incubated for 20 h at 20°C with 0.3 ml sodium acetate buffer (0.2 M) pH 4.5± 2-ME (3 mM) and 0.1 ml MEP-1 (1.2 μg). The samples were partially desalted before application to IEF gels in a Centricon® TM 10 device, containing 1 ml of distilled water, for 1 h at 5,000 *g*.

4.2.6 Inhibition of α-amylase and β-amylase in malt samples.

To inhibit α-amylase, malt samples were incubated for 16 h at 20°C with EDTA (0.025 M) and NaCl (0.1M) according to the method of Sopanen and Laurière (1989). β-Amylase was inhibited by heating a malt extract at 70°C for 15 min. The samples were partially desalted before application to IEF gels in a Centricon® TM 10 device, containing 1 ml of distilled water, for 1 h at 5,000 *g*.

4.2.7 α-Amylase and β-amylase assays.

α-Amylase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenol maltoheptaoside, blocked at its non-reducing end (BPNPG7), in the presence of excess glucoamylase and α-glucosidase at A 410 nm.

β-Amylase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenol maltopentaoside (PNPG5) in the presence of excess α-glucosidase at A 410 nm. One unit of activity is defined as the amount of enzyme required to release one μmole of *p*-nitrophenol in one minute at 40°C. The α- and β-amylase assays were supplied in kits (Biocon, Australia) and are based on methods described by McCleary and Sheehan (1987) and Mathewson and Seabourn (1983) respectively.

4.2.8 Characterisation of amylases by IEF.

Total malt amylases were extracted as described in Section 4.2.3. The β-amylase sample was prepared by inhibiting α-amylases in a total malt amylase sample with EDTA and the α-amylase sample by inhibiting the β-amylase component with heat as described in Section 4.2.6. Gels were prepared as described in Section 2.2.2.2. The pH range was achieved by mixing LKB ampholine ranges 3.5 - 10.0 and 4.0 - 6.0 in a ratio of 2:1 respectively. The anode solution was (DL) glutamic acid (0.04 M) and the cathode solution was histidine (0.2 M).

4.2.9 Starch overlay procedure.

Soluble starch (2 g) (supplied by Sigma, USA) was dissolved in 50 ml deionised water, boiled and then cooled to 40°C. The solution was poured over the IEF gel and incubated for 30 min at 37°C. After incubation the gel was washed with deionised water and stained with a solution of iodine (0.025 M); potassium iodine (0.05 M). Gels were fixed overnight in 10 % (v/v) acetic acid before air drying.

4.2.10 Determination of pI values of proteins separated by IEF.

All pI values given are apparent and were estimated with reference to the pI markers. The pI markers (broad range (4.6 - 9.6) IEF standards supplied by BioRad, Australia) were loaded onto the IEF gels in 5 µl aliquots. After electrophoresis lanes containing the pI markers were cut away and fixed in TCA (10 % (w/v)); sulphosalycylic acid (3.5 % (w/v)) for 15 min at 4°C. The presence of the gel bond backing prevented shrinking or stretching of the gel during staining procedures. After fixing the markers were stained with Coomassie Brilliant Blue R-250 (CBB) (0.25 % (w/v)) in a solution of methanol (50 % (v/v)) and acetic acid (5 % (v/v) for 1 h with gentle shaking. The gels were then destained with several changes of methanol (20 % (v/v)) and acetic acid (5 % (v/v)) until the background cleared and air dried. Amylase activity in the gel was detected using the starch overlay procedure described in Section 4.2.9.

Gels were reassembled after staining and the pI values of the amylase bands were estimated by comparison with the pI markers.

4.2.11 Endopeptidase enzymes.

4.2.11.1 Purified MEP-1.

The purified MEP-1 fraction was prepared as described by Phillips and Wallace (1989). The protein content (12 µg/ ml) was measured using the protein assay described in Section 2.2.4.

4.2.11.2 Separation of CMA endopeptidases.

Preparation of the CM-Sephadex adsorbed (CMA) protein fraction described by Phillips and Wallace (1989) is briefly outlined as follows: green malt (4 d) was extracted with sodium acetate (0.1 M); 2-ME (2 mM); insoluble polyvinylpolypyrrolidone (PVPP) (2.5 % (w/v)) pH 4.5 and centrifuged for 10 min at 20,000 *g*. The supernatant was loaded onto a Sephadex G-25 column equilibrated and eluted with sodium acetate (0.1 M); 2-ME (1 mM). The eluent containing endopeptidase activity was treated with ammonium sulphate ($(NH_4)_2SO_4$) at 35 % saturation and centrifuged as above. The supernatant was then treated with 70 % ammonium sulphate saturation and centrifuged again. Endopeptidase activity was contained in the pellet which was resuspended into sodium acetate (0.1 M); 2-ME (1 mM); NaCl (50 mM) pH 4.5 and loaded onto a Sephacryl S200 column equilibrated and eluted with the resuspension buffer. Endopeptidase fractions were pooled and concentrated and loaded onto a CM-Sephadex C-50 column equilibrated with the resuspension buffer. The fraction containing MEP-1 was washed through the column with this buffer and purified to homogeneity on a Pharmacia FPLC system using a Mono Q (HR 5/5) column.

The CMA endopeptidases adsorbed onto the CM-Sephadex column (2.1 x 3 cm) and were eluted with sodium acetate (0.1 M) pH 5.2; 2-ME (1 mM); NaCl (0.2 M). The endopeptidase pool (44 ml) was further separated on a 5 x 90 cm Sephacryl S200 gel filtration column with the above buffer. The active fractions were pooled (total volume 150 ml) and concentrated to 15 ml, with an Amicon ultrafiltration cell using a YM 10 membrane at 350 k Pa, then dialysed

into sodium acetate (50 mM) pH 5.2; 2-ME (1 mM). β-Amylase activity had been removed at this stage and this fraction was used as the endopeptidase sample (CMA) used to release bound β-amylase (Section 4.2.4).

The concentrate was loaded onto a Pharmacia FPLC cation exchange Mono S (HR 5/5) column equilibrated with the dialysis buffer. Proteins were eluted from the Mono S column in step-wise fashion with the dialysis buffer containing NaCl (0.5 M). The gradient was increased from 0 to 10 % of the elution buffer after 24 ml and maintained at that level for 17 ml before slowly increasing to 40 % over 75 ml. All steps, except for those carried out on the Pharmacia FPLC system were undertaken at 4°C. Endopeptidase activity was measured against azocasein (as described in Section 5.2.2).

4.2.12 Inhibition of the CMA fractions.

CMA endopeptidase fractions 1, 2 and 3, eluted from the Mono S column, were incubated in the presence of the inhibitors pepstatin (12.5 μM), leupeptin (5 μM) or control solutions (water for leupeptin, 6.6 % methanol for pepstatin) for 20 min at 37°C. The CMA fractions were also treated with 3 mM DDT/ 3 mM EDTA, or water as a control, for 1 h at 37° C. After incubation 40 μl of each sample was tested for endopeptidase activity against azocasein (described in Section 5.2.2).

4.2.13 SDS-PAGE gel transfer to PVDF membrane.

SDS-PAGE was performed as described in Section 2.2.2.1. Protein samples separated by SDS-PAGE were transferred to PVDF membrane for 1 h at 100 V in glycine (0.2 M), Tris-HCl (0.025 M), methanol (20 % (v/v)) in a BioRad mini transblot unit (BioRad, Australia). The initial buffer temperature was 4°C.

4.2.14 Estimation of total thiol concentration.

The methods used for estimating thiol concentrations were based on those described by Sedlak and Lindsay (1968). Barley and malt flour samples (1 g) were extracted with 4 ml EDTA (0.02 M) for 2 h on ice on a horizontal shaker at 50 osc/min. The sample (0.5 ml) was

then mixed with 1.5 ml Tris-HCl (0.2 M) pH 8.2; 0.1 ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) or Ellman's reagent (0.01 M in methanol) and 7.9 ml methanol. Colour was allowed to develop over 15 min at room temperature with occasional mixing before the mixture was centrifuged for 15 min at 3,000 *g*. The absorbance of the supernatant was measured at 412 nm and compared with a 2-ME standard curve (Figure 4.8). Two blanks were measured for each sample, a reagent blank substituting EDTA (0.02 M) for the sample and a sample blank where the DTNB solution was replaced with methanol. The readings for both these blanks were subtracted from the sample reading.

4.2.15 Estimation of non-protein bound thiol concentration.

Samples (2.5 ml) were extracted as above and incubated on ice with H_2O (2 ml) and 1.0 ml TCA (50 % (w/v)) for 10 min with occasional mixing before a 10 min centrifugation at 3,000 *g*. An aliquot of the supernatant (2.0 ml) was mixed with 4 ml Tris-HCl (0.2 M) pH 2.8 and 0.1 ml DTNB (0.01 M in methanol). The absorbance of this solution was read at 412 nm within 5 min of mixing. Reagent blanks and sample blanks were made for each sample as above.

4.3 RESULTS

4.3.1 Effects of MEP-1 and 2-ME on bound β-amylase.

Release and activation of bound β-amylase prepared from quiescent barley seeds (*Hordeum vulgare* L. cv. Clipper) was demonstrated with 2-ME and a cysteine-endopeptidase (MEP-1), isolated from green malt. This endopeptidase is activated by 2-ME (Phillips and Wallace, 1989). Both 2-ME and MEP-1 treatments result in an increase in β-amylase activity in the supernatant fraction (Table 4.1). In combination the effect is slightly more than additive presumably due to the interaction between MEP-1 and 2-ME resulting in increased MEP-1 activity.

The amount of β-amylase released was proportional to the level of MEP-1 added (Figure 4.1). The concentration of 2-ME was constant in all the assays (1 mM) which explains the high initial β-amylase activity.

4.3.2 Identification of α-amylases and β-amylases in malt.

In the isozyme studies on malt samples it was necessary to distinguish α- from β-amylases and inhibition procedures established by Sopanen and Laurière (1989) were followed. In a green malt extract the β-amylase was inhibited 93 % by heat treatment and α-amylase was inhibited 99 % after incubation with EDTA (Table 4.2). Using this inhibition procedure the α- and β-amylases could be identified in a starch overlay after their separation by IEF (Figure 4.2.) Lane 2 (control) illustrates the range of α- and β-amylases in a 4 d green malt, however the lowest band at pI 4.8 is not clearly seen in the photograph. In lane 3 (heat treated) the following α-amylases are apparent, α-amylase I at pH 5.1, α-amylase II at 6.4 and 6.5 and α-amylase III at 7.1. The faint bands at 5.2 and 7.5 are likely to be due to residual β-amylase activity. Lane 4 (EDTA treated) shows the following β-amylases, a low pI form at 4.8, a pronounced group between 5.0 and 5.3, single bands at 5.5, 5.7, 6.6 and 7.0, a major band at 7.5 and another band at 8.0. α-Amylase I which has been reported to be insensitive to EDTA (Jacobsen, 1983) may contribute to the 5.1 band.

Table 4.1 Effect of 2-ME and MEP-1 on bound β-amylase.

Bound β-amylase was incubated with and without 2-ME (1 mM) and MEP-1 (2.4 μg) for 4h at 37°C. β-Amylase activity in the 12,000 *g* supernatant was measured as described in Section 4.2.7. The activity at the start of incubation was 4.3 units/g.

β-Amylase activity (units/g)

2-ME	MEP-1 +	MEP-1 -
+	52.8 ± 1.3	42.8 ± 0.5
-	21.2 ± 0.2	15.6 ± 0.4

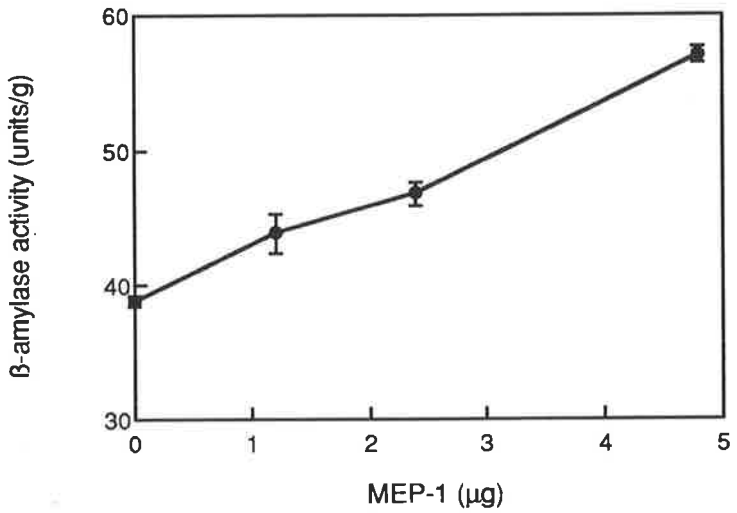

Figure 4.1 Effect of a malt endopeptidase on bound β-amylase from barley flour.

Bound β-amylase was incubated with 2-ME (1 mM) and MEP-1 for 4 h at 37°C. β-Amylase activity was measured in the 12,000 *g* supernatant as described in Section 4.2.7.

Table 4.2 Inhibition of malt α- and β-amylases in barley malt.

The α- and β-amylase activities in malt extracts were inhibited using EDTA and heat treatments. The respective amylase activities were measured with the appropriate assays described in Section 4.2.7.

	Treatment	α-amylase activity (units/g)	β-amylase activity (units/g)
1)	nil	82	391
2)	70°C 15 min	56	26
3)	EDTA (25 mM) 20°C 16 h	1	430

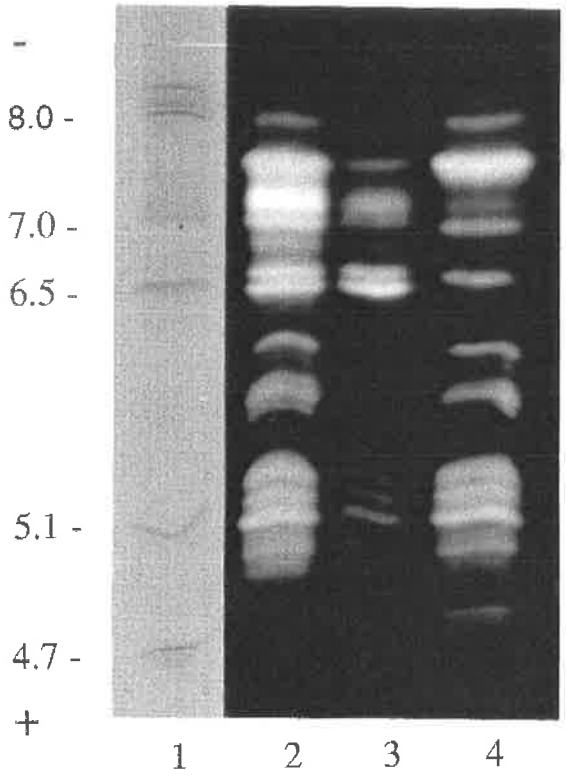

Figure 4.2 Characterisation of α- and β-amylases in malt on an IEF activity gel.

Malt amylases were applied to an IEF gel and identified with a starch overlay. The following samples are shown: 1) pI markers, 2) malt, 3) malt heated 15 min at 70°C (α-amylases), 4) malt treated with EDTA (β-amylases).

4.3.3 Separation of the CMA endopeptidases.

The CMA endopeptidase fraction was eluted from the S200 Sephacryl column in one main peak (Figure 4.3). The fractions indicated, containing most of the azocasein degrading activity, were pooled and loaded onto the Mono S column. The active fractions were separated as shown in Figure 4.4. Sample 1 was eluted with 40 mM NaCl; sample 2 with 50 mM NaCl and sample 3 with 140 mM NaCl.

4.3.4 Inhibition of CMA endopeptidases.

The CMA endopeptidase samples 1, 2 and 3 (described in Section 4.2.11.2) were treated with the inhibitors pepstatin, leupeptin and DTT/EDTA, which inhibit aspartic acid, cysteine and metallo endopeptidases respectively (Table 4.3). Endopeptidase activity was completely inhibited after preincubation with leupeptin in fractions 1 and 2 and fraction 3 was strongly inhibited. This suggests that the endopeptidase activity in the CMA fractions is mainly due to cysteine endopeptidases. Both the pepstatin and DTT/EDTA treatments had no inhibitory effect on the enzyme samples; the DTT/EDTA treatment actually had a stimulatory effect on all three samples. DTT and EDTA are both inhibitors of metallo-endopeptidases but the activating effect of this treatment is probably due to DTT which is known to stimulate the activity of thiol-endopeptidases (Barrett, 1986). Low molecular weight thiol compounds protect the essential thiol group of thiol-endopeptidases from reacting with numerous compounds which inhibit the enzyme (Barrett, 1986).

4.3.5 Immunological relationship between the CMA endopeptidases and MEP-1.

Protein bands bearing an immunological relationship to MEP-1 in the endopeptidase samples 1 and 3 were identified after separation using SDS-PAGE and immunoblotting (Figure 4.5). Crude extracts of Schooner (lanes 2 and 3) show two main bands with Mr 30,000 and ~36,000. The total CMA sample (lane 6) appears to contain a double band at ~30,000 Mr and a band at 37,000 Mr. The lower 30,000 band has an equivalent Mr with the major MEP-1 band but the upper band, which is most prominent, appears to be the major band in fractions

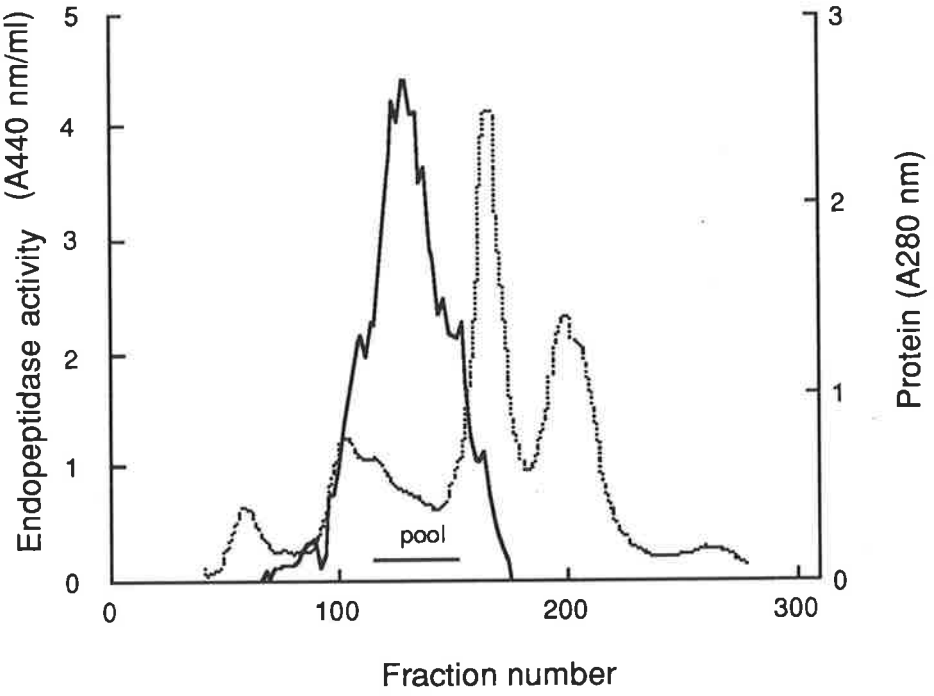

Figure 4.3 Elution profile of CMA proteins from the S200-Sephacryl column.

CMA proteins were eluted from the CM-Sephadex column, concentrated and applied to a S200-Sephacryl column. The protein elution from the S200 Sephacryl column was monitored at 280 nm (·····), and azocasein activity (Section 5.2.2) at 440 nm (——). Fractions 118-155 were pooled and retained for application to the Mono S column (Figure 4.4).

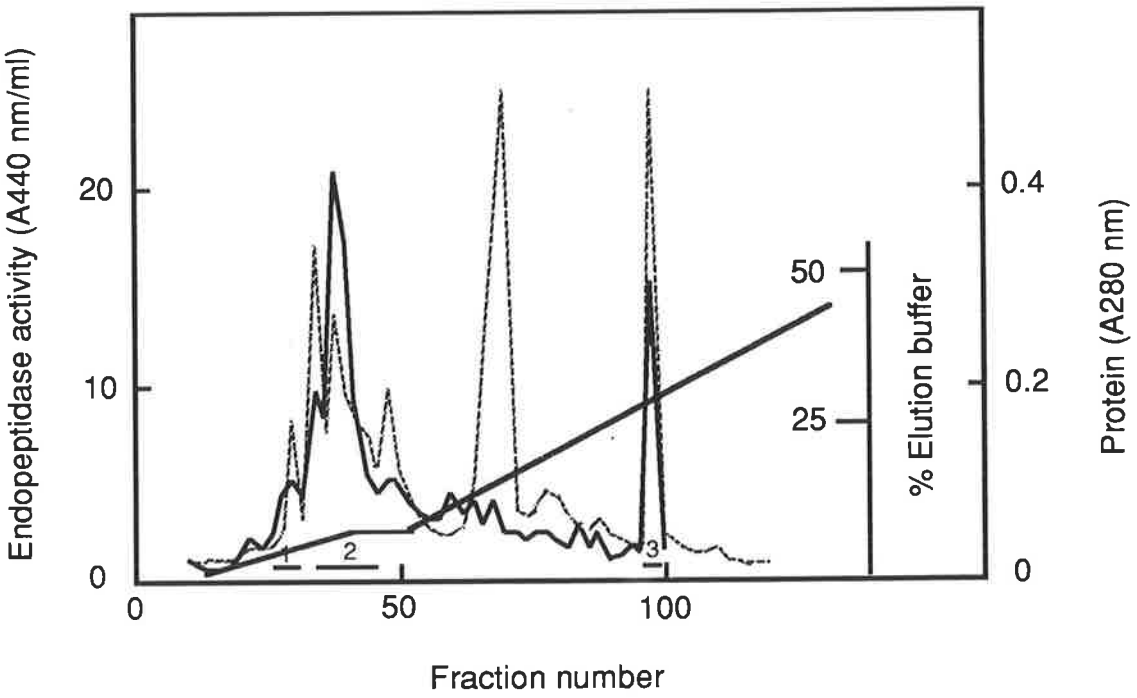

Figure 4.4 Separation of CMA proteins by cation exchange chromatography.

The main endopeptidase activity eluted from the S200 column (Figure 4.3) was pooled and applied to a Mono S cation exchange column connected to a Pharmacia FPLC system. The protein elution was monitored at 280 nm (.....). Azocasein activity (Section 5.2.2) was measured at 440 nm (——). Fractions 33-35, 37-42, and 97-98 were pooled as shown and are referred to as fractions 1, 2 and 3 respectively.

Table 4.3 Inhibition of the CMA endopeptidase fractions separated by cation exchange chromatography.

CMA fractions 1, 2 and 3 were preincubated in the presence of pepstatin (12.5 μM), leupeptin (5 μM), DTT/EDTA (3 mM/3 mM) or control solutions. Endopeptidase activity was measured against azocasein (Section 5.2.2) and is expressed as a % of the control assay.

	% ACTIVITY		
FRACTION	LEUPEPTIN	PEPSTATIN	DTT/EDTA
1	0	96	118
2	0	105	112
3	26	123	154

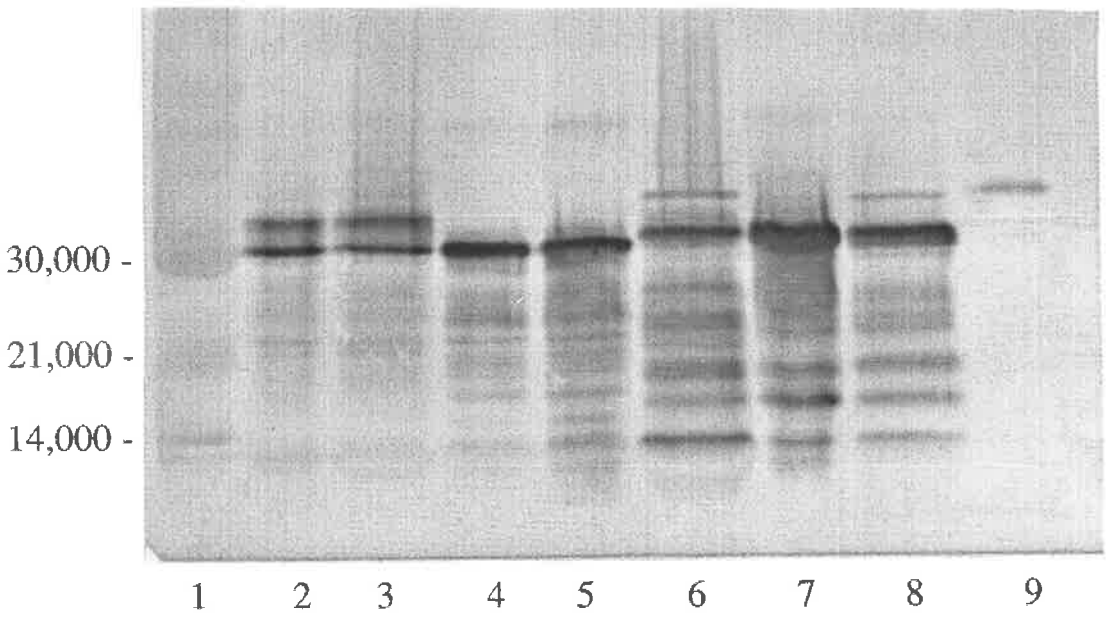

Figure 4.5 Immunoblot of CMA endopeptidase fractions.

The CMA endopeptidase fractions were separated using SDS-PAGE and proteins with immunological identity to MEP-1 were detected using immunoblotting.
Lane 1) rainbow standards (supplied by Amersham) ; 2) Schooner crude extract (10 µl); 3) Schooner crude extract (20 µl); 4) MEP-1 (5 µl); 5) MEP-1 (10 µl); 6) total CMA proteins (15 µl); 7) CMA fraction 1 (5 µl); 8) CMA fraction 3 (20 µl); 9) 37,000 endopeptidase (20 µl).

1 and 3 (lanes 7 and 8). The 37,000 Mr band appears as a faint band in fraction 1 (lane 7) and is more strongly present in fraction 3 (lane 8).

Several less defined bands run in the region below the 30,000 marker in the crude extract samples (lanes 2 and 3). It is postulated that these lower bands are either breakdown products of MEP-1 or related proteins. Bands in the MEP-1 sample which run below the 30,000 region (lanes 4 and 5) are breakdown products as the enzyme was isolated to homogeneity but had been stored at -20°C for several weeks. The total CMA proteins and the CMA fractions had also been stored at -20°C for several weeks and also show several bands in the region below 30,000 Mr. However, the degradation products of MEP-1 (lanes 4 and 5) do not have the same banding pattern as the products in the CMA samples (lanes 6 to 8) which further indicates that the CMA endopeptidases are distinct from MEP-1. The fractions 1 and 3 (lanes 7 and 8) have the same banding pattern apart from the presence of the 37,000 Mr band in fraction 3. The 37,000 Mr enzyme (lane 9) does not show any degradation products indicating that it is either not susceptible to autolysis or that there are no stable, intermediate steps in this process.

4.3.6 Modification of bound β-amylase by malt endopeptidases.

Treatment of the bound β-amylase sample for 3 days at 20°C in 0.2 M acetate pH 4.5 resulted in an increase in the activity of the free enzyme from 4.3 to 62 units/g (Figure 4.6). In the presence of 2-ME (3 mM) the activity was 146 units/g. In both cases the isoform pattern was the same as the free enzyme (see Figure 4.6 lanes 2 and 3 and Figure 4.7 lane 2). Treatment with the malt endopeptidase MEP-1 or another partially purified malt endopeptidase fraction (CMA) and 2-ME increased free β-amylase activity to 205 ± 8.0 and 155 ± 6.0 units/g respectively. The endopeptidase treatment resulted in the generation of several β-amylase isoforms with higher pI values (5.7, 6.6, 7.0 and 7.5) and a decrease in the amount of the lower pI isoforms. A malt sample (free β-amylase 430 units/g) contained all the β-amylase isoforms generated by treatment of the bound enzyme with malt endopeptidases and 2-ME plus additional isoforms with pI values 4.7 and 8.0.

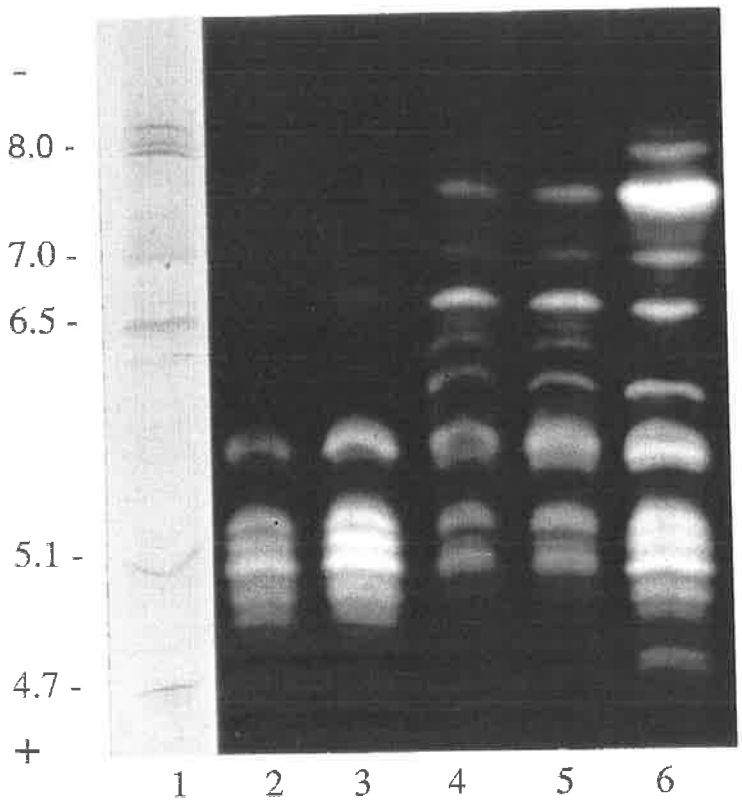

Figure 4.6 Effect of malt endopeptidases and 2-ME on bound β-amylase in comparison with malt β-amylases.

Amylase samples were applied to an IEF gel and identified with a starch overlay. The endopeptidase samples used (CMA and MEP-1 (2.4 μg)) had similar activities when tested against azocasein (Section 5.2.2).
The samples shown are : 1) pI markers, 2) bound β-amylase incubated 3 d at 20°C, 3) with 3 mM 2-ME, 4) 3 mM 2-ME + CMA endopeptidase, 5) 3 mM 2-ME + MEP-1, 6) malt sample treated with EDTA.

Sample	2	3	4	5	6
β-Amylase activity (units/g)	62 ± 0.1	146 ± 21	155 ± 6.0	205 ± 8.0	430 ± 9.2

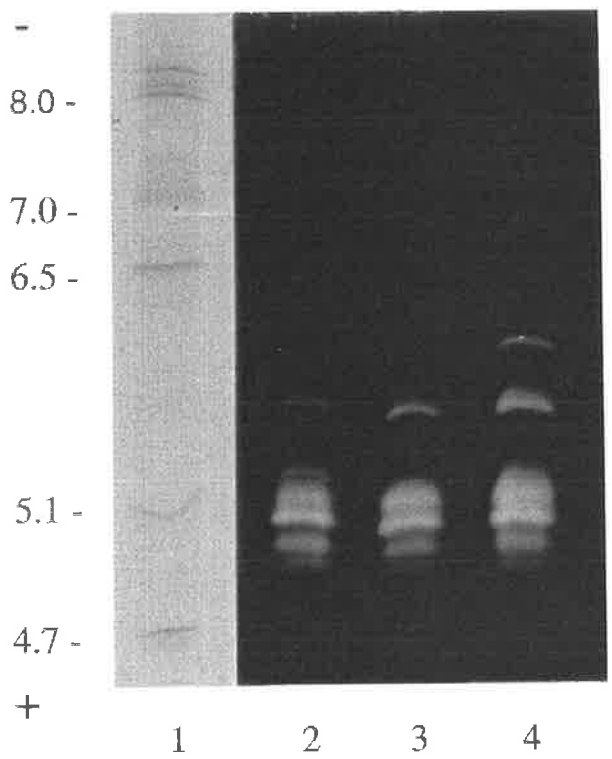

Figure 4.7 Effect of MEP-1 on free β-amylase.

Amylase samples were applied to an IEF gel and identified with a starch overlay.
The samples shown are : 1) pI markers, 2) Free β-amylase incubated 20 h at 20°C, 3) with 3 mM 2-ME, 4) 3 mM 2-ME + MEP-1.

Sample	2	3	4
β-Amylase activity (units/g)	27 ± 0.1	41 ± 4.7	69 ± 0.6

4.3.7 Modification of free β-amylase by malt endopeptidases.

Free β-amylase comprised the following pI isoforms, 5.0 - 5.3, 5.5 and 5.7 (Figure 4.7), however, the 5.7 band is not clear in the reproduction of the photograph. Incubation with 2-ME (3 mM) increased β-amylase activity from 27 units/g to 41 units/g but no additional isoforms were detected. Treatment with MEP-1 and 2-ME resulted in an in increase β-amylase activity to 69 units/g plus the appearance of an additional band appearing at pI 6.4, and an enhancement of the band at pI 5.7. The band present at 6.4 is also difficult to detect in the reproduction of the photograph. Prolonged incubation times (as used with the bound β-amylase) resulted in the loss of free β-amylase activity.

4.3.8 Estimation of thiol concentration in barley and malt flour.

Both total thiol content and non-protein thiol content were determined in barley and malt flour, the amount of protein bound thiol present in each sample being estimated from the difference in the former two parameters. The thiol level was estimated with Ellman's reagent and 2-ME used for the standard curve (Figure 4.8). Malt flour contains considerably more thiol than barley flour (Table 4.4). Most of the SH groups found in barley flour (91 %) are protein bound but only 45 % of the SH groups in malt flour appear to be protein bound. A recovery test of 2-ME added to barley and malt samples demonstrated 94 % of the 2-ME was recovered from barley and 97 % for malt using the total thiol assay.

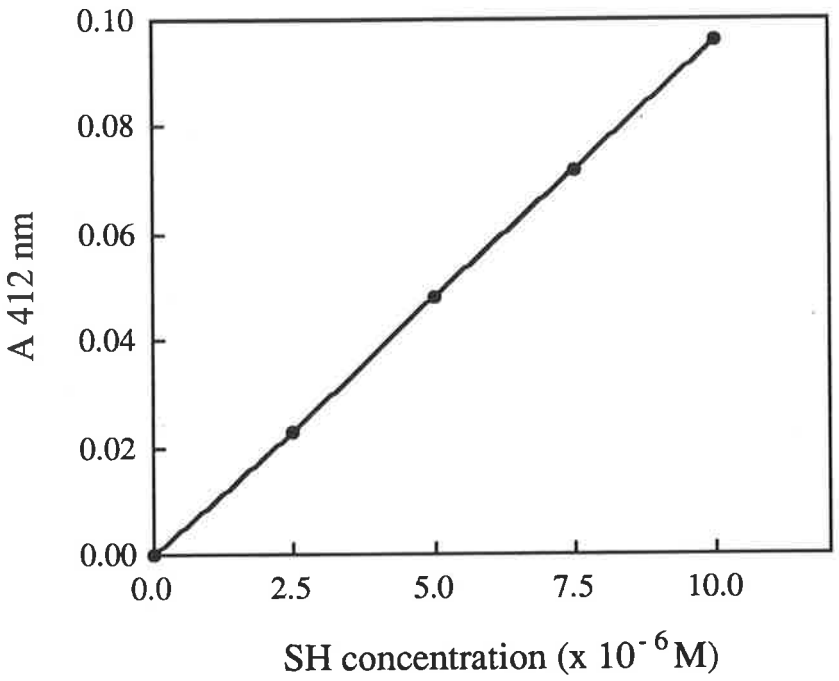

Figure 4.8 2-ME standard curve.

Samples of 2-ME (0.5 ml) were tested for total thiol content as described in Section 4.2.14. Concentrations shown are final SH concentrations in the colour reaction mixture.

Table 4.4 Estimation of total and non-protein bound thiol in malt and barley flour.

Thiol contents in barley and malt samples were measured as described in Sections 4.2.14 and 4.2.15.

Sample	Total SH	Non-protein SH	Protein Bound SH	% Bound SH
		(μmoles SH/g flour)		
Barley	1.1	0.1	1.0	91
Malt	4.0	2.2	1.8	45

4.4 DISCUSSION

β-Amylase accumulates, as the barley seed develops, in a relatively inactive bound form which is released and consequently activated during germination. Thiol compounds and endopeptidases appear to have a vital role in the activation process. It is still not clear what proportion of the bound and salt insoluble β-amylase complex in the barley seed is the result of self aggregation of this albumin or its association with other seed proteins (Gupta *et al.*, 1991) and starch (Hara-Nishimura *et. al.*, 1986). Such complexes are likely to form during the desiccation phase of seed development (Hara-Nishimura *et. al.*, 1986). During the germination process a range of isoforms differing in pI and Mr values are generated from the β-amylases present in the quiescent seed.

Treatment of barley flour with thiols or endopeptidases has been shown to release β-amylase and result in the generation of many of the isoforms observed in malt (LaBerge and Marchylo, 1986). In the present study it is shown that there is a distinct difference between the action of thiols and the endopeptidases. While incubation at pH 4.5, especially in the presence of 2-ME, promotes the release of β-amylase, the isoforms of the released enzyme are the same as that of the free enzyme in the seed. Addition of an endopeptidase is necessary to obtain the isoform pattern of β-amylase characteristic of malt.

In earlier studies it was predicted that the use of papain, to produce malt type forms of β-amylase, mimicked the role of malt endopeptidases. The main components of these are cysteine dependent enzymes that are synthesised during germination. Sopanen and Laurière (1989) used an endosperm extract from germinating barley to convert β-amylase from the barley seed to the malt type enzyme and showed that it was inhibited by the cysteine-endopeptidase inhibitors leupeptin and antipain. We have used two cysteine endopeptidase samples (MEP-1 and CMA), extracted from germinating barley seeds, and have shown that they both could be responsible for the main modifications of β-amylase observed in malt. MEP-1 is a cysteine-endopeptidase, which accounts for most of the endopeptidase activity in 4 day barley seedlings (Phillips and Wallace, 1989). Both the MEP-1 and CMA

endopeptidase samples generated a range of isoforms with higher pI values. The higher pI isoforms that are generated by proteolysis are likely to result from a limited cleavage of a fragment of Mr up to 5,000 from the carboxy-terminal end of the β-amylase protein (Sopanen and Laurière, 1989; Lundgard and Svensson, 1987). This modification has been shown to have no effect on the activity or kinetic properties of the enzyme (Lundgard and Svensson, 1987). The two isoforms present in malt, but not detected in our *in vitro* studies with endopeptidases, may have been generated by carboxypeptidases that are present at high activities in malt (Winspear *et al.*, 1984), or by the action of endopeptidases which are yet to be characterised.

The activity of free β-amylase also increased with thiol and endopeptidase treatments. The present study demonstrates that the effect with 1 mM 2-ME and MEP-1 (+156 %) was greater than 2-ME alone (+52 %). Sopanen and Laurière (1989) reported that dithiothreitol and papain produced a similar activation of the free β-amylase (20-30 %) but no additional effect when both agents were added together. However they used a higher level of thiol (maximum effect with 25 mM) and a shorter incubation time.

The activation of free β-amylase could be the result of its dissociation from small homopolymers or a heterodimer with protein Z. Niku-Paavola *et al.* (1973) showed that purified β-amylase monomers spontaneously formed a series of polymers in the absence of reducing agents. Hejgaard and Carlsen (1977) examined salt extracts of barley prepared with 1 mM 2-ME for β-amylase complexes. The only polymer detected was a β-amylase-protein Z heterodimer which accounted for about 30 % of the β-amylase activity, however the thiol content may have dissociated any β-amylase homopolymers. Treatment with 100 mM 2-ME split the β-amylase-protein Z complex but gave no increase in β-amylase activity.

An alternative explanation for the activation of free β-amylase is that a modification of its conformation is triggered by either reduction of an intrachain dithiol bond(s) or proteolytic cleavage. Lundgard and Svensson (1987) showed that there was no change in activity during the proteolytic modification of β-amylase but the sample they used had been extracted with

0.1 M monothioglycerol and may have been fully activated by the thiol prior to their proteolysis treatments.

The proportion of free to bound β-amylase is controlled by a simple inheritance factor (Bendelow, 1964). In most studies free β-amylase has been measured in salt extracts and bound β-amylase has been estimated by the increase in activity after treatment with papain or thiol (Pollock and Pool, 1958). Our study has shown that in any evaluation of the relative amounts of free and bound β-amylase, consideration must be given to the activation of both components.

The full range of endopeptidases present in germinating barley is yet to be determined, the number of enzymes present and their biochemical class is still under investigation. Although it was not a primary aim of the present study to characterise the cysteine endopeptidases in germinating barley some progress was made with the separation of the CMA endopeptidases. Protein bands possessing immunological identity with MEP-1 were separated by SDS-PAGE (Figure 4.5). It is assumed that these are responsible for the endopeptidase activity detected and from inhibitor studies they appear to be mainly cysteine endopeptidases (Table 4.3). The MEP-1 sample used to raise antibodies was essentially a 30,000 Mr protein but it would be expected that breakdown would occur in the rabbit's blood and that antibodies would also be raised against these degradation products. Therefore the polyclonal antibody population would contain IgG molecules that react with various conformations of the native MEP-1 protein as well as partially denatured forms. It is also likely that MEP-1 and other related endopeptidase species would share common epitopes.

Two main bands reacting with the anti-MEP-1 polyclonal antibodies were observed in crude extract separated by SDS-PAGE (Figure 4.5 lanes 2 and 3). A similar pattern was also observed by Jones and Poulle (1989) on western blots of crude extracts. The CMA fractions 1 and 3 contain an identical major band as well as a very similar pattern of degradation products. It is possible that these are isoforms of the same enzyme which are differently charged and are therefore separated during ion exchange chromatography. The cysteine

endopeptidases MEP-1 (Phillips and Wallace, 1989), EP-B (Koehler and Ho, 1990a) exhibit a single band after separation by SDS-PAGE but more than one species when separated on the basis of charge. The differently charged forms may also be the products of post-translational modification of MEP-1. Recent reports have shown that α-amylase is modified after translation by carboxypeptidases (Søgaard *et al.*, 1991) and deamidase (Sticher and Jones 1991) giving rise to several new forms of the enzyme. Koehler and Ho (1990b) reported a small gene family of four or five cysteine endopeptidases that are related to MEP-1. Wrobel and Jones (1992) used an electrophoretic technique and found four cysteine endopeptidase species present in malt.

Although there is not substantial information on the thiol level in the endosperm during germination (Wallace and Lance, 1988) it does not appear to be present at the level used in most *in vitro* studies. Malt flour has approximately four times the total amount of bound SH in barley flour. The SH group in the latter are predominantly protein bound (91 %), whereas in malt less than half (45 %) is protein bound (Table 4.4). During germination the protein bound SH is nearly doubled and nonprotein bound SH is increased more than 20 fold in the malt flour sample. It appears that SH groups are generated during germination particularly in the nonprotein bound fraction. Several mechanisms of thiol generation have been proposed which may account for this increase in thiol content. NADP-thioredoxin reductase and thioredoxin *h* have been shown to reduce the major storage proteins in wheat (Kobrehel *et. al.* 1992) and it has been suggested that reduced thioredoxin may function as a signal in germination to increase the mobilisation of storage proteins as well as activating several enzymes. There is increasing evidence that the thiol level in seed endosperm has a regulating influence on proteolysis and other metabolic events. Regulation of endopeptidase activity by the intracellular redox state has been reported in erythroleukemia cells (Tsukahara *et al.*, 1990). Indeed the activity of MEP-1 against barley proteins is enhanced by the presence of thiol (Phillips and Wallace 1989) and the full activation of β-amylase requires both the thiol and endopeptidase components.

β-amylase is activated by the action of endopeptidases, the bulk of which are synthesised *de novo* (Hammerton and Ho, 1986), and also by the presence of thiol. However, the present study has confirmed that the isoforms of β-amylase found in malt are generated only after treatment with endopeptidases and not by thiol treatment alone. Recently, Koehler and Ho (1990a) reported that they have unpublished data which indicates that cysteine endopeptidases, isolated in their laboratory, are also capable of modifying β-amylase. Limit dextrinase has recently been reported to be activated by endogenous barley cysteine endopeptidases (Longstaff and Bryce, 1993). The endopeptidases in germinating barley appear to have an important regulatory role in the activation of other proteins and hydrolytic enzymes during germination.

CHAPTER 5

THE EFFECTS OF ENDOPEPTIDASE ACTIVITY AND BARLEY STORAGE PROTEINS ON MALTING QUALITY

5.1 INTRODUCTION

The composition of barley storage proteins and their modification by proteolytic enzymes during malting influence the final balance of nitrogenous compounds in wort. Both the amount of protein in barley and how effectively it is degraded during malting influences the overall modification of the endosperm. The catabolism of proteins is necessary to provide amino acids for yeast metabolism and to degrade proteins which interfere with starch hydrolysis and filtration. However, high levels of soluble nitrogen compounds in the wort result in the formation of hazes in beer (Asano *et al.*, 1982). The action of malt endopeptidases is important as it limits the rate of proteolysis during malting and mashing (Burger and Schroeder, 1976).

Endopeptidase activity has been shown to influence a variety of malting quality characteristics yet it is not routinely measured as a part of malting quality evaluation or considered in barley breeding programs. There have been difficulties in choosing a suitable technique for measuring the activity of malt endopeptidase enzymes as it depends upon the nature of the substrate (Baxter, 1976). Relatively little is known about the levels of activity required for optimum malting quality in comparison to other hydrolytic enzymes such as the amylases and β-glucanases. Endopeptidase activity has been shown to be high in better malting varieties (Morgan *et al.*, 1983; Baxter, 1976) and accounts for 20% of the variability of malt extract. However only a limited number of varieties were compared in these studies.

It is well known that the amount of total protein in barley seeds is generally inversely correlated with malting quality (Hulton, 1922) and much research has been undertaken trying to relate individual protein components to poor quality. Studies attempting to associate hordein composition and malting quality have demonstrated a weak relationship between the two. Insoluble proteins containing cysteine form disulphide bridges with other proteins and cell wall components during mashing (Baxter and Wainwright, 1979). These compounds trap starch granules and contribute to filtration problems. Soluble sulphur compounds may also be responsible for characteristic 'off' flavours and aromas in beer. High molecular weight aggregates of B and D hordeins, known as 'gel protein', cause problems during mashing and are inversely related to malting quality (Smith and Lister, 1983). Recently the hordein composition of Japanese varieties of two row barleys was examined by SDS-PAGE (He *et al.*, 1993). The banding pattern of the B and C hordeins was found to be closely related to malting quality parameters such as extract yield, diastatic power, soluble nitrogen content and Kolbach index.

The effect of hordein banding pattern on malting quality was studied in this Chapter using near isogenic lines differing in hordein banding patterns (the Pallas lines). The lines were originally developed to study the interaction of powdery mildew and powdery mildew resistance genes (*Mla* locus) in barley (Kølster *et al.*, 1986). Donors of resistance genes were crossed and backcrossed 5 times to spring barley cv. Pallas. Two linked hordein loci (*Hor 1* and *Hor 2*), on the short arm of chromosome 5, were used to select for genetic similarity in the region of the resistance gene. The degree of similarity between the isogenic lines and the parent Pallas was high according to morphological and physiological observations. The allocation of hordein genes in some of the near isogenic lines described by Kølster *et al.* (1986) and is summarised in the following table. Parantheses indicate that the allelle is identical in Pallas and the donor Parent.

Near isogenic line	Allele in locus *Hor 1* C hordein	*Hor 2* B hordein	Donor	Origin
Pallas	Pallas	Pallas		
P03	donor	Pallas	Iso 20R	Franger
P04A	donor	(Pallas)	Nordal	Heine 4808
P07	donor	Pallas	Mona	Monte Cristo
P08A	donor	donor	Senat	Triple Awn Lemma
P09	(Pallas)	donor	Iso 12R	Durani

The isolines P03, P04A and P07 contain donor C hordeins, isoline P09 contains donor B hordein and P08A has donor B and C hordein. The advantage of these lines is that the genetic background of each isoline should be similar for the content of protein degrading activity but the banding patterns of the B and C hordeins will vary. Therefore any difference in malting quality between the lines should be related to hordein composition and the interaction with a common set of peptidase enzymes. Many factors are involved in the production of quality malt extract, thus it is difficult to assess the effect a single factor may have on extract value. The degradation of hordeins for instance will be affected by the environment, type and quantity of the hordeins as well as the action of endopeptidases and other hydrolytic enzymes. The Pallas lines are therefore useful for studying the effect of hordein on malting quality.

The general aims of the following study are to investigate the effects of endopeptidase activity and hordein composition on malting quality. The specific aims are as follows:

i) To determine the most accurate method of measuring endopeptidase activity.

ii) To study endopeptidase activity and degradation of barley storage proteins during the malting and kilning processes.

iii) To investigate the effect of barley storage proteins on malting quality using near isogenic barley lines which differ in hordein banding pattern.

iv) To investigate the effect of endopeptidase activity on malting quality in several barley varieties.

5.2 EXPERIMENTAL PROCEDURE

5.2.1 Preparation of enzyme extracts for comparison of endopeptidase assays.

Kilned malt was ground to a flour in a (Udy) mill with a 0.5 mm screen and extracted with sodium acetate pH 4.5 (100 mM), 2-ME (1 mM), NaCl (50 mM); insoluble PVPP (2.5%) at a concentration of 0.75 g/ 3.75 ml. The samples were shaken on ice for 1 h on a horizontal shaker at 50 osc/min before centrifugation at 10,000 *g* for 10 min. The supernatants (2.4 ml) were desalted using PD10 columns (Pharmacia, Sweden) equilibrated with sodium acetate pH 4.5 (100 mM), 2-ME (1 mM) to remove amino acids which interfere with the ninhydrin assay. The volume collected for each sample was 3 ml.

5.2.2 Endopeptidase assay using azocasein.

Purified MEP-1 or enzyme extract (75 µl) was incubated with 400 µl of azocasein (0.6 % (w/v)) in citrate (0.063 M)/ phosphate (0.063 M) buffer pH 5.5 and 25 µl 2-ME (0.1 M) for 2 h at 37°C. The reaction was stopped with the addition of 1 ml Hagihari reagent [acetic acid (0.3 M), TCA (0.1 M) and sodium acetate (0.2 M)]. Proteins in the mixture precipitated during a 10 min incubation on ice followed by 10 min centrifugation at 12,000 *g*. The supernatant was decanted into a fresh tube, mixed with 0.1 ml NaOH (10 M) to stop the reaction. The absorbance was read at 440 nm and the units are given as Δ A 440 nm /h /g.

5.2.3 Hordein preparation.

Pearled barley flour (30 g) from cv. Clipper was extracted with 150 ml NaCl (0.5 M); $HgCl_2$ (0.1 M) at room temperature for 1 h with vigorous shaking. The extract was centrifuged for 10 min at 13,000 *g* and the supernatants containing water soluble proteins were discarded. The pellets were then re-extracted with the same solution and centrifuged again. The resulting pellets were resuspended in iso-propanol (50 % (v/v); 2-ME (0.6 % (v/v)), shaken for 1 h, centrifuged (as

above) and the supernatants retained. The extraction was repeated and the supernatants pooled with those from the first extraction. Insoluble PVPP (1 % (w/v)) was added to the pool, the mixture was stirred at room temperature for 30 min and then filtered through Whatman No 1 paper. The PVPP treatment was repeated then the solution was concentrated to a volume of approximately 100 ml by rotary evaporation at 40°C. The suspension was dialysed exhaustively against RO water, freeze dried under vaccuum and stored at 4°C.

5.2.4 Endopeptidase assay using hordein.

Enzyme extract (30 µl) was incubated with 30 µl hordein suspension (50 mg/ml) and 30 µl sodium acetate (0.2 M) pH 4.5; 2-ME (0.15 % (v/v)) for 1 h at 37°C. The reaction was stopped with 90 µl cold trichloroacetic acid (TCA) (10 % (w/v)) and placed on ice for 10 min. Precipitated proteins were removed by centrifugation at 12,000 *g*. An aliquot (0.1 ml) of the supernatant was mixed with 1.4 ml of ninhydrin reagent (Shannon and Wallace, 1979), placed in a boiling water bath for 12 min and then plunged into an ice slurry to stop further colour development. The absorbance was read at 570 nm at room temperature. Units given are Δ A 570 nm /min /g.

5.2.5 Enzyme-linked immunosorbent assay (ELISA).

96 well microtitre plates (Nunc, Denmark) were coated with a 1:1000 dilution of poly clonal antibodies raised against MEP-1, in sodium carbonate buffer (0.1 M) pH 9.6 (200 µl per well) overnight at 4°C. The plates were washed five times, including a 5 min soak after the third wash, with phosphate buffered saline pH 7.4 (PBS) containing Tween 20 (0.05 % (v/v)) then blocked with 200 µl BSA (1 % in PBS) per well for 1 h at 37°C. The plates were washed as above. Serial dilutions of MEP-1 were prepared in BSA (1 % (w/v) in PBS) starting at 50 ng/ml then diluted by a factor of 0.75 nine times until a final concentration of 5 ng/ml was reached; 100 µl of each solution was added per well. Likewise 100 µl aliquots of sample diluted in BSA (1 % (w/v) in PBS) by factors of 50, 100, 500, 1000, and 5000 were added to appropriate wells. At this

stage plates were incubated at 37°C for 1.5 h. After another wash (as above), 100 µl of antiMEP-1 IgG conjugated with horse-radish peroxidase (BioRad, Australia) diluted 1:200 with BSA (1 % (w/v) in PBS) was added to each well and incubated as above. The plates were washed (as above) and 50 µl of substrate solution (prepared by adding 10 µl hydrogen peroxide (30 % (v/v)) to 100 ml of 2, 2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) solution (540 mg ABTS in 2 mM citrate buffer) was added to each well. The reaction was stopped with the addition of 50 µl oxalic acid (3 % (w/v)) and after 10 min the absorbance of each well was measured at 414 nm using an ELISA plate reader (Titertek Multiscan, Flow Laboratories, Sweden). Control wells containing no antigen, no antibody and no substrate were included on each plate and used as a blank. Units given are equilavent to ng MEP-1/100 µl as determined from the standard curve applicable to each run.

5.2.6 Carboxypeptidase assay.

Enzyme extract (0.2 ml) was incubated with 0.5 ml *N* -carbobenzoxy-*L* -phenylalynyl-*L* -alanine (N-CBZ phe-ala) 1 mg/ml in sodium acetate (0.1 M) pH 5.2 for 30 min at 37°C. The reaction was stopped with the addition of 2.8 ml ninhydrin reagent (Shannon and Wallace, 1979). The mixture was placed in a boiling water bath for 12 min and then plunged into an ice slurry to stop further colour development. The absorbance was read at 570 nm when the mixtures had returned to room temperature. Units given are Δ A 570 nm /min /g.

5.2.7 Inhibition of carboxypeptidases.

Crude extracts were incubated with the inhibitor PMSF (4 mM in 20% (v/v) isopropanol), or a control solution (20% isopropanol (v/v)) for 1 h at 37°C at a 3:1 ratio. The extracts were then tested for endopeptidase activity against azocasein and hordein and for carboxypeptidase activity described in Sections 5.2.2, 5.2.4 and 5.2.6 respectively.

5.2.8 Experiments using the Pallas isolines.

The Pallas lines were grown at Charlick Experimental Station, Strathalbyn in a randomised block design with 3 replicates. Malt samples were micromalted in the Waite Barley Quality Evaluation Laboratory as described in Section 2.2.1.2.

5.2.9 SDS extraction of malt and barley samples and size exclusion (SE) HPLC.

Barley or malt flour from the Pallas isolines was extracted with sodium phosphate (50 mM); SDS (2% (w/v)) pH 6.9 at a concentration of 20 mg/ml. The suspension was sonicated for 30 sec and mixed (with a vortex) for 30 min before centrifugation at 12,000 *g*. The supernatants were filtered through a 0.2 µm low protein binding Durapore membrane, (Millipore, Australia) and loaded onto a Protein Pak 300 SW HPLC column (Millipore-Waters, USA), run at a pump speed of 0.5 ml/min in an isocratic gradient of acetonitrile (50 % (v/v)); trifluoroacetic acid (TFA) (1 % v/v)). This analysis was undertaken on a Waters HPLC at C. S. I. R. O. Division of Plant Industry, Grain Quality Research Laboratory, Sydney. The eluted protein was measured by absorbance at 210 nm and the peak areas are expressed in arbitrary HPLC units (millivolts x centimetres).

Samples of Schooner barleys were supplied by the Australian Barley Board. These samples varied in both protein content and % malt extract. Protein samples were prepared in the same way as for the Pallas isolines (described above) but were separated on a Varian TSK 4000 size exclusion column using a Varian Vista Series 5000 Liquid Chromatograph at the Waite Institute Campus.

5.2.10 Protein extraction for SDS-PAGE.

Barley proteins were prepared for SDS-PAGE based on the method of Doll and Andersen (1981). Barley or malt flour was extracted with isopropanol (50 % (v/v)); Tris-HCl (100 mM) pH 8.5 at a

concentration of 50 mg/ml, samples were shaken (on a vortex) for 30 min then subjected to centrifugation for 10 min at 12,000 *g*. The supernatants were decanted into fresh tubes and the proteins were reduced by incubation with 10 µl of dithiothreitol (DTT) (0.2 M) for 1 h at 50°C. After reduction the proteins were alkylated by the addition of 10 µl iodoacetamide (0.5 M) and kept at 50°C for a further 15 min. The proteins were then precipitated with 600 µl of cold, distilled water and kept on ice for 30 min. The samples were subjected to centrifugation as above, the pellets resuspended in 750 µl loading buffer (2-ME (5 % (v/v)), SDS (0.2 % (w/v)), glycerol (10 % (v/v)), bromophenol blue (0.002 % (w/v)) and Tris-HCl (0.06 M) pH 6.8) and separated by SDS-PAGE as described in Section 2.2.2.1.

5.2.10.1 Protein extraction with SDS and sonication in preparation for SDS-PAGE.

Barley flour from the Pallas isogenic lines was extracted in sodium phosphate (50 mM); SDS (2% (w/v)) pH 6.9 at 20 mg/ml and sonicated for 30 sec. The suspensions were centrifuged for 10 min at 12,000 *g* and the supernatants boiled in the presence of loading buffer (described in Section 5.2.10) and 2-ME (final concentration 40 mM). Samples were then loaded onto a SDS-PAGE gel.

5.2.11 'Gel protein' extraction for SDS-PAGE.

Barley flour (50 mg) was mixed with 1 ml of SDS (1.5 % (w/v)) for 2 h before centrifugation at 12,000 *g* for 15 min. The supernatant was decanted and the pellet dissolved in 400 µl of SDS (1.5 % (w/v)); 2-ME (10 % (v/v)) N, N'-dimethylformamide (16% (v/v)) at 55°C for 2 h with intermittent vortexing. The samples were centrifuged for 15 min at 12,000 *g* before separation by SDS-PAGE (Section 2.2.2.1).

5.2.12 Endopeptidase activity in different barley varieties.

Twenty barley varieties grown at a single site (Charlick Experimental Station) were used in the pilot study of this experiment. For the main experiment fourteen different barley varieties were grown at 3 sites (Crystal Brook, Warooka and Lameroo) each with replicate plots in randomised block designs.

The barley samples were micromalted in the Waite Barley Quality Evaluation Laboratory (Section 2.2.1.2) and malt flour was ground in a Udy mill with a 0.5 mm screen. Endopeptidase activity was extracted from malt flour (0.1 g) on a horizontal shaker at 200 osc/min with 0.5 ml sodium acetate (0.1 M); 2-ME (1 mM) pH 4.5 for 30 min at room temperature. The samples were centrifuged for 10 min at 12,000 *g* and the supernatants were decanted and kept on ice. Endopeptidase activity in each supernatant was measured against azocasein (Section 5.2.2).

5.3 RESULTS

5.3.1 Comparison of methods used to measure endopeptidase activity.

5.3.1.1 Comparison of ELISA, azocasein and hordein assays in several varieties.

Endopeptidase activity against hordein, azocasein and by ELISA was measured in several malt samples to select the most appropriate assay for screening a large number of samples. The endopeptidase activities shown in Figure 5.1 are divided by the activity against azocasein (Δ A 440 nm/ h/ g dry wt) to emphasise the difference in activities of each of the assays. The activity against azocasein therefore always equals 1 and the hordein and ELISA are a ratio of the azocasein activity. There appears to be no constant relationship between the activities of the assays over the different varieties tested. The azocasein assay was the most convenient to use. Green malt extracted from Clipper barley was used for 20 azocasein assays and the mean activity and standard error were 16.05 ± 0.1 (Δ A 440 nm /h /g fresh wt).

5.3.1.2 Effect of carboxypeptidases on endopeptidase substrates.

The endopeptidase assays using hordein and azocasein as substrates were tested to determine whether carboxypeptidases interfered with the degradation of these substrates. The effect of PMSF, an inhibitor of the serine dependent carboxypeptidases on azocasein, hordein and carboxypeptidase assays is shown in Table 5.1. The PMSF treatment inhibited carboxypeptidase activity by 67%, endopeptidase activity against hordein by 20% and endopeptidase activity against azocasein by 0.7%. The results indicate that carboxypeptidases contribute to the degradation of hordein when crude extracts are used as a source of endopeptidase enzyme. The azocasein assay, however, was not significantly affected by the action of carboxypeptidases.

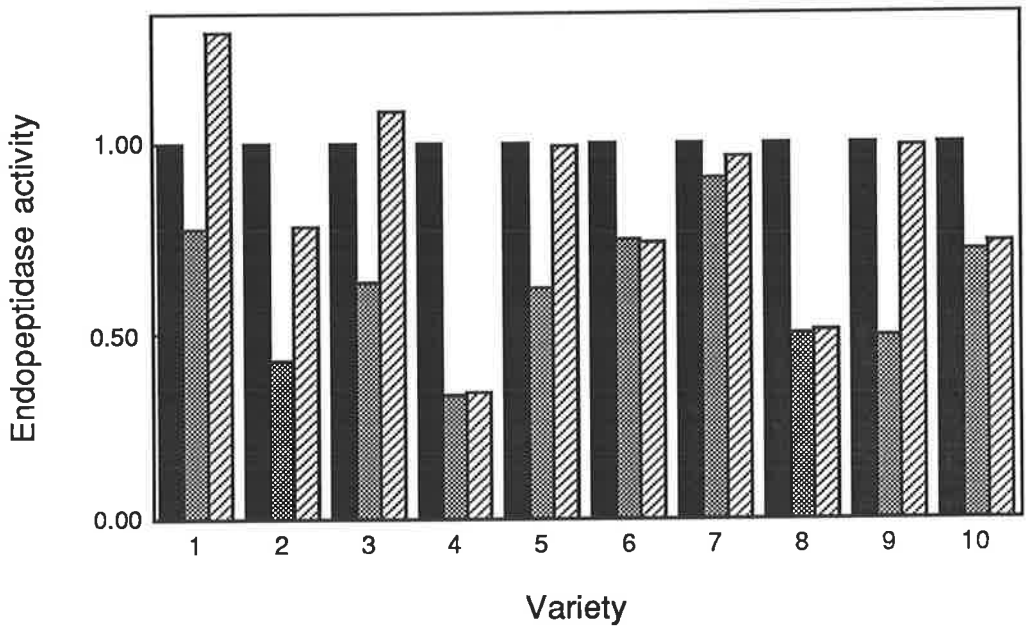

Figure 5.1 Comparison of three endopeptidase assays on several malt samples.

The varieties 1) WI-2691; 2) Nordal ant; 3) Klages; 4) Triumph; 5) Skiff; 6) WI-2737; 7) WI-2736; 8) WI-2738; 9) Forrest and 10) Stirling were tested for endopeptidase activity against azocasein (■) and hordein (▨) and with the ELISA (▩). Activities measured against azocasein (Δ A 440 nm /h/g dry wt), hordein (Δ A 570 nm x10 /min /g dry wt) and using an ELISA (ng MEP-1) were divided by the activity against azocasein to emphasise the differences in activities measured between the varieties.

Table 5.1 The effect of PMSF on the hordein and azocasein assays.

Crude extracts preincubated with (4 mM) PMSF or a control solution for 1 h at 37°C and then tested for activity against hordein, azocasein and CBZ-phe-ala.

Substrate	% Inhibition
hordein	20
azocasein	0.7
CBZ-phe-ala	67

5.3.2 The effect of malting and kilning on endopeptidase activity and hordein degradation.

5.3.2.1 Changes in endopeptidase and carboxypeptidase activity during malting and kilning.

The profile of endopeptidase activity throughout malting and kilning was examined by testing the activity of samples of Schooner and Clipper at several stages during the malting and kilning cycles. Figure 5.2 shows the rise and fall of endopeptidase activity during micromalting. The activity curve is similar for both barley varieties; activity increases until day 5 when kilning starts, then declines as the kilning temperature increases. Carboxypeptidase activity also peaks on day 5 then decreases as the temperature rises (Figure 5.3). Endopeptidase and carboxypeptidase activities during micromalting were compared and it is seen that endopeptidase activity decreases more rapidly during kilning (Figure 5.3).

5.3.2.2 Hordein degradation during micromalting.

Barley proteins were extracted from Clipper samples taken daily from the micromalter and separated by SDS-PAGE. Figure 5.4 shows the barley protein extracts and degradation of hordein groups during the malting process. The D hordeins are rapidly degraded and have disappeared by day 4 (lane 5). The B and particularly the C hordeins appear to resist total degradation and are still present at the end of the malting process (lane 7).

5.3.3 The effect of hordein composition on malting quality.

The Pallas lines were used to determine the effect of hordein composition on the malting quality of barley. Although differing in hordein banding pattern these isogenic lines should have similar peptidase components. The type of hordein present and its susceptibility to degradation may influence malt extract. However, the null hypothesis is that there is no difference between endopeptidase activity or malt extract values between the Pallas lines.

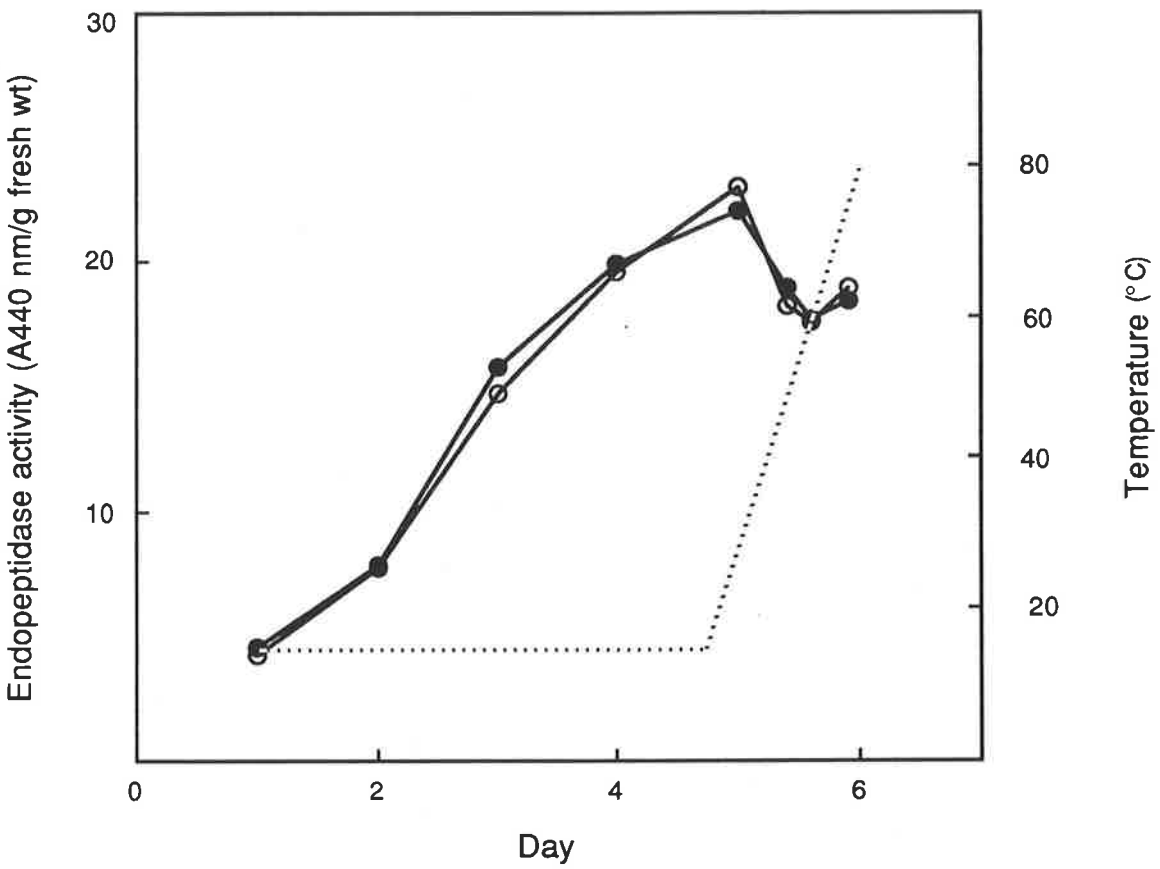

Figure 5.2 Endopeptidase activity during malting and kilning.

Samples of barley cvs. Schooner (—o—) and Clipper (—●—) were removed during micromalting and tested for endopeptidase activity using the azocasein assay. The temperature ramp during kilning is also indicated (.....).

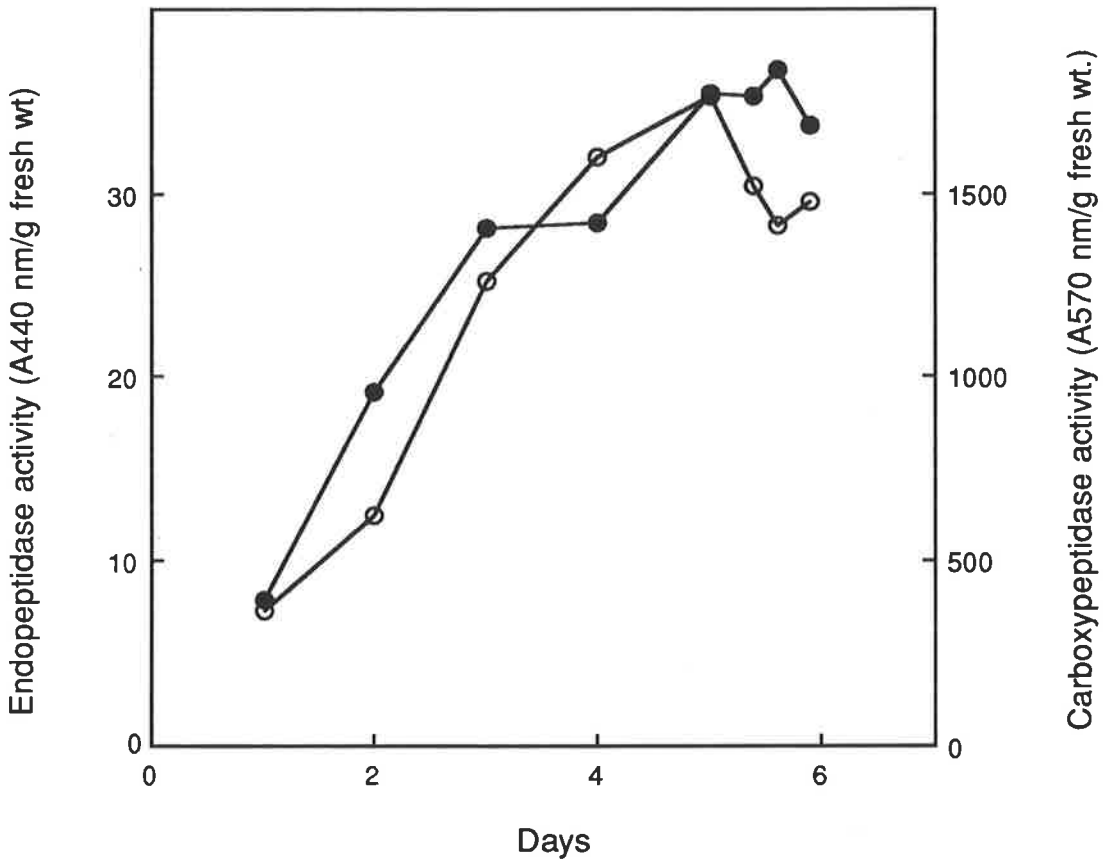

Figure 5.3 Endopeptidase and carboxypeptidase activity in barley cv. Clipper during malting and kilning.

Carboxypeptidase (—●—) and endopeptidase (—⊖—) activities were measured in samples of Clipper barley that were removed at time intervals during micromalting, kilning temperature ramp is shown in Figure 5.2.

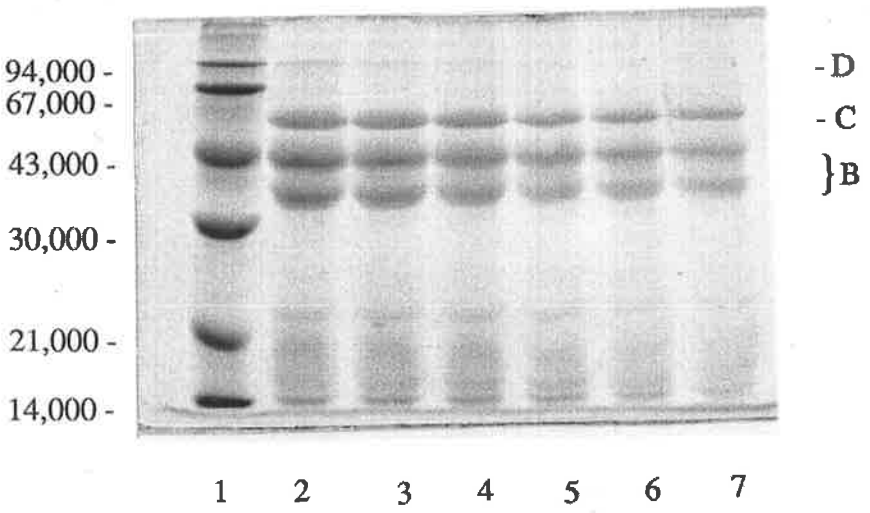

Figure 5.4 Hordein degradation during malting and kilning.

Barley proteins were extracted (described in Section 5.2.10) from Clipper samples taken from the micromalter during the malting process and separated by SDS-PAGE. The hordein groups are as indicated.

Lane 1) standards, 2) hordeins extracted on day 1, 3) day 2, 4) day 3, 5) day 4, 6) day 5, 7) day 6.

5.3.3.1 Hordein and 'gel protein' banding patterns in the Pallas isogenic lines.

The variation of hordein banding patterns in the Pallas isogenic lines is shown in Figure 5.5. The recurrent parent Pallas (lane 2) has 4 main C hordein bands and 3 main B hordein bands. Isoline P03 (lane 5) differs from Pallas in the C hordein region, it has 5 C hordein bands in a pattern distinct from Pallas. Isolines P04A (lane 6), P07 (lane 9) and P08A (lane 10) have identical C hordein banding patterns which differ from Pallas in that the lowest band is missing. P08A also has at least three extra B hordein bands with major bands at ~ Mr 35,000 and 43,000. Isoline P09 (lane 12) differs from Pallas only in the B region where an extra band is present at ~ Mr 38,000. The isolines P03 and P08A differ the most from the parent Pallas in their banding patterns. 'Gel protein' was also extracted and separated by SDS-PAGE (Figure 5.6). Isoline P08A (lane 10) shows a distinctly different banding pattern than the others. It appears that more 'gel protein' was extracted from isoline P03 (lane 5) than the others, however, this experiment was not repeated and therefore the result was not confirmed.

5.3.3.2 Endopeptidase activity, extract values and % grain protein levels of the Pallas isogenic lines.

Endopeptidase activity was measured in 4 day green malts of the parent Pallas and the isolines which contain donor hordein genes using the azocasein assay. Table 5.2 shows a summary of the means of the endopeptidase activities, malt extracts and % grain protein values measured in the Pallas lines. The activities differed significantly between the lines ($p = 0.001$) and the mean differences are shown in Table 5.3. The high level of activity in the parent Pallas differed from all of the isolines tested.

Hot water extract values and % grain protein levels were measured in the Pallas isolines in the Waite Barley Quality Evaluation Laboratory. The extract values varied significantly between the isolines ($p = 0.003$) but there was no significant variation between the % grain protein levels ($p =$

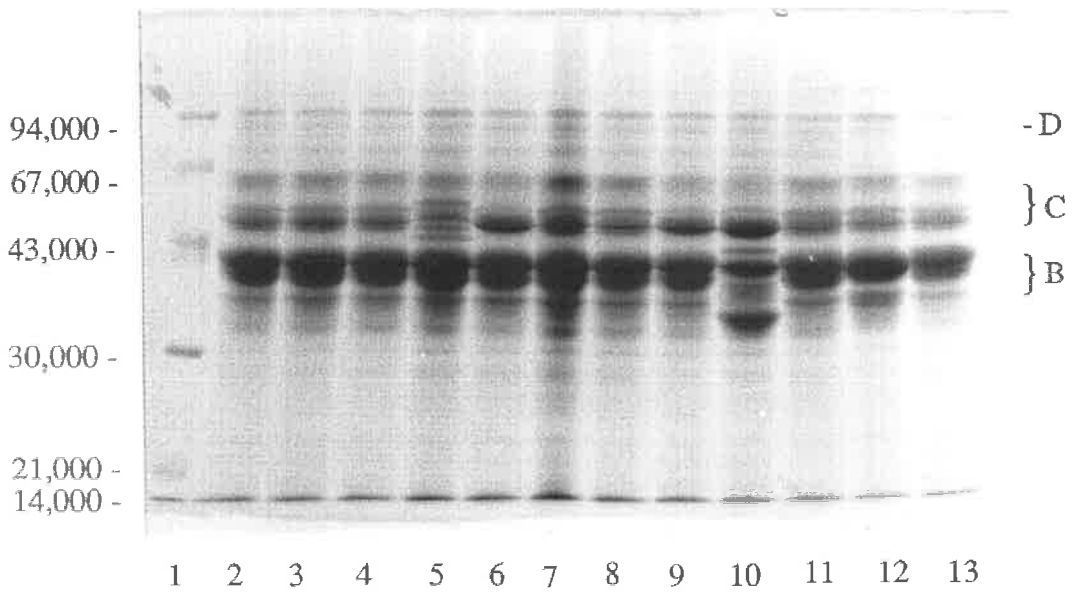

Figure 5.5 Hordein profiles of the Pallas isolines.

Barley flour samples (20 mg) were extracted with 1 ml of Na PO4 (50 mM); SDS (2% (w/v) pH 6.9 and 30 sec sonication. The mixture was centrifuged for 10 min at 12,000 *g* and the 50 µl of the supernatants were separated by SDS-PAGE. The hordein groups aare as indicated.
Lane 1) standards, 2) parent Pallas sample, 3) isoline P01, 4) P02, 5) P03, 6) P04A, 7) P04B, 8) P06, 9) P07, 10) P08A, 11) P08B, 12) P09, 13) P011.

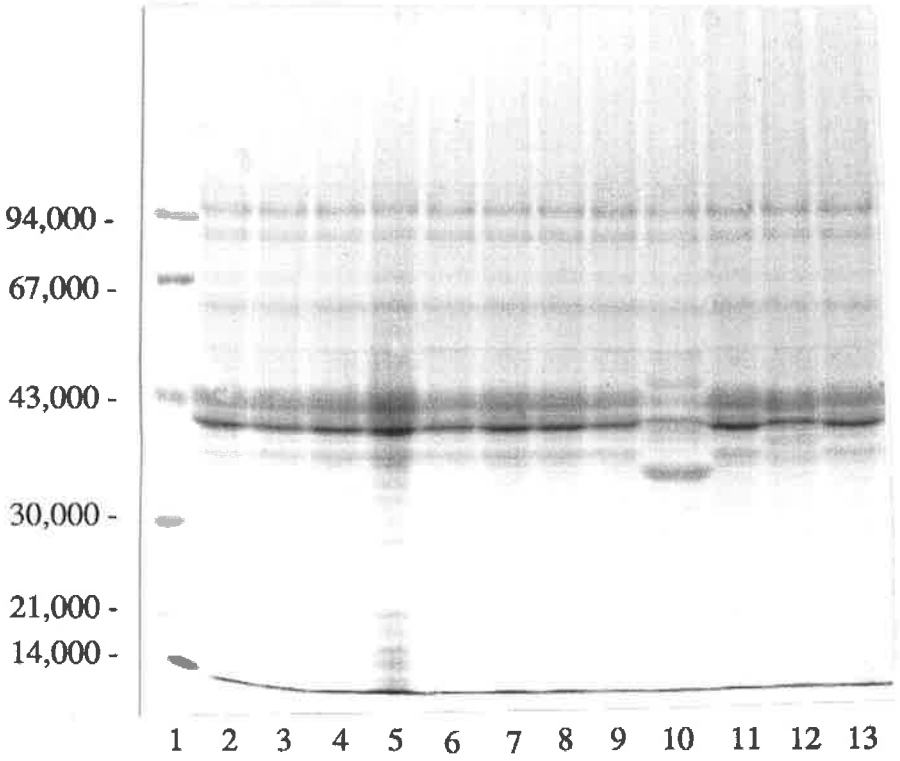

Figure 5.6 'Gel protein' extracted from the Pallas isolines.

'Gel protein' was extracted from the Pallas isolines and separated by SDS-PAGE.
Lane 1) standards, 2) Pallas, 3) P01, 4) P02, 5) P03, 6) P04A, 7) P04B, 8) P06, 9) P07, 10) P08A, 11) P08B, 12) P09, 13) P011.

Table 5.2 Table of means of endopeptidase activities, extract values and % grain protein values of the Pallas isolines.

Endopeptidase activity was measured against azocasein (described in Section 5.2.2). Malt extract and grain protein were measured in the Waite Barley Quality Evaluation Laboratory.

	endopeptidase activity Δ 440 nm /h /g fresh wt.	malt extract (%)	grain protein (%)
Pallas	16.03	74.41	15.59
P03	14.52	73.85	15.04
P04A	13.49	74.72	15.54
P07	13.32	74.57	15.50
P08A	14.35	73.78	15.31
P09	13.78	74.51	14.80
LSD	1.05	0.46	0.63

Table 5.3 Table of mean differences between the endopeptidase activities of the Pallas isolines.

Endopeptidase activities in Pallas malts were measured against azocasein (described in Section 5.2.2), units are Δ A 440 nm /h /g fresh wt. The differences between the means of each variety are shown below. Least significant difference = 1.05.

	Pallas	P03	P04A	P07	P08A	P09
means	16.03	14.52	13.49	13.32	14.35	13.78
Pallas	0.00	1.52*	2.54*	2.71*	1.68*	2.25*
P03		0.00	1.03	1.20*	0.17	0.74
P04A			0.00	0.17	-0.86	-0.29
P07				0.00	-1.03	-0.46
P08A					0.00	-0.57
P09						0.00

* significant at 95%

0.120). The analysis of mean differences (Table 5.4) shows that the extract values are significantly lower in isolines P03 and P08A.

5.3.3.3 Analysis of barley and malt hordeins in the Pallas isolines using size exclusion HPLC.

Extracts of barley and malt flour were subjected to size exclusion chromatography on an HPLC Protein Pak 300 SW column to quantify the differences in hordeins. The chromatographs of the parent Pallas barley and malt are shown in Figure 5.7. The graph consists of seven peaks which were integrated, although it is the first three peaks that are of interest. The protein content of these peaks was determined by comparison with similar work on wheat proteins (Singh *et al.*, 1990) indicating that the first peak should comprise hordein aggregates, the second peak mainly C and some B hordeins with albumins and globulins making up the third peak. In the barley samples the average areas of peaks 1, 2 and 3 [in HPLC units x 10^{-6} (described in Section 5.2.9)] were 8.446, 5.599 and 3.583 whereas in malt samples the average areas were 8.079, 4.796 and 4.265. The proteins in the first two peaks appear to have been degraded in the malt samples but the third peak increased either due to the accumulation of products from aggregates being degraded or due to the increase of metabolic proteins which are synthesised during germination.

Analysis of variance of the peak areas showed that only peak 2 differed significantly between isolines in both barley and malt data sets. Differences in the mean areas of peak 2 for barley and malt were compared (Table 5.5). The data from barley shows that isoline P08A differs significantly from P03 and P09 in that peak 2 was smaller in P03 and P09 but larger in P08A. In the malt sample the highest peak mean (isoline P07) was significantly different than the lowest peak mean (isoline P03).

The relationship between peak 2 areas and extract values was investigated using a correlation test. The two isolines P03 and P08A had significantly low extract values compared to the other

Table 5.4. Table of mean differences between the malt extract values of the Pallas isolines.

Malt extract was measured in the Waite Barley Quality Evaluation Laboratory. The differences between the means of each line are shown below. Least significant difference = 0.46.

	Pallas	P03	P04A	P07	P08A	P09
means	74.4	73.9	74.7	74.6	73.8	74.5
Pallas	0.00	0.57*	-0.31	-0.20	0.63*	-0.10
P03		0.00	-0.87*	-0.72*	0.07	-0.66*
P04A			0.00	0.15	0.94*	0.21
P07				0.00	0.79*	0.06
P08A					0.00	-0.73*
P09						0.00

* significant at 95%

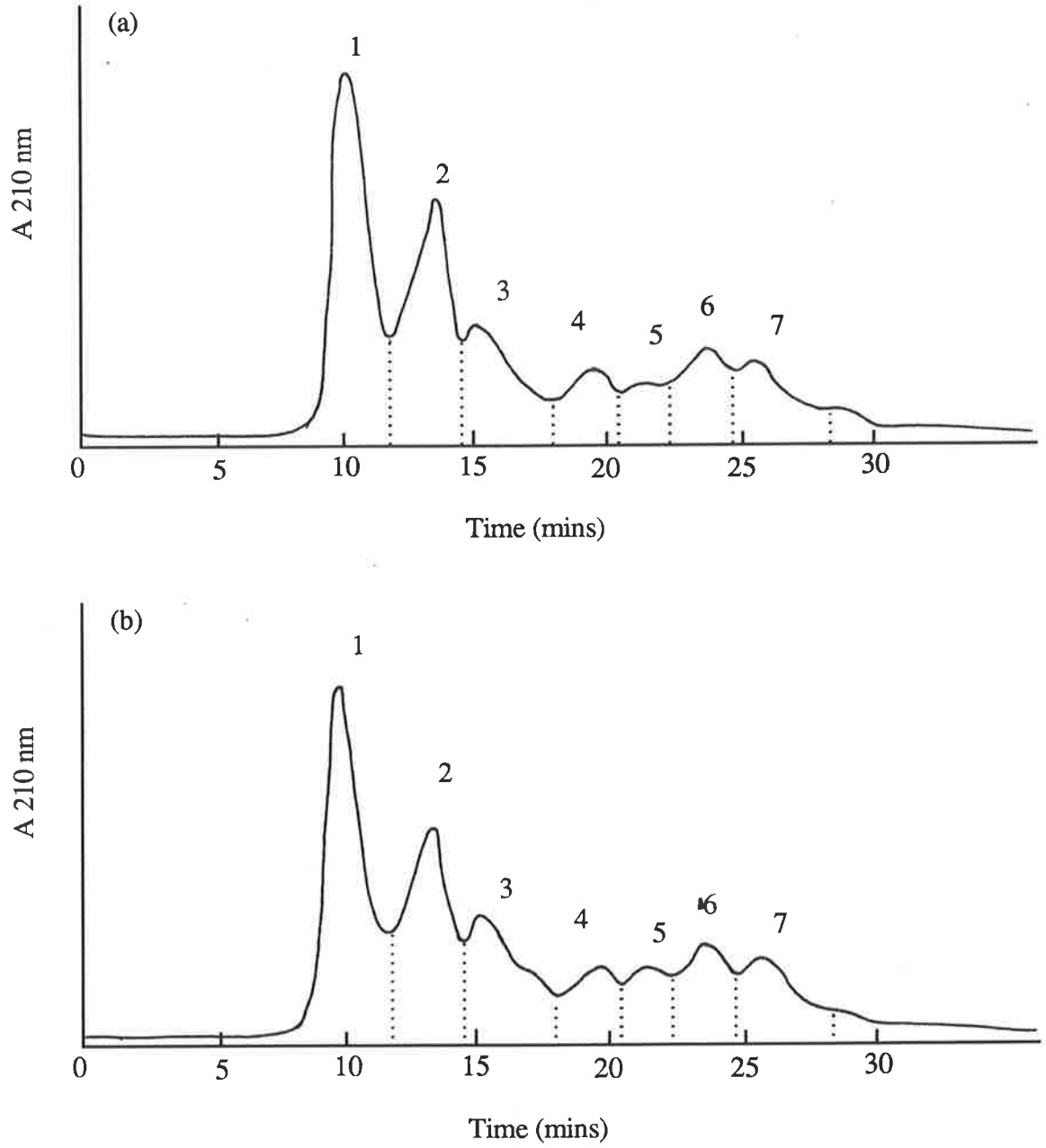

Figure 5.7 SE-HPLC of Pallas barley and malt.

Flour from barley (a) and malt (b) samples of the Pallas lines was extracted with $NaPO_4$ (50 mM); SDS (2% (w/v) pH 6.9. Proteins in the 12,000 *g* supernatants were separated by SE-HPLC with a Waters HPLC system.

Table 5.5 Table of mean differences of the size exclusion (SE) HPLC peak 2 areas for the Pallas isoline barley and malt data sets.

Flour from barley (a) and malt (b) samples of the Pallas isolines was extracted with $NaPO_4$ (50 mM); SDS (2% (w/v) pH 6.9. Proteins in the 12,000 *g* supernatants were separated by SE-HPLC. The means of the areas of the second protein peak (containing the C and some high Mr B hordeins) eluted from the column are shown in HPLC units x 10^{-6} described in Section 5.2.9.

(a) Data from barley set.

	Pallas	P03	P04A	P07	P08A	P09
means	5.47	4.71	5.70	5.95	6.53	5.23
Pallas	0.00	0.79	-0.23	-0.48	1.06	0.24
P03		0.00	-0.99	-1.24	-1.82*	-0.52
P04A			0.00	-0.25	-0.83	0.47
P07				0.00	-0.58	0.72
P08A					0.00	1.30*
P09						0.00

(b) Data from malt set.

	Pallas	P03	P04A	P07	P08A	P09
means	4.74	4.34	4.66	4.99	4.78	4.86
Pallas	0.00	0.40	0.08	-0.25	-0.04	-0.12
P03		0.00	-0.32	-0.64*	-0.44	-0.52
P04A			0.00	-0.33	-0.12	-0.20
P07				0.00	0.20	0.13
P08A					0.00	-0.75
P09						0.00

* significant at 95 %.

isolines yet their peak areas are low and high respectively. Hence there was no significant correlation between malt extract and peak area.

The conclusions of this study with the Pallas isolines are as follows. The genetic differences between the isolines affected extract value, endopeptidase activity and the area of the second SE-HPLC protein peak. None of the parameters measured were significantly correlated with malt extract. The two isolines (P03 and P08A) which differed most in their 'gel protein' content and hordein banding patterns (Figures 5.5 and 5.6) were the only isolines to have significantly low extract values. However, hordein banding pattern and 'gel protein' were not quantified in this study and therefore cannot be correlated with malt extract.

5.3.3.4 Analysis of Schooner barley proteins using size exclusion HPLC.

Barley flour from 15 different samples of Schooner was extracted in duplicate with SDS and sonication and analysed by SE HPLC. The Schooner samples chosen varied in % grain protein contents and % malt extract values. The proteins were separated into three main peaks of interest (Figure 5.8) that were integrated. The resolution of proteins by the Varian HPLC system was not as clear as the Pallas isoline study when the Waters HPLC was used.

Analysis of variance showed that the peak areas differed significantly between the batches of Schooner barleys. The peak areas were correlated with % malt extract and % grain protein (Table 5.6). Peak 2 and % grain protein are significantly and negatively correlated with malt extract whereas peaks 1 and 3 were not.

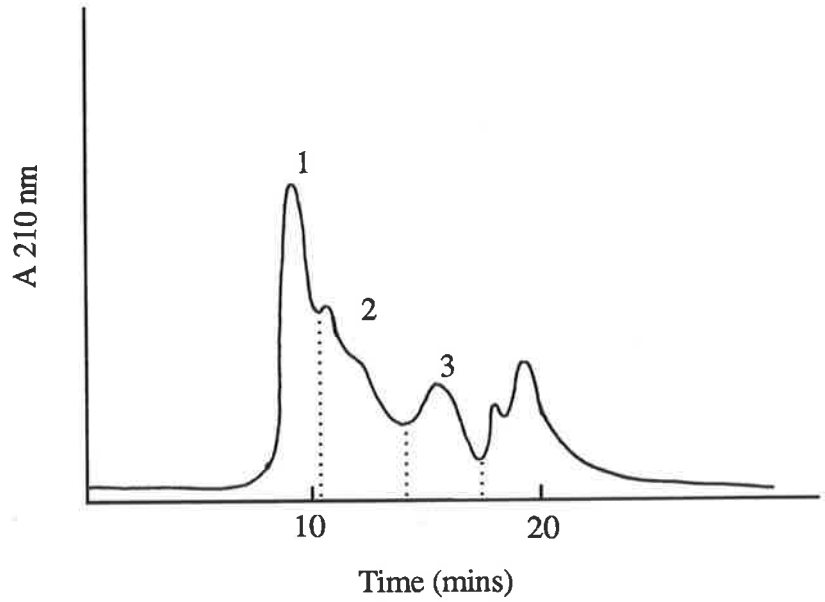

Figure 5.8 SE-HPLC of Schooner barley.

Flour from Schooner barleys was extracted with $NaPO_4$ (50 mM); SDS (2% (w/v) pH 6.9. Proteins in the 12,000 *g* supernatants were separated by SE-HPLC with a Varian HPLC system.

Table 5.6 Correlation table between % malt extract, % protein and SE-HPLC peak areas for Schooner samples.

Malt extract and grain protein were assessed in the Waite Barley Quality Evaluation Laboratory and correlated with the SE-HPLC peak 2 areas of the Schooner samples.

	% Extract	% Protein	Peak 1	Peak 2	Peak 3
% Extract	1.000	-0.856*	-0.377	-0.749*	-0.333
% Protein		1.000	0.319	0.741*	0.188
Peak 1			1.000	0.845*	0.891*
Peak 2				1.000	0.845*
Peak 3					1.000

* significant at 95%.

5.3.4 The effect of endopeptidase activity on malting quality.

5.3.4.1 Pilot study on endopeptidase activity in different barley varieties.

An initial study was undertaken measuring endopeptidase activity in 20 different varieties grown at the same site. Diastatic power (DP), α-amylase activity, endo-β-glucanse activity and % malt extract were measured in the Waite Barley Quality Evaluation Laboratory.

Analysis of variance (Table 5.7) showed that the variation in malt extract was significantly affected by endopeptidase activity ($p = 0.055$). Variation in other enzymes measured (Table 5.7) did not relate to variation in % malt extract nor did they relate to variation observed in endopeptidase activity.

5.3.4.2 Study of endopeptidase activity in different barley varieties.

The results of the pilot experiment (above) justified a larger scale experiment using 14 different cultivars of differing malting quality grown at three different sites. The malts were analysed in the Waite Barley Quality Evaluation Laboratory for α- and β-amylase activities, malt β-glucan, free amino nitrogen (FAN), % grain protein and % malt extract - routine measurements important in the assessment of malting quality barleys. The variation between samples and individual parameters was estimated using a two way analysis of variance model using site and cultivar as X variables against the individual parameters as Y variables. The analysis was undertaken using the program JMP version 2.0 (SAS Institute Inc U S A). Analysis of variance revealed that β-amylase activity, % malt extract, % protein, FAN and endopeptidase activity all varied significantly between cultivars.

Table 5.7 Pilot variety study: correlation between malt parameters.

Malt extract, α-amylase activity, diastatic power (DP) and endo-β-glucanase activity were assessed in 20 barley varieties by the Waite Barley Quality Evaluation Laboratory and correlated with endopeptidase activity measured against azocasein (as described in Section 5.2.2).

	% Extract	Endopeptidase	DP	α-Amylase	endo-β-glucanase
% Extract	1.000	0.439*	-0.039	0.191	-0.369
Endopeptidase		1.000	-0.294	-0.113	-0.004
DP			1.000	0.542*	0.967*
α-Amylase				1.000	0.316
glucanase					1.000

* significant at 95%.

The parameters were then compared to each other using a regression model to determine if the variation in % malt extract could be accounted for by the parameters measured. However, the effects of variety and site were so large that the effects of the parameters measured were non significant. The relationship between parameters was therefore estimated by examining the correlation between the parameters without the effects of site and variety (Table 5.8). By this analysis it is shown that % extract has a significant relationship with endopeptidase activity, FAN and % protein.

Table 5.8 Variety study: correlation between malt parameters.

The % malt extract, amylase activities, free amino nitrogen (FAN) and % grain protein were assessed in 14 different barley varieties grown at 3 different sites by the Waite Barley Quality Evaluation Laboratory and correlated with endopeptidase activity measured against azocasein (as described in Section 5.2.2). The data set means are shown in Appendix 5.1.

	% Extract	Endopeptidase	α-Amylase	β-Amylase	FAN	% Protein
% Extract	1.000	0.387*	0.115	0.138	0.283*	-0.270*
Endopeptidase		1.000	0.137	-0.043	0.409*	-0.317*
α-Amylase			1.000	-0.110	-0.046	0.046
β-Amylase				1.000	0.349*	-0.072
FAN					1.000	-0.201
% Protein						1.000

* significant at 95%.

5.4 DISCUSSION

5.4.1 Analysis of endopeptidase assays.

The discrepancies between the assays tested on the same varieties shown in Figure 5.1 are not surprising. The ELISA quantifies amount of MEP-1 present, with possible interference from other immunologically related proteins, whereas the hordein and azocasein assays measure the range of endopeptidase activities present in the malt sample. The assays employing hordein and azocasein estimate different levels of endopeptidase activity in the different cultivars. The endopeptidases present in these cultivars may have different specificities for the different substrates. Furthermore the hordein assay was shown to have interference from carboxypeptidase activity (Table 5.1). Additionally, amino acids must be removed from malt samples used as a source of endopeptidase for the hordein assay as they interfere with the ninhydrin colour development. Baxter (1976) expounded the use of hordein for measuring endopeptidase activity as it is the natural substrate for barley endopeptidases. Endopeptidase activities estimated using hordein as a substrate varied significantly from those obtained using non-barley substrates such as gelatin or haemoglobin as was found in the present study. The hordein endopeptidase assay used by Baxter (1976) involved a correction for exopeptidase activity and may therefore be more accurate than the hordein assay used for the present study.

The ELISA is easy to use for large samples and gives useful information in tandem with a more general endopeptidase assay. However, the ELISA used in the present study was not reproducible between dilutions and the rate of colour development varied between plates. These problems as well as the cross reaction of antibodies with other proteins in barley crude extracts (demonstrated in Western blots in previous Chapters) indicated that this ELISA may give misleading results. The azocasein assay was chosen for the larger studies as it was convenient, reproducible and was not confounded by carboxypeptidase activity.

5.4.2 The effect of malting and kilning on endopeptidase activity and hordein degradation.

The carboxypeptidase and endopeptidase activity profiles throughout malting and kilning (Figure 5.3) show the relative temperature sensitivities of the enzymes. The carboxypeptidase component is more thermo-stable to increasing temperatures and retained more activity after kilning. Endopeptidase action limits the rate of protein breakdown during malting (Burger and Schroeder, 1976; Sopanen *et al.*, 1980) and therefore becomes more limiting during kilning and mashing as its activity is reduced more than the carboxypeptidase enzymes. Baxter (1978) purified three carboxypeptidase fractions and suggested that they were likely to be responsible for most of the amino acids released during mashing due to their thermostable nature. Jones and Poulle (1989) demonstrated that a 30,000 Mr endopeptidase is present during mashing but its activity is destroyed at temperatures above ~70°C.

The degradation of D, B and C hordeins extracted with 50 % isopropanol is shown in Figure 5.4. Even after the peak of endopeptidase activity (day 5) a high percentage of extractable hordein remains. However, the D hordeins have disappeared by the end of malting. Several reports have previously demonstrated that D hordeins are degraded first during malting (Skerritt, 1988; Smith and Simpson, 1983; Moonen *et al.*, 1987). However, other workers have found that a D hordein fraction extracted with 8 M urea and 1% (w/v) DTT persists throughout malting (Marchylo *et al.*, 1986). They proposed that this fraction is tightly bound to the endosperm and is less susceptible to proteolytic degradation.

5.4.3 The effect of hordein composition on malting quality.

The analysis of hordein and its relationship to malting quality has been the subject of many studies. The advantage of finding properties in unmalted barley which would predict the quality of the malt would be of tremendous benefit to maltsters trying to predict the quality performance of barley and to breeders attempting to improve the quality of barley varieties. Barley proteins

associated with malt quality could easily be used as a criteria for selection. At present the best test for predicting malt extract in unmalted grain is to measure the % grain protein which is generally negatively correlated with malt extract. The technique of SDS extraction and separation by SE-HPLC has been used successfully for predicting the dough quality in wheat (Singh *et al.*, 1990). However, the present study has shown that the same technique was not as effective for predicting the malt extract values of barley as % grain protein *per se*.

The SE-HPLC studies of hordein from the Pallas lines and Schooner samples presented in this Chapter are in contrast. The Pallas lines differ in hordein banding pattern yet in the Schooner samples the banding patterns are identical, but the amount of each hordein group is not. The study using Schooner barleys showed significant, negative correlation between the second SE-HPLC peak and malt extract. However, the individual protein peaks were not more strongly correlated with extract than the % grain protein. The SE-HPLC technique clearly detected the different amounts of the hordein groups in the Schooner samples and the variation of peak areas between samples were highly significant. In the Pallas study however the variation between hordein banding patterns was resolved more clearly using SDS-PAGE than the SE-HPLC technique. SE-HPLC is useful because it quantifies the amount of hordeins extracted but it does not appear to be very sensitive to small differences in banding pattern. Separation of barley proteins by reversed-phase HPLC (RP-HPLC) has been used to identify different barley varieties and is even more sensitive to differences in hordein composition than SDS-PAGE (Marchylo and Kruger, 1985). This could be a more useful technique for quantifying the hordein subgroups in the (near) isogenic Pallas lines.

It was assumed that as a result of backcrossing that the hordein alleles would be different from Pallas but the complement of endopeptidases would be the same. However, significant differences in endopeptidase activity were detected between the Pallas isolines. The *Hor1* and *Hor2* genes are located on the short arm of chromosome 5 whereas the endopeptidase gene is situated on the long arm of chromosome 3 (Guerin *et al.*, 1993). The main difference in

endopeptidase activity between the Pallas isolines is due to the high activity in the parent Pallas, which could suggest that the introduction of foreign genes has deleteriously affected the endopeptidase activity of the isogenic lines. Some genetic factor responsible for regulating endopeptidase expression or activity may be linked to *Hor1* or *Hor2* resulting in a reduction of endopeptidase activity in the backcrossed lines.

The extract values also varied significantly between the Pallas isolines and it is conceivable that hordein composition could affect extract values. He *et al.* (1993) identified specific banding patterns of both C and B hordeins that were associated with malting quality. The isolines P03 and P08A which gave significantly low extract values also had the most unique hordein banding patterns. How hordein composition affects malt extract is not known. The composition of the malt extracts need to be analysed to determine which components were altered. Other malting quality parameters which are more directly related to protein degradation such as soluble nitrogen content and Kolbach index and free amino nitrogen (FAN) also need to be assessed. The low extract values from P03 and P08A cannot be attributed to variation in any one hordein group as P03 has a different C hordein component from the parent and P08A differs in both B and C components. However, the C hordein banding pattern in isoline P08A is identical to isolines P07 and P04A. The way in which the protein groups interact during malting and mashing could have a strong level of influence on malt extract. Skerritt and Janes (1992) showed that in a set of seven barley cultivars, grown at two sites, neither total protein or SDS-extractable protein was significantly correlated with extract yet the 'gel protein' fraction was.

5.4.4 The effect of endopeptidase activity on malting quality.

The correlation between endopeptidase activities and other malt parameters (Tables 5.7 and 5.8) confirms earlier work proposing that endopeptidase activity tends to be higher in better malting varieties (Baxter, 1976; Morgan *et al.*, 1983). However, the present study used more varieties grown at three sites giving more weight to the statistical evidence. In the pilot study the 20

varieties tested were grown at the same site and the variation in malt extract values was significantly affected by endopeptidase activity (at the 6% level). The main study, testing 14 varieties grown at three different sites, revealed that environment followed by variety accounted for most of the variation in any of the parameters measured. It is interesting that endopeptidase had a significant correlation with malt extract but the activities of the amylases and endo-β-glucanase did not. This observation was also reported by Morgan *et al.* (1983). The relationship between endopeptidase activity and malt extract suggests that the dissolution of the protein matrix is an important step which influences the degradation of starch and other macromolecules in the endosperm.

Barley enzymes such as β-amylase, α-amylase and endo-β-glucanase are routinely tested in breeding programs aimed at improving malting quality. These enzyme activities are known to be important but are not necessarily related to any one parameter such as % malt extract. The main difficulty with assessing the effects of endopeptidase activity on aspects of quality is that the enzymes may affect the quality of the malt and beer in many ways. Endopeptidase activity effects all aspects of protein catabolism during germination as well as other processes such as starch degradation and enzyme activation. Problems in the finished product of beer such as chill haze or unstable foam may also be attributed to endopeptidase action in malt and wort. Although the present study has mainly compared malt extract as a measure of quality other aspects are also important. Excessive protein breakdown causes problems later in the brewing process and may result in lack of foam stability. Testing protein levels during the production of beer in relation to endopeptidase activity during malting would also help to establish the ideal level of malt endopeptidase activity. It is important to establish the level at which endopeptidase activity reduces foam stability and how it relates to chill haze in conjunction with the effects of protein breakdown during malting.

The requirements for quality in terms of protein content and catabolism are not fully understood. They are also often in contradiction with each other, for instance a high level of protein

modification during malting results in trouble free pocessing in the brewery but may impair foam stability, beer taste and colour (Shildbach and Burbidge, 1992). Furthermore the quality parameters tested on a malt will not necessarily predict the behaviour of that malt during brewing and processing (Shildbach and Burbidge, 1992). MacGregor (1992) stated that while it is important that adequate protein modification takes place during malting, the desired specifics of protein modification are not known. He ascertained that Kolbach Index and free amino nitrogen are the best measures of protein modification at the present time. On going research into the behaviour of malt endopeptidases and barley proteins during malting and brewing will further our understanding of quality requirements and lead to the development of more accurate and predictive quality tests.

CHAPTER 6

GENERAL DISCUSSION

Several aspects of endopeptidases and their action during germination have been investigated in this project. MEP-1 was characterised genetically by N-terminal amino acid sequencing and chromosome mapping, the action of endopeptidases on β-amylase was studied and ways of measuring enzyme activity in malt and the effects of activity and protein composition on malting quality were examined. The research presented in this thesis has contributed to the knowledge of endopeptidases and their function during germination in several ways.

Establishing the role of a purified malt endopeptidase (MEP-1) in the release of β-amylase demonstrates an important regulatory function in the activating a hydrolytic enzyme whereas it was previously thought that the only role of endopeptidases was to catabolise storage proteins. Recently limit dextrinase has also been reported to be activated by endogenous barley cysteine endopeptidases (Longstaff and Bryce, 1993). Endopeptidases may be responsible for the post translational modification of any number of proteins present in the grain and throughout germination. They must also be important in the degradation of other enzymes and may affect their rate of turnover.

Amino acid sequencing and gene mapping of the endopeptidase MEP-1 described in Chapter 3 will help to reconcile any differences between the cysteine-endopeptidases that have been isolated from germinating barley as well as contributing to the construction of the barley gene map. The discovery of an allele at the *CepB* locus encoding the MEP-1 enzyme may be a valuable tool for learning about enzyme structure-function relationships by comparing the form of MEP-1 with that of the variant. The discovery of additional alleles may also be a highly productive pursuit in the search for ways to improve the malting quality of barley varieties.

A major research goal in the investigation of barley endopeptidases is to purify, characterise and sequence the other endopeptidases which are present in the germinating barley grain. Two of the cysteine endopeptidases have been purified out of the four or five which clonal studies indicate are present in barley (Koehler and Ho, 1990b). The other enzymes have not yet been purified due to technical difficulties, however some progress was made in this study (Section 4.3.3). The lability of the enzymes appears to be one of the main difficulties encountered in isolating these enzymes. Immuno-affinity techniques using the polyclonal antibodies which appear to cross react with other (putative) endopeptidases (Figure 4.5) may be a successful strategy for isolating other cysteine endopeptidases. Similarly cross hybridisation of endopeptidase clones could be utilised to sequester other endopeptidase clones which, once sequenced and appropriate primers designed, could be amplified utilising the polymerase chain reaction (PCR). Analysis of the structure, function and substrate specificities of these enzymes is required for developing a clear picture of the cysteine-endopeptidases present in malt and their roles during germination. Other groups of endopeptidases have also been reported in barley, such as aspartic acid endopeptidases (Sarkkinen *et al.*, 1992), metallo-endopeptidases (Sundblom and Mikola, 1972) and others which could not be characterised by inhibition (Wrobel and Jones, 1992). The whole assembly of endopeptidases need to be examined in order to reach a full understanding of the individual enzymes and how they complement each other in their actions.

Another important objective is to establish the level of endopeptidase activity in malt that is optimal for the requirements of the brewer. In assessing optimum malt endopeptidase activity it is essential to test the effects of endopeptidase activity throughout malting to the final stages of brewing. Problems which arise later in the processing of beer such as lack foam retention and chill haze formation are likely to be affected by the level of endopeptidase activity in the malt. Protein Z is one of the major proteins isolated from beer which is responsible for foam retention (Yokoi and Tsugita, 1988). β-Amylase and protein Z are aggregated as heterodimers in the barley endosperm (Hejgaard, 1976). Both proteins exist in salt soluble forms or are extracted with thiol and/or proteolysis (Hejgaard, 1976;

Hejgaard and Carlsen, 1977). Therefore protein Z is likely to be solubilised by malt endopeptidases along with β-amylase.

One of the main problems with assessing endopeptidase activity in malt is finding a suitable assay. Baxter (1976) showed that endopeptidase activity measured against hordein was significantly different from that measured against non-barley substrates. However, the hordein assay used in this project was tedious for screening large numbers of varieties, also at least 20 % of the estimated activity was due to carboxypeptidases (Section 5.3.1.2). It is very difficult to mimic the conditions in the germinating seed in an assay system. The hordein fraction used as a substrate is subjected to relatively harsh conditions during extraction, precipitation and lyophilisation and it is debatable whether it remains in the same form as exists in the seed. The activity of MEP-1 against hordein is affected by the extraction conditions of the substrate (Wallace and Batt unpublished data). The state of reduction of the hordeins appears to be a major factor influencing endopeptidase activity (Phillips and Wallace, 1989). NADP-thioredoxin reductase and thioredoxin *h* have been shown to reduce wheat storage proteins (Kobrehel *et al.*, 1992) and it has been suggested that reduced thioredoxin may function as a signal in germination to increase the mobilisation of storage proteins as well as activating several enzymes.

The ELISA is a better method for measuring endopeptidase activities as the problems of choosing a suitable substrate are avoided. The use of monospecific antibodies raised against the different endopeptidases present in germinating barley would be of tremendous benefit in examining the roles of these enzymes. The activities of the individual enzymes could be measured simultaneously as germination progresses. *In situ* hybridisation similar to the techniques used by Marttila *et al.* (1993) utilising monospecific antibodies would show the location of the enzymes and their patterns of secretion into the endosperm. The chromosomal location of the other endopeptidase genes could be determined using monospecific antibodies and the wheat-barley addition lines as used in this project (Section 3.3.3), if a difference between wheat and barley could be demonstrated. The detection of enzyme variants and establishment of linkages with other isozymes and molecular genetic

markers could be employed to map the endopeptidase loci, thereby providing genetic evidence for the number of different endopeptidase genes.

Recombinant DNA technology is rapidly developing and transformation of many higher plants, including cereals, is now practised. Rice (Shiamamoto *et al.*, 1989; Christou *et al.*, 1991), maize (Gordon-Kamm *et al.*, 1990; D'Halluin *et al.*, 1992) wheat (Vasil *et al.*, 1992) and oats (Somers, *et al.*, 1992) have all been successfully transformed within the last few years. Plant regeneration (Jähne *et al.*, 1991) and transient expression of foreign genes in barley endosperm protoplasts have been reported (Lee *et al.*, 1991). Stable transformation of barley is highly likely to be achieved in the near future. This type of technology will prove useful in clarifying the roles of the endopeptidases during germination as endopeptidases and their activities could be manipulated within common genetic backgrounds. Transgenic barley carrying modified endopeptidases could then be directly compared with unaltered barley. The functions of the endopeptidases as well as the effects of activity levels, thermo-stability and regulation of expression on malting quality might be further elucidated by generating and testing this type of material.

Appendix 3.1 *H. spontaneum* lines tested for endopeptidase genotype on IEF gels.

The lane number refers to lanes on the IEF gels in Figures 3.4, 3.5 and 3.6.

Lane	CPI number	Location
		Israel
1	77138	Mt. Meron
2	77136	Maalot
3	77132	Caesarea
4	77134	Mt. Hermon
5	77147	Yeroham
6	77146	Tel Shoqet
7	77143	Tabigha
8	77141	Sede Boker
9	77135	Wadi Qilt
10	77137	Mehola
11	77140	Zefat
12	77285	Bor Mashash
		Iran
13*	109844*	Pole Zohab
14*	109827*	Shahabad Ilam 1
15	109829	Ilam Mehram 2
16	109830	Ilam Mehram 1
17*	109828*	Ilam Mehram 3
18	109832	Shahabad Dejful 2
		Turkey
19*	109853*	43 km N Diyarbakir
20	109851	60 km S Bitlis
21*	109861*	9 km E Siverek
22	109858	10 km N Mordin
23	109860	20 km E Siverek
24	109846	9 km E Gazientep

* = lines containing the variant endopeptidase band.

Appendix 5.2 Table of means of malting quality parameters measured.

Cultivar	Endopeptidase	% Extract	D P	Alpha	Beta	FAN	% Protein
Clipper	43.8	78.3	404	157	245	127	11.3
Forrest	32.6	78.6	254	123	133	-	12.0
Galleon	31.1	77.3	289	103	184	-	11.4
O'Connor	40.1	79.0	344	153	191	-	11.9
Schooner	48.3	79.4	483	133	351	158	11.1
Skiff	35.2	77.6	448	134	315	129	12.2
Stirling	40.4	77.7	454	123	276	107	11.5
WI-2692	39.8	78.5	481	152	329	121	11.9
WI-2727	43.3	76.8	409	132	277	126	11.6
WI-2736	38.6	77.4	416	127	290	143	11.5
WI-2737	44.2	77.1	436	133	304	142	11.7
WI-2785	31.5	76.7	378	113	266	118	11.6
WI-2808	45.1	77.5	396	137	172	114	10.7
Weeah	43.7	77.6	443	105	329	137	11.8

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