# Novel Alleles From Wild Barley for Breeding Malting Barley (*Hordeum vulgare* L.)

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy at the University of Adelaide

By

Jason K. Eglinton, B.Sc. (Hons), Flinders University

School of Agriculture and Wine
Waite Campus
University of Adelaide
South Australia

November, 2003

# STATEMENT OF ORGINALITY

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Jason K. Eglinton
November, 2003

# ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the supervision and guidance during this work from my supervisors, Dr Reg Lance, Dr Lesley MacLeod, Dr Tony Brown, Professor Peter Langridge, and Dr Evan Evans. The determination of Peter and Evan has contributed significantly to the completion of this thesis. In particular I thank Evan for his support, technical advice, and also for encouraging me to pursue a very wide range of research within my PhD studies. I would also like to thank Dr Andrew Barr and Dr Steve Jefferies for their support and enthusiasm, and the staff of the South Australian Barley Improvement Program for their assistance.

Special thanks to Dr Neil Shirley and Mr Jelle Lahnstein who provided me with significant technical assistance and also an education in protein chemistry. A number of colleagues have also made contributions to this work through technical advice, assistance and collaboration. In particular I would like to acknowledge Mike Dalton, Richard Stewart, Yu Fang Ma, Doug Stewart, Stewart Coventry, Patricia Warner, Rebecca Fox, Nigel Steinborner, Anne Marshall, and Max Paris. I would also like to thank the staff and students of the Waite Campus of the University of Adelaide for providing such a highly skilled and collegial environment. I also appreciate and acknowledge the long term support of the GRDC.

I would like to thank my family and friends for their encouragement. In particular I would like to thank my parents, Carol and Arthur Eglinton, for their enthusiasm and tireless support. Finally, but most importantly, my deepest thanks to my wife Penny and children Connor, Mitchell, and Jonah, for their exceptional support, patience, and understanding during frequent father absenteeism.

# ABSTRACT

*Hordeum vulgare* ssp. *spontaneum* is recognised as the progenitor of cultivated barley, and crosses readily with *Hordeum vulgare* resulting in fully fertile progeny. Wild barley exhibits significantly greater genetic diversity than current barley varieties due to the bottlenecks of domestication and modern plant breeding. Wild barley is therefore a source of novel genetic variation that could be exploited to develop superior barley varieties. The current study aims to identify and characterise novel alleles for key malting quality genes from wild barley. A review of the published literature is used to identify the key target genes, and an extensive collection of barley varieties and wild barley is screened for variation in ß-glucanase, protein Z, and ß-amylase. In addition to identifying genetic variation for protein Z, the genetics of protein Z is investigated, facilitating the molecular mapping of the protein $Z_7$ locus. The basic biochemical properties of the variant forms of the protein are characterised.

Three ß-amylase alleles were identified in cultivated barley, and a further three novel alleles were identified in wild barley. The allelic enzymes are shown to have differences in thermostability and expression levels. The differences in thermostability are shown to have a significant impact on wort fermentability, a key malting quality parameter. The amino acid sequence differences between the three ß-amylase enzymes from cultivated barley and a highly thermostable form from wild barley are identified. The amino acid substitutions are localised within the three-dimensional structure of ß-amylase and the structural basis of improved thermostability is characterised. A range of marker systems are developed and validated for the selection of specific ß-amylase alleles, and marker assisted selection is applied to introgress the novel thermostable ß-amylase from wild barley into elite barley breeding germplasm. The success of this approach is used to discuss broader opportunities to utilise wild relatives for plant breeding and crop improvement.

# PUBLICATIONS AND CONFERENCE PROCEEDINGS

**Eglinton J.K.** and Evans, D.E (1997) Identification of the *beta*-amylase isoelectric focusing band patterns in barley (*Hordeum vulgare* L.). Barley Genetics Newsletter. 27:8-12.

**Eglinton, J.K.** and Evans, D.E. (1998). ELISA for barley ß-amylase. Proceedings of the Cereal Chemistry Division Cereal Carbohydrates Workshop, Cairns, Australia. P32-35.

**Eglinton, J.K.**, Evans, D.E., Brown, A.H.D, Lance, R.C.M. (1995). Techniques for the examination of isozyme variation in malting quality enzymes of barley. Proceedings of the 45th Australian Cereal Chemistry Conference, Adelaide, Australia. P530-533.

**Eglinton, J.K.**, Evans, D.E., Brown, A.H.D., Lance, R.C.M., MacLeod, L.C. and Langridge, P. (1996) Preliminary characterisation of two forms of barley ß-amylase. Proceedings of the V International Oats Conference and VII International Barley Genetics Symposium, Saskatoon, Canada. P8-10.

**Eglinton, J.K.**, Evans, D.E., Brown, A.H.D., Langridge, P., McDonald, G., Jefferies, S.P. and Barr, A.R. (1999). The application of wild barley (*Hordeum vulgare* ssp. *spontaneum*) in breeding for quality and adaptation. Proceedings of the 9th Australian Barley Technical Symposium, Melbourne, Australia. P2.29.1-2.29.6.

**Eglinton, J.K.**, Langridge, P. and Evans, D.E. (1998). Thermostability variation in barley ß-amylase. Proceedings of the Cereal Chemistry Division Cereal Carbohydrates Workshop, Cairns, Australia. P15-17.

**Eglinton, J.K.**, Langridge, P. and Evans, D.E. (1998) Thermostability variation in alleles of barley *beta*-amylase. J.Cer.Sci. 28: 301-309.

Evans, D.E., Lance, R.C.M., **Eglinton, J.K.**, Logue, S.J., Barr, A.R. (1995). The influence of ß-amylase isoform pattern on ß-amylase activity in barley and malt. Proceedings of the 45th Australian Cereal Chemistry Conference, Adelaide, Australia. P357-364.

Evans, D.E., MacLeod, L.C., **Eglinton, J.K.**, Gibson, C.E, Zhang, X., Wallace, W., Skerritt, J.H. and Lance R.C.M. (1997). Measurement of *Beta*-amylase in malting barley (*Hordeum vulgare* L.). Part 1: Development of a quantitative ELISA for *Beta*-amylase. J.Cer.Sci. 26(2): 229-239.

Fox, R.L., **Eglinton, J.K.**, and Logue, S.J. (2001). Fermentable sugar profile as an alternative to Apparent Attenuation Limit as a selection tool in a Barley Improvement Program. Proceedings of the 10th Australian Barley Technical Symposium, Canberra, Australia.

Logue, S.J., **Eglinton, J.K.**, Margach, C., Roumeliotis, S., Tansing, P., Evans, D.E. and Barr, A.R. (1999). Application of ß-amylase thermostability analysis in a barley breeding program. Proceedings of the 9th Australian Barley Technical Symposium, Melbourne, Australia. P3.4.1-3.4.5.

Ma, Y., Stewart, D.C, **Eglinton, J.K.**, Logue, S.J., Langridge, P. and Evans, D.E. (1998). A kinetic study of two forms of ß-amylase from barley grain and malt. Proceedings of the 48th Australian Cereal Chemistry Conference, Cairns, Australia. P291-295.

Ma, Y., Stewart, D.C, **Eglinton, J.K.**, Logue, S.J., Langridge, P. and Evans, D.E. (2000). Comparative enzyme kinetics of two allelic forms of barley (*Hordeum vulgare* L.) *beta*-amylase. J.Cer.Sci. 31(3): 335-344.

Ma, Y., **Eglinton, J.K.**, Evans, D.E., Logue, S.J. and Langridge, P. (2000). Removal of the four C-terminal glycine-rich repeats enhances the thermostability and substrate binding affinity of barley ß-amylase. Biochemistry 39 (44): 13350-13355.

Paris, M., Jones, M.G.K., and **Eglinton, J.K.** (2002). Genotyping single nucleotide polymorphisms for selection of barley beta-amylase alleles. P.Mol.Biol.Rep. 20(2): 149-159.

# TABLE OF CONTENTS

# ABBREVIATIONS AND ACRONYMS

| | | |
|---|---|---|
| Å | : | Angstrom |
| AAL | : | Apparent Attenuation Limit |
| ABA | : | Abscisic Acid |
| AFLP | : | Amplified Fragment Length Polymorphism |
| amu | : | Atomic Mass Units |
| ASBC | : | American Society of Brewing Chemists |
| BASI | : | Bifunctional α-Amylase/Subtilisin Inhibitor |
| $BC_x$ | : | Backcross |
| bp | : | Base Pair |
| BSA | : | Bovine Serum Albumen |
| BSZ4 | : | Barley Serpin Z4 |
| BSZ7 | : | Barley Serpin Z7 |
| CAPS | : | Cleaved Amplified Polymorphic Site |
| CI | : | Chymotryptic Inhibitor |
| cM | : | Centimorgan |
| cm | : | Centimetre |
| CPI | : | Commonwealth Plant Introduction |
| cv. | : | Cultivar |
| Da | : | Dalton |
| DH | : | Doubled Haploid |
| DP | : | Diastatic Power |
| DPTU | : | Diphenylthiourea |
| DTT | : | Dithiothreitol |
| EDTA | : | Ethylene Diamene Tetra-acetic Acid |
| ELISA | : | Enzyme Linked Immunosorbant Assay |
| ESI-MS | : | Electrospray Ionisation Mass Spectrometry |
| EST | : | Expressed Sequence Tagged |
| g | : | Gram |
| GA | : | Gibberelic Acid |
| $H_e$ | : | Nei's Genic Diversity |

| | | |
|---|---|---|
| HPAEC | : | High Performance Anion Exchange Chromatography |
| HPLC | : | High Performance Liquid Chromatography |
| HRP | : | Horse Radish Peroxidase |
| HWE | : | Hot Water Extract |
| IEF | : | Isoelectric Focusing |
| IOB | : | Institute of Brewing |
| KDa | : | Kilodalton |
| KJ | : | Kilojoule |
| LD | : | Limit Dextrinase |
| LOD | : | Log-likelihood |
| LTP | : | Lipid Transfer Protein |
| LysC | : | Endo LysC Proteinase |
| M | : | Molar |
| mA | : | Milliamp |
| MALDI | : | Matrix Assisted Laser Desorption Ionisation |
| mg | : | Milligram |
| min | : | Minute |
| mL | : | Millilitre |
| mM | : | Millimolar |
| mm | : | Millimetre |
| $M_r$ | : | Molecular Mass |
| MTG | : | Monothioglycerol |
| N | : | Nitrogen |
| $N_a$ | : | Observed Number of Phenotypes |
| $N_e$ | : | Effective Number of Alleles |
| NIR | : | Near Infrared Reflectance |
| nm | : | Nanometre |
| nmol | : | Nanomole |
| PCR | : | Polymerase Chain Reaction |
| pI | : | Isoelectric Point |
| pmol | : | Picomole |
| PTH | : | Phenylthiohydantoin |
| RFLP | : | Restriction Fragment Length Polymorphism |

| | | |
|---|---|---|
| RMS | : | Root Mean Square |
| RP-HPLC | : | Reversed Phase High Performance Liquid Chromatography |
| SDS-PAGE | : | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SNP | : | Single Nucleotide Polymorphism |
| SnuPE | : | Single Nucleotide Primer Extension |
| $TC_x$ | : | Topcross |
| TFA | : | Trifluoroacetic Acid |
| µL | : | Microlitre |
| V | : | Volt |
| W | : | Watt |

# Chapter 1: Literature Review

## 1.1 Introduction

This investigation aims to assess the genetic variation in wild and cultivated barley for proteins important in malting and brewing, and to characterise these alternative alleles to determine if they may confer an advantage in malting quality. The identification of preferred alleles for malt quality enzymes could find immediate application within barley breeding programs. The exploitation of allelic variation for specific genes is discussed as one approach to breeding barley for improved malt quality.

The level of genetic knowledge of crop species is increasing at a rapid rate, through mapping studies, EST databases, the completion of genome sequences for arabidopsis and rice, and the application of syntenic approaches to gene discovery. These resources provide a growing number of candidate genes that may underpin key economic traits. These candidate genes are potential targets for allele discovery, mutation breeding, or genetic engineering approaches to crop improvement. While these approaches provide significant opportunities to address abiotic stress tolerance, disease resistance, and quality, one of the key limitations for reverse genetics is in the identification of those candidate genes that are likely to have a significant phenotypic effect. A relatively detailed understanding of the physiological and biochemical basis of the target trait is required in order to determine which genes, proteins or enzymes could be used to positively influence the phenotype.

Within the current study, a detailed literature review is used to identify the opportunities to improve the malting quality of barley through targeted allele discovery. The malting and brewing processes are outlined with emphasis on the role of the barley enzymes in various stages of malting and brewing. The key barley enzymes and proteins involved in malting and brewing are reviewed. The biochemistry and genetics of each is discussed in

addition to its impact on the malting and brewing processes, with emphasis on whether improvements in quality could be made through manipulating the levels or properties of each protein. The level of genetic variation that has already been identified within both the *Hordeum vulgare* and *Hordeum vulgare ssp. spontaneum* gene pools is also considered.

## 1.2 The Malting Process

Malting is essentially controlled germination. The schematic diagram in Figure 1.1 outlines the malting process. The first step in malting is steeping of the grain, which initiates metabolism in the embryonic and aleurone tissues, and aims to provide uniform germination. During the early stages of germination, hydrolytic enzymes are secreted from the scutellum and aleurone under the control of gibberellic acid (GA) and abscisic acid (ABA). The enzymes diffuse through the endosperm, degrading the cell walls and protein matrix, and exposing the starch granules for subsequent hydrolysis. Recent reviews provide a detailed discussion of the biochemistry of germination (Briggs 1989, Fincher and Stone 1993). The process of cell wall and protein degradation is referred to as 'modification', which leaves the grain friable and easily milled. The barley is allowed to germinate until the desired level of modification is achieved, while minimising starch degradation. At this point the malt is kilned to arrest growth and reduce moisture, facilitating storage.

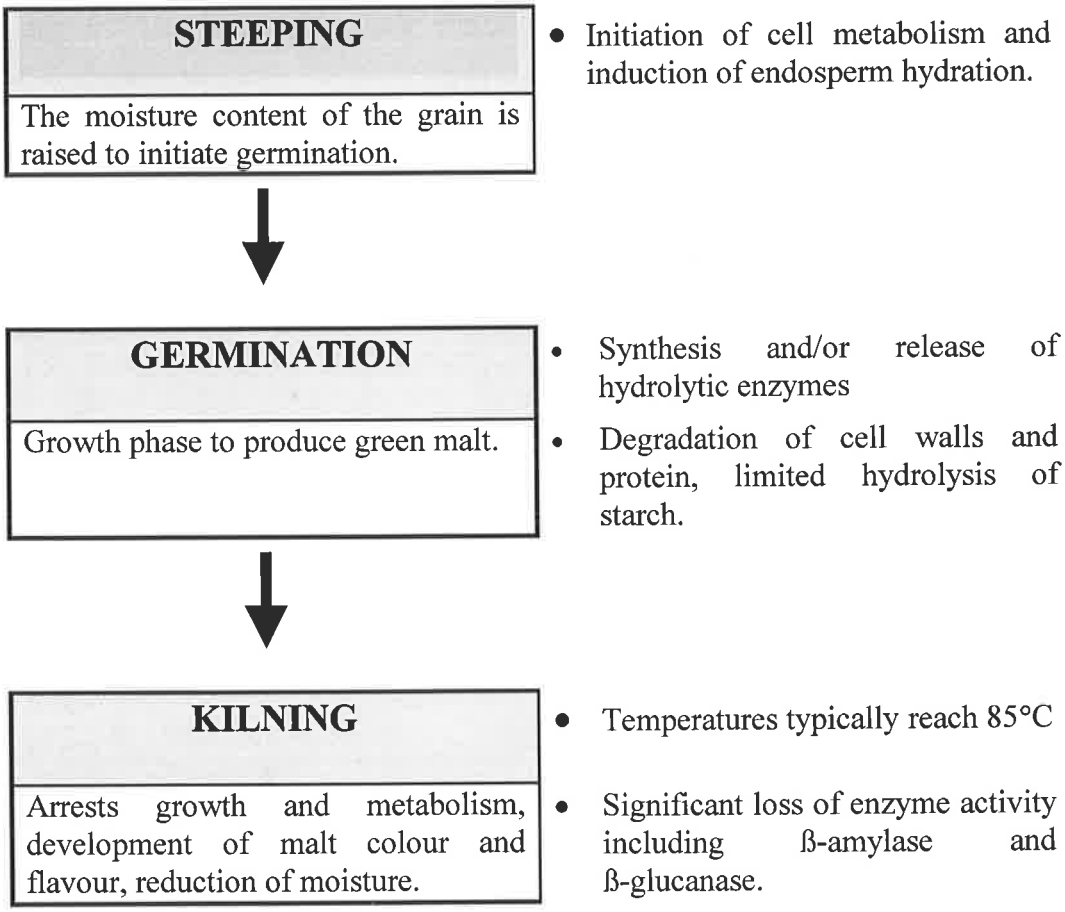



**Figure 1.1:** Schematic representation of the malting process and the impact of malt enzymes in each step.

A summary of the major characteristics used to assess malt quality is described in Table 1.1. The actual specifications for these characteristics vary significantly depending the style of beer to be brewed. Most of the procedures used to assess malt quality are recommended methods, either of the Institute of Brewing (IOB 1991), the American Society of Brewing Chemists (ASBC 1976), or the European Brewery Convention (EBC 1987).

**Table 1.1:** A summary of the major characteristics used to assess malt quality.

| PARAMETER | DEFINITION |
|---|---|
| Moisture (%) | Percentage of water in the malt. |
| Extract, Fine (%) | Level of water soluble material extracted from the malt after different levels of milling. |
| Extract, Coarse (%) | |
| Fine/Coarse Extract Difference (%) | Provides a simple index of modification. |
| Wort Colour | The intensity of the colour of the wort, reflecting the internal colour of the grain. |
| Diastatic Power (DP) | Total activity of the enzymes involved in starch hydrolysis. |
| α-amylase | Enzyme activity extracted from malt |
| ß-amylase | Enzyme activity extracted from malt |
| Fermentability (AAL%) | The level of fermentable sugars obtained from the malt. |
| Total Nitrogen | Total nitrogen content of the malt, this is also a measure of total protein. |
| Soluble Nitrogen | The level of nitrogen extracted into the wort. |
| Kolbach Index | Ratio of soluble N to total N, providing a measure of the extent of modification. |
| Wort Viscosity | Reflects the level of ß-glucan and other soluble high molecular weight material. |
| Wort ß-glucan | Total (1-3),(1-4)- ß-glucan in the wort. |

## 1.3 The Brewing Process

Beer production is a major end use of barley malt, and a significant amount of malt is also used by the food industry and in whiskey production. A schematic representation of the brewing process highlighting the impact of malt enzymes is shown in Figure 1.2. The milled malt is mixed with water to allow extraction of the soluble malt components and further production of fermentable sugars from starch degradation, while continuing the breakdown of proteins and cell wall material that occurred during malting. The diastatic enzymes hydrolyse gelatinised starch more efficiently than raw starch granules, therefore mash temperatures must reach at least 60°C to gelatinise the barley starch granules (Bamforth and Barclay 1993). Malt may be supplemented with starch containing adjuncts such as flaked or roasted barley, wheat flour, rice or corn syrup. Adjuncts are used either to impart specific flavour or colour characteristics on the beer, or more commonly due to cost savings relative to malt. The ratio of malt to starch adjunct may range from 75:25 to 25:75 (Inoue 1996), with the malt diastatic enzymes required to convert the additional starch into fermentable sugars. In the Australian the typical adjunct material is cane sugar that does not require degradation by the malt enzymes.

Mashing requires that the hydrolytic enzymes function at high temperatures. With the exception of α-amylase, the hydrolytic enzymes are relatively thermolabile, and are rapidly inactivated under mash conditions. It is considered that much higher enzyme activity is required for brewing than would be needed during normal barley germination. The use of starch containing adjuncts further increases the performance required of the malt enzymes. The degree of breakdown of the malt and adjunct material at this point impacts upon the remaining steps in the brewing process.

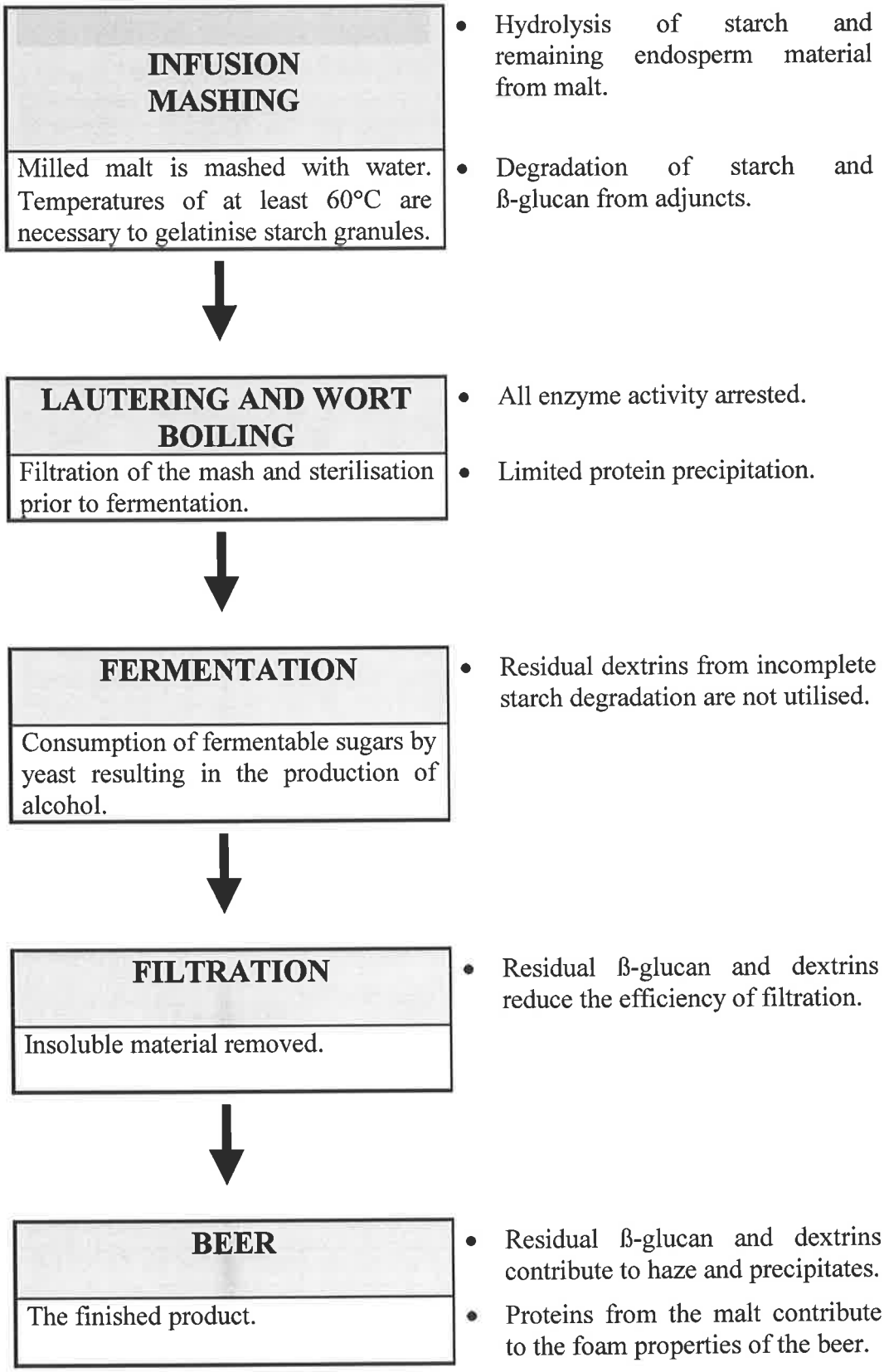



**Figure 1.2:** Schematic representation of the brewing process highlighting the involvement of malt enzymes.

The wort is separated from the spent grains by lautering or mash filtration. Residual ß-glucan resulting from incomplete degradation of cell walls increases the viscosity of the wort resulting in low rates of wort separation and beer filtration (Bamforth 1982). ß-glucan can also contribute to hazes and precipitates in finished beer (Letters 1977, Yamashita *et al.* 1989).

The wort is then boiled with hops to dissolve and isomerise hop components, to precipitate protein/polyphenol complexes (hot break), and to volatilise undesirable aromas. Boiling also served to inactivate the malt enzymes and sterilise the wort. The wort is then cooled resulting in the precipitation of additional protein/polyphenol material (cold break). The wort is then pitched with yeast. During primary fermentation the yeast converts fermentable sugars into ethanol and produces various flavour compounds such as esters. Fermentation may proceed at temperatures from 8-20°C for 5-10 days depending on the beer style and fermenter design.

Secondary fermentation serves to mature the flavour of the beer. Some beer styles employ bottle conditioning, where the beer is packaged and matured in contact with the yeast. More commonly beer is brewery matured, then filtered free of yeast and any solid material prior to packaging.

In 100% malt mash as much as 25% of the starch degradation products may be unfermentable dextrins (Enevoldsen and Bathgate 1969, Enevoldsen 1978). Dextrins are the most abundant high molecular weight compounds in beer, present in much larger amounts than ß-glucans or proteins, and can cause beer filtration and clarification problems (Schur 1985). Dextrins have been assumed to be important contributors to mouthfeel and body characteristics of finished beer. However the concentration of dextrins in beer ranges from 10-50 g $L^{-1}$ depending on the beer style, and an increase of 52 g $L^{-1}$ is required to produce an increase viscosity of light beer detectable by sensory testing (Ragot *et al.* 1989).

Attempts to address the problems associated with incomplete hydrolysis of malt components have taken a number of directions. The use of exogenous enzymes from bacteria and fungi has been used to supplement malt enzymes. However the use of commercial enzymes represents additional cost, and there are legal restrictions preventing enzyme addition in certain countries.

Genetic engineering of brewers yeast to express glucoamylase (Kim *et al.* 1994) and endo-ß-(1→4) glucanase (Xie *et al.* 1995) have been important research advances. However genetically engineered yeast are yet to be used in commercial brewing. Barley has also been transformed with a thermostable bacterial ß-glucanase (Jensen *et al.* 1996). Methods in protein engineering have also been used to increase the thermostability of barley (1→3),(1→4)-ß-glucanase (Stewart *et al.* 1996) and ß-amylase (Yoshigi *et al.* 1995), however transgenic barley is not currently used in commercial beer production.

The malting process is controlled to produce malt with the required specifications, however most of the characteristics of the finished malt are limited by the intrinsic properties of the barley. The challenge for barley breeders is to generate new varieties with improved potential for malt quality. Traditional approaches in barley breeding have sought to increase the levels of key enzymes by using this as a selection tool within the relatively limited germplasm used by barley breeding programs. This investigation aims to extend this strategy by assessing the genetic variation in both cultivated and wild barley for proteins important in malting and brewing, and assessing the potential of alternative alleles for application in breeding barley for improved malt quality.

## 1.4 The Key Proteins and Enzymes

The processes of malting and mashing are essentially the enzymic degradation of the barley endosperm. One of the first stages is the hydrolysis of the crushed cell layer and the cell walls within the endosperm. The principal enzyme in this process is (1→3,1→4)-endo-ß-glucanase (Woodward and Fincher 1982a and 1982b). Its activity facilitates access to the contents of the endosperm for the proteases which degrade the protein matrix, releasing the starch granules. Importantly, proteolysis does not result in the complete degradation of all the storage proteins. Protein Z, lipid transfer protein, and fragments of the hordeins survive malting and brewing to become major determinants in the foam properties of the finished beer (Hejgaard 1977, Sorensen *et al.* 1993, Kauffman *et al.* 1994, respectively).

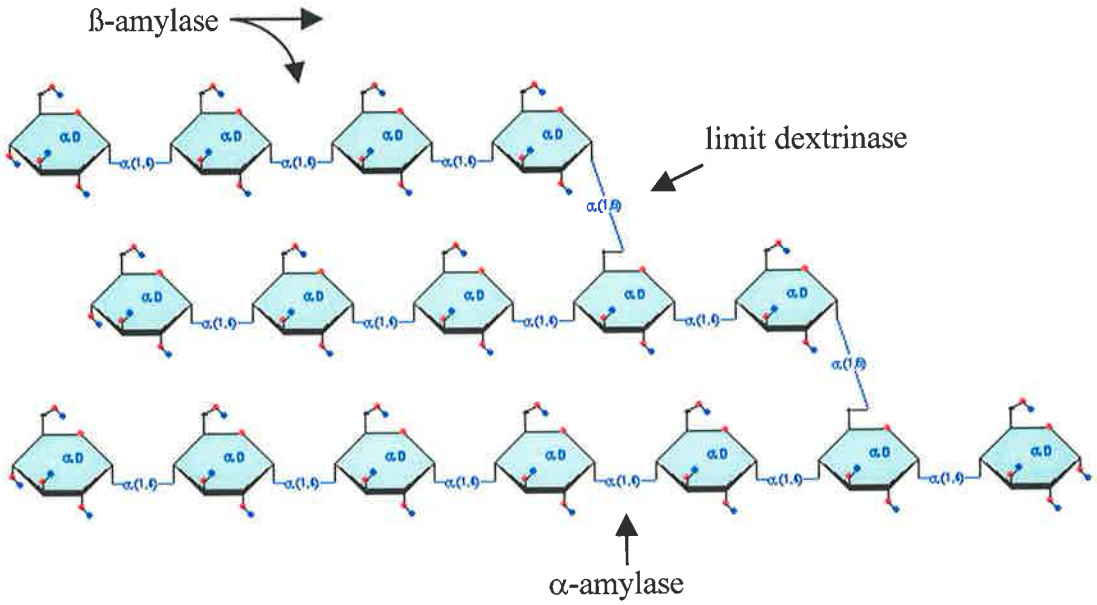



**Figure 1.3:** Simplified schematic diagram of the enzymes involved in the hydrolysis of starch.

The degradation of the starch and protein reserves is critical to yeast nutrition and alcohol production. Starch hydrolysis is achieved by the concerted action of four enzymes as

illustrated in Figure 1.3. The long chains of sugar residues are cleaved internally by α-amylase to yield branched and linear dextrins. The branch points are removed by limit dextrinase and the exo-action of ß-amylase degrades linear dextrins to maltose. Maltose represents the major fermentable sugar produced, however there is also hydrolysis of maltose to glucose which is mediated by α-glucosidase. The complete breakdown of starch involves a number of different enzymes, therefore attempts to increase the effectiveness of starch hydrolysis should be aimed at either the rate limiting steps, or all of the enzymes involved.

The following sections examine the biochemistry, genetics, and role of each of the key barley proteins involved in malting and brewing. The opportunity to improve malt quality by altering the levels or properties of each protein is discussed.

### 1.4.1 (1→3,1→4)-ß-glucanase

One of the key processes during germination is the production of hydrolytic enzymes in the aleurone layer that subsequently degrade the storage compounds in the endosperm. Before this can proceed the cell walls in the crushed cell layer and within the endosperm must be broken down to allow these enzymes access to the starch granules.

The major component of starchy endosperm cell walls is (1→3,1→4)-ß-glucan which can account for up to 70% (w/w) of the wall (Fincher 1975). This polysaccharide can be hydrolysed by three classes of glucanase, including (1→4)-ß-glucanase (cellulase) and to a lesser extent (1→3)-ß-glucanase. However the most important class is the (1→3,1→4)-ß-D-glucan 4 glucanohydrolases, and this group will be the focus of this discussion.

### 1.4.1.1 Biochemistry of ß-glucanase

(1→3,1→4)-ß-D-glucan-4 glucanohydrolase (EC 3.2.1.73) catalyses the hydrolysis of (1→4)-ß-glucosyl linkages in (1→3,1→4)-ß-glucans, but requires adjacent (1→3)- and (1→4)-ß-glucosyl residues in the substrate as shown in Figure 4. The major oligosaccharides released from the hydrolysis of the mixed linkage ß-glucan are 3-O-ß-cellobiosyl-D-glucose and 3-O-ß-cellotriosyl-D-glucose.

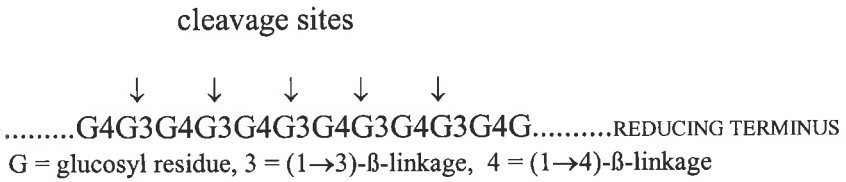



**Figure 4:** Schematic diagram of (1→3,1→4)-ß-glucanase cleavage sites in mixed linkage ß-glucan.

There are two forms of (1→3,1→4)-ß-D-glucan 4 glucanohydrolase present in germinating barley and these are designated as isoenzyme EI and isoenzyme EII (Woodward and Fincher 1982a). The two isoenzymes have very similar physical and chemical properties that are summarised in Table 1.2. In addition to their functional properties, the primary structure of the two isoenzymes is very similar with both consisting of 306 amino acids with a positional identity of 91%, and the bulk of these amino acid substitutions are of a conservative nature (Fincher *et al.* 1986, Slakeski *et al.* 1990).

**Table 1.2:** Properties of the two (1→3,1→4)-ß-glucanase isoenzymes[a].

| Property | Isoenzyme EI | Isoenzyme EII |
|---|---|---|
| Apparent Molecular Mass | 28,000 Da | 33,000 Da |
| pI | 8.5 | >10 |
| $V_{max}$ | $7.06 \times 10^3$ | $11.62 \times 10^3$ |
| $K_m$ | 3.0 | 3.4 |
| pH Optimum | 4.7 | 4.7 |

[a] Values from Woodward and Fincher (1982a) and Woodward and Fincher (1982b).

The difference in apparent molecular weight between the two isoenzymes has been attributed to glycosylation. Isoenzyme EII contains one site for N-linked glycosylation whereas this site is not present in isoenzyme EI (Slakeski *et al.* 1990). Analysis of glycan structures released by hydrazine treatment shows five different (although related) glycans can be covalently linked to the enzyme (Harthill and Thomsen 1995). The major glycan structure identified is shown in Figure 4. It has been shown that the presence of this carbohydrate contributes to the relative thermal stability of isoenzyme EII with respect to EI (Doan and Fincher 1992).

### 1.4.1.2 ß-glucanase in Malting and Brewing

One of the key steps during malting is the depolymerisation of cell walls facilitating access of starch and protein degrading enzymes to their substrates within the cells of the endosperm. Incomplete degradation of cell walls during malting reduces the yield of hot water extract (HWE) that can be derived during mashing (Bamforth 1982). This relationship leads to a strong positive correlation between HWE and total ß-glucanase activity ($r = 0.85$ in Stuart *et al.* 1988, and $r = 0.92$ in Barber *et al.* 1994).

Although (1→3,1→4)-ß-glucanase levels in green malt may be adequate to hydrolyse any remaining (1→3,1→4)-ß-glucan, the thermolabile nature of the two isoenzymes can result in residual ß-glucan in wort and beer. A high level of residual ß-glucan typically increases the viscosity of wort, resulting in slow rates of wort separation and beer filtration (Bamforth 1982). Investigations into the survival of ß-glucanase during kilning have shown isoenzyme EI is reduced to negligible levels (Loi *et al.* 1987, Henry 1990, Edmunds *et al.* 1994), and levels of isoenzyme EII range from 20% to 40% of the activity in green malt (Bamforth and Martin 1983, Loi *et al.* 1987, Edmunds *et al.* 1994). Compounding the loss of activity during kilning is the rapid inactivation of the remaining ß-glucanase in simulated mashing, with no enzyme activity detected after only 10 minutes at 60-65°C (Loi *et al.* 1987, Barber *et al.* 1994).

The sensitivity of ß-glucanases to the temperatures routinely used in kilning and mashing has led to research in genetic and protein engineering aimed at overcoming this problem. One approach has been to exploit naturally occurring thermostable (1→3,1→4)-ß-glucanases from other organisms. Suspension cultures of barley cells have been transformed with a thermostable ß-glucanase from *Trichoderma reesei* (Aspegren *et al.* 1995). A number of thermostable enzymes have been engineered through the construction of hybrid genes from the bacterium *Bacillus* spp. (Olsen *et al.* 1991), and transformed barley plants that synthesise thermostable ß-glucanase during germination have now been produced with this approach (Jensen *et al.* 1996). An alternative method has been to utilise protein engineering to increase the thermostability of the barley (1→3,1→4)-ß-glucanase. The introduction of an N-glycosylation site in isoenzyme EI has produced a small but significant increase in thermostability (Doan and Fincher 1992). Site directed mutagenesis has been used to generate mutant forms of isoenzyme EII containing single amino acid substitutions that enhance thermostability (Stewart *et al.* 1996).

### 1.4.1.3 Genetics of (1→3,1→4)-ß-glucanase

The gene for isoenzyme EI (*Glb* 1) is tightly linked to the malate dehydrogenase (*Mdh* 1) locus on chromosome 5H (MacLeod *et al.* 1991) and the gene for (1→3,1→4)-ß-glucanase isoenzyme EII has been mapped to the long arm of chromosome 1H (Loi *et al.* 1988, Slakeski *et al.* 1990). Both EI and EII genes have been isolated, and they exhibit 92% sequence identity (Litts *et al.* 1990, Slakeski *et al.* 1990, Wolf 1991, Wolf 1992). The coding region for the signal peptide in both genes is interrupted by a large intron of 2505 bp in EI and 2952 in EII. The intron is in the 25th codon of the signal peptide, and this intron position appears to be conserved among plant ß-glucanases (Wolf 1992).

The two (1→3,1→4)-ß-glucanase genes are subject to separate genetic control systems. Isoenzyme EI is expressed in young leaves and the scutellum and aleurone of germinating grain, however isoenzyme EII expression is restricted to the aleurone layer of germinating grain (Slakeski *et al.* 1990). Expression of isoenzyme EII is enhanced by $GA_3$ and is suppressed by ABA, however levels of EI mRNA in germinating grain are not significantly stimulated by $GA_3$ (Slakeski and Fincher 1992a). The response of isoenzyme EI expression to phytohormones is different in each tissue, suggesting a more specialised role in cell wall metabolism in addition to its function in cell wall degradation during germination.

In the mature barley grain (1→3,1→4)-ß-glucanase activity is very low to absent. Enzyme activity increases to a maximum 4 to 6 days after the initiation of germination (Ballance *et al.* 1976, Stuart and Fincher 1983). One day after germination begins the levels of EI mRNA in the scutellum are relatively high, but decrease after 3 days. In contrast, the levels of EII mRNA in the aleurone increase significantly between 1 and 3 days (Slakeski and Fincher 1992b). The expression of EII is restricted to the aleurone layer and follows a similar developmental pattern to isoenzyme EI in this tissue (Slakeski and Fincher 1992b). Analysis

of the levels of each form of the protein by immuno-blotting and ELISA have shown essentially the same pattern, with isoenzyme EI expressed first and the levels of both enzymes increasing for 3 days after the initiation of germination (Hoj *et al.* 1990, Edmunds *et al.* 1994).

Significant variation in total ß-glucanase activity exists between different barley varieties, with genotype influencing the rate of development of both isoenzymes and the absolute levels of EI (Edmunds *et al.* 1994). After 48 hours of germination isoenzyme EI is the dominant form, and it ranges from 63% to 82% of total ß-glucanase activity depending on the variety (Henry 1990). Despite this genetic variation in enzyme activity, there are no published reports of alternative alleles for either isoenzyme from within the *H. vulgare* gene pool.

Twenty lines of *H. spontaneum* have been examined for variation in (1→3),(1→4)-endo-ß-glucanase isoenzyme EI using isoelectric focusing combined with immunoblotting (MacLeod *et al.* 1991). Although this collection represented significant genetic diversity, only one enzyme variant was identified. The enzyme variant was identified by a shift from pI 8.5 in the normal EI isoenzyme to pI 8.0 (MacLeod *et al.* 1991). Other properties of the variant such as molecular weight, glycosylation status, expression levels or specific activity were not determined. There are no published investigations into variation in (1→3),(1→4)-endo-ß-glucanase isoenzyme EII.

Studies on 5 day germinated *H. spontaneum* samples have shown significant variation in total (1→3),(1→4)-endo-ß-glucanase activity, with some accessions exceeding cv. Bomi by 2-3 times. The increase in ß-glucanase activity was correlated with increased barley ß-glucan levels (Ahokas and Naskali 1990a and 1990b). Backcrossing these high activity lines with cv. Adorra as the recurrent parent resulted in few lines with increased ß-glucanase activity,

however lines were identified with up to 1.7 times more activity than the recurrent parent (Ahokas and Erkkila 1992).

Total (1→3),(1→4)-endo-ß-glucanase activity is the combined action of isoenzymes EI and EII, therefore increased activity may be the result of allelic variation at either locus. Since the backcross lines were not derived by selection for specific ß-glucanase alleles, the changes in enzyme activity may also be the result of factors acting indirectly on the enzyme. One possibility is that differences in the mechanisms involved in the gibberellic acid mediated control of gene expression could lead to variation in ß-glucanase levels.

Within the current study, wild barley is surveyed for natural variation in (1→3,1→4)-ß-glucanase isoenzyme EI. The only allelic variant identified was that reported by MacLeod *et al.* (1991), therefore the results are not presented in detail. The distribution of the variant allele within the wild barley collection is summarised in Appendix 1.

### 1.4.2 Proteases

During germination, barley proteins are hydrolysed to peptides and amino acids providing nutrients for the developing embryo. From a malting standpoint this is important because it results in the degradation of the endosperm protein matrix, facilitating hydrolysis of the starch granules, and providing amino acids for yeast nutrition. The extent of protein hydrolysis is an important malt quality parameter, and is assessed as the ratio of soluble to total nitrogen. In addition to the breakdown of storage proteins, the proteases play important roles in posttranslational modifications of other malt enzymes including protein Z, α-amylase, and ß-amylase (see sections 1.4.3, 1.4.4, and 1.4.7).

Over 40 different proteolytic activities have been identified in malt (Zhang and Jones 1995), however few enzymes have been isolated and characterised. The most detailed studies

have examined members of the endopeptidases (EC 3.4.21), which includes the cysteine endopeptidases that mediate the release of the bound fraction of ß-amylase (Guerin *et al.* 1992a) and limit dextrinase (Longstaff and Bryce 1993). Additional work has partially characterised five members of the serine carboxypeptidases (EC 3.4.16.1) in germinating barley (Mikola 1983).

Proteinase inhibitors have been implicated in mediating protein hydrolysis during germination, and a number of these inhibitors have been characterised. Lipid transfer proteins (LTP1 and LTP2) suppress the activity of many of the cysteine endopeptidases from green malt (Jones and Marinac 1995 and 1997). The protein Z family are serine proteinase inhibitors (Dahl *et al.* 1996), and two chymotryptic inhibitors (CI-1 and CI-2) have been purified from barley (Jonassen 1980).

While the proteinases are very important to malt quality, there role is far more complex than just the degradation of storage proteins. They are involved in post-translational modifications of key malt proteins and there is a requirement for proteins important in foam formation and head retention to survive through to the finished beer. The presence of a large number of discrete proteinase enzymes, and the association of inhibitors, means that changing the complement of these enzymes in malt is not feasible without a more detailed understanding of how each enzyme impacts upon malt and beer quality. While the identification of alternative alleles for these enzymes may provide some information on their role and significance, this will not be pursued within this study.

### 1.4.3 Protein Z

Protein Z comprises a family of barley endosperm albumens that belong to the superfamily of serine proteinase inhibitors known as serpins. Serpins are sacrificial protease inhibitors that are cleaved and bind to the protease to achieve their function of protease regulation. The complex can subsequently dissociate into active protease and inactive serpin (Bjork *et al.* 1987). Protein Z comprises a major component of the mature kernel, accounting for between 0-5% of the albumen fraction (Hejgaard 1982). The relatively high lysine content of protein Z can contribute up to 5% of the total lysine in barley, and this has led to the suggestion that it may have a role as a lysine sink (Hejgaard 1982).

#### 1.4.3.1 Biochemistry of Protein Z

Two immunogenically related forms of protein Z have been isolated in barley, the major form is designated protein $Z_4$ (Nielson *et al.* 1983) and the minor form protein $Z_7$ (Hejgaard 1984). Amino acid sequences have been determined for protein $Z_4$ (Brandt *et al.* 1990) and a 39,000Da protein that probably corresponds to protein $Z_7$ (Lundgard and Svensson 1989). The protein $Z_7$ isolated by Lundgard and Svensson inhibits α-chymotrypsin, while protein $Z_4$ inhibits trypsin, chymotrypsin, and cathepsin G (Dahl *et al.* 1996).

Protein Z is present in free, bound and latent forms in mature grain. The bound fraction is extracted in the presence of reducing agents or papain (Hejgaard 1982), while the latent fraction is extracted by a combination of detergent and reducing agent (Evans and Hejgaard 1999), and both forms are released into solution after germination (Hejgaard 1978). Results from cross-linked immunoelectrophoresis have suggested the bound fraction is due to the formation of heterodimers with ß-amylase (Hejgaard 1976, Hejgaard and Carlsen 1977, Hejgaard 1978). The heterodimers were extracted in the presence of 1mM ß-mercaptoethanol

and these aggregates could be split using 100mM ß-mercaptoethanol. These heterodimers could be expected to possess novel $M_r$ and pI values, and contribute to the heterogeneity seen when crude extracts of ß-amylase are separated by chromatofocusing and isoelectric focusing (Hejgaard 1976, Hejgaard and Carlsen 1976). Significant heterogeneity is seen in protein $Z_4$ when subjected to isoelectric focusing with four major bands ranging from pI 5.0 to pI 5.8 (Hejgaard 1982, Evans *et al.* 1995).

More recent evidence has challenged the view that bound ß-amylase is the result of aggregation with protein Z. The cultivar Pirrka does not express protein $Z_4$, the major protein in this family (Hejgaard 1978, Brandt *et al.* 1990,). Therefore it could be expected that all the ß-amylase would be in the free form, however it was found that Pirrka has 58% of its ß-amylase in the bound form (Evans *et al.* 1995). Significantly, the involvement of other related forms of protein Z in aggregation with ß-amylase was not excluded (Evans *et al.* 1995).

### 1.4.3.2 Protein Z in Malting and Brewing

Protein Z is one of the few barley proteins to survive the malting and brewing processes to be present as up to 10% of the protein in beer (Hejgaard and Kaersgaard 1983). Protein Z in beer retains its basic structure and antigenic properties, but exhibits microheterogeneity due to the addition of carbohydrate moities through glycation reactions during malting and brewing (Hejgaard and Kaersgaard 1983, Curioni *et al.* 1995). Another significant property of the beer form of protein Z is that it has a tendency to aggregate with itself and other proteins. Protein Z is present in beer haze (Hejgaard 1977), and it has been suggested that it may contribute to haze formation (Hejgaard and Kaersgaard 1983).

Protein Z may also have a significant role in foam stability in beer, since it is also a major antigen in this fraction (Hejgaard 1977). The role of protein $Z_4$ in beer foam has been investigated with pilot brews of the variety Pirrka which is a protein $Z_4$ null. Results indicated head retention was not significantly affected by the absence of protein $Z_4$, however possible compensatory effects by other forms of protein Z could not be ruled out (Gibson *et al.* 1995). More recent and comprehensive brewing trials have shown that protein Z4 is significantly correlated with foam stability (Evans *et al.* 1999).

### 1.4.3.3 Genetics of Protein Z

Three barley genes encoding protein $Z_4$ (BSZ4), protein Zx (BSZx), and protein $Z_7$ (BSZ7) have been sequenced (Brandt *et al.* 1990, Rasmussen 1993, Rasmussen *et al.* 1996). Barley protein $Z_4$ is encoded by a small multigene family on the short arm of chromosome 4H, designated *Paz1* (Nielsen *et al.* 1983, Rasmussen *et al.* 1984). Protein $Z_7$ is the product of a gene on chromosome 7, but has not been the subject of linkage analysis (Hejgaard 1984). Protein Zx is not expressed in the barley grain (J. Hejgaard - personal communication). The deduced amino acid sequences of the barley serpins show 70% homology, and approximately 30% similarity to serpins from animal sources (Rasmussen 1993).

Protein Z is synthesised in the developing endosperm from 10-25 days after fertilisation, in parallel with the hordein storage proteins (Giese and Hejgaard 1984, Sorensen *et al.* 1989). The Hiproly high-lysine barley variety contains a mutation in the *lys*1 gene on chromosome 5H, and exhibits significantly enhanced expression of protein Z4 (Sorensen *et al.* 1989). Protein Z4 expression is downregulated by the *lys3a* mutation of Riso 1508 (Hejgaard and Biosen 1980). The *lys3a* gene on chromosome 5H exerts its effects on protein

expression through promoter hypermethylation (Sorensen *et al.* 1996). Environmental effects also influence protein Z expression in the same way as storage proteins, with improved nitrogen nutrition generating increased levels of protein Z in the mature grain (Geise and Hopp 1984, Giese and Hejgaard 1984).

While the physiological function of protein Z is not well understood, its presence in high levels in barley, malt, beer, beer hazes and beer foam mean that is of significant interest to the malting and brewing industry. The null Paz4 line Pirrka has provided opportunities to investigate the role of protein $Z_4$, and additional serpin mutants could offer further opportunities. Therefore genetic variation for protein Z will be examined in both cultivated and wild barley.

### 1.4.4 α-Amylase

α-Amylase, 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1), is synthesised during germination in response to the plant hormone gibberellin (Filner and Varner 1967) and catalyses the cleavage of internal (1→4)-α-glucosyl linkages of amylose and amylopectin. α-Amylase consists of two discreet families, AMY1 and AMY2, and the two groups are distinguished by differences in isoelectric points, with pI 4.7-5.2 for AMY1 and pI 5.9-6.1 for AMY2 (Svensson *et al.* 1985). The properties of the two α-amylase isoenzymes are summarised in Table 1.3. The AMY1 and AMY2 families share 80% amino acid identities (Rogers and Milliman 1983, Rogers 1985), and have similar substrate specificities and action patterns, however the AMY1 isoforms degrade raw starch granules faster than the high pI group (MacGregor and Ballance 1980, Sissons and MacGregor 1994).

**Table 1.3:** Physical properties of the two α-amylase isoenzymes.

| Property | AMY1 | AMY2 |
|---|---|---|
| Apparent M.W. | 45,000 Da[1] | 45,000 Da[1] |
| Calculated M.W. | 45,447 Da[2] | |
| pI | ~4.5[1] | ~5.8[1] |
| No. Isoforms | 6[3] | 8[4] |
| Gene Symbol | *α-amy*1 | *α-amy*2 |
| No. Genes | 3[5] | 6[6] |

References: 1- Callis and Ho (1983), 2- Rogers and Milliman (1983), 3- Sogaard *et al.* (1991), 4- Simon and Jones (1988), 5- Muthrukrishnan *et al.* (1984), 6- Khurseed and Rogers (1988).

The AMY2 isoforms are expressed earlier in germination and are also the most abundant, accounting for up to 80% of the total α-amylase activity in malt (MacGregor *et al.* 1988). The activity of the AMY2 group of isoforms is also modified by the bifunctional α-amylase/subtilisin inhibitor (BASI). BASI is a 19,865 Da protein synthesised during grain filling (Munck *et al.* 1985), and is a fast reacting, tight binding inhibitor of AMY2 (Sidenius 1995). Henson and Stone (1988) have shown the wide range seen in α-amylase activity in germinating barley is the result of variation in the levels of both the enzyme and BASI. The genetic variation for α-amylase has been assessed in cultivated barley, and three alleles for *α-amy2* have been reported (Takano and Takeda 1985). There is no significant relationship between each allele and total α-amylase activity (Takano *et al.* 1988).

In contrast to other hydrolytic enzymes of malt, α-amylase is a relatively thermostable enzyme, with only 4-18% of enzyme activity lost during kilning (Runkel 1975, Sjoholm *et al.* 1995). In isothermal mashes using temperatures up to 65°C no α-amylase activity is lost, and

approximately 50% of activity remains after 1 hour at 70°C (Sjoholm *et al.* 1995). While α-amylase is certainly the key enzyme in the degradation of raw starch granules during germination, in brewing the starch is gelatinised during mashing, and it is only ß-amylase that is significantly correlated with the hydrolysis of boiled soluble starch (Sun and Henson 1991). Dextrin profiles in wort and beer suggest that α-amylolysis of starch is usually complete by the end of mashing (MacGregor 1995). It has also been demonstrated that the level of α-amylase in most malts is sufficient to enable maximum production of soluble dextrins not only from the starch of the malt itself, but also from the starch of relatively large proportions of adjuncts (Norris and Lewis 1985).

The biochemistry and genetic control of α-amylase have been well characterised. The combination of high α-amylase levels in malt and the thermostability of the enzyme mean that it is not normally a rate limiting enzyme in the degradation of starch during mashing. This suggests that increases in α-amylase activity may not yield significant advances in increasing the rate or efficiency of starch degradation. In addition, the genetic variation for α-amylase has been assessed and the three alleles do not exhibit significant differences in activity (Takano *et al.* 1988). Accordingly, α-amylase will not be the subject of investigation in this study.

### 1.4.5 Limit Dextrinase

Limit dextrinase, α-dextrin 6-glucanohydrolase, (EC 3.2.1.41) hydrolyses α-1,6-linkages in amylopectin or derived limit dextrins. The enzyme does not hydrolyse α-1,6 bonds linking a single glucosyl residue to a maltosaccharide, as it requires at least one α-1,4 linkage an each side of the susceptible bond. Limit dextrinase is the only malt enzyme

capable of hydrolysing α-1,6 bonds therefore it complements the activity of the amylases in starch hydrolysis.

Limit dextrinase has been purified to homogeneity from malt and is a monomeric protein of 104 kDa consisting of six isoforms with apparent pI's ranging from 4.2-4.6 and has optimal activity at pH 5.5 (Sissons *et al.* 1992a). Limit dextrinase is only slightly less thermostable than α-amylase during kilning, with between 0-25% of activity lost under standard kilning conditions (Sjoholm *et al.* 1995). Under mashing conditions limit dextrinase exhibits a similar thermostability profile to ß-amylase. Approximately 50% of activity remains after 1 hour at 62.5°C, 50% of activity remains after only 30 minutes at 65°C, and there is rapid inactivation as temperatures approach 70°C (Lee and Pyler 1984, Sjoholm *et al.* 1995).

Synthesis of limit dextrinase begins around six days after anthesis and the enzyme is stored in the mature grain as a bound protein but is present in relatively low levels (Manners and Yellowlees 1973, Lenoir *et al.* 1984, Sissons *et al.* 1993). The first five days of germination see a rapid increase in the level of bound limit dextrinase due to additional enzyme synthesis (Longstaff and Bryce 1993). As germination proceeds the bound enzyme is converted to an active free form through proteolytic modification by cysteine endoproteinases (Longstaff and Bryce 1993).

Two inhibitors of limit dextrinase are present in both mature grain (MacGregor *et al.* 1994) and at lower levels in germinated barley (Macri *et al.* 1993). The two inhibitors are heat stable proteins of approximately 15 kDa and have isoelectric points of 6.7 and 7.2 (Macri *et al.* 1993). Both inhibitors are active over a wide pH range, and they are most effective at the pH optima of their target enzyme (MacGregor *et al.* 1994).

Wort and beer contain significant levels of branched dextrins (Enevoldsen and Bathgate 1969, Enevoldsen and Schmidt 1974), suggesting incomplete hydrolysis by limit dextrinase. Increasing the effectiveness of limit dextrinase during mashing could therefore lead to higher levels of fermentable sugars in the wort. The significance of the recently identified LD inhibitors during mashing requires further investigation, lower levels of these proteins may also be a viable approach to increasing the effectiveness of limit dextrinase during brewing.

The lack of a detection method for limit dextrinase activity in native electrophoresis gels, and the lack of antibodies specific for the enzyme or its inhibitors, limits the opportunities to identify qualitative variation that may affect the efficiency of hydrolysis of α-1,6-linkages. Genetic variation for limit dextrinase and its inhibitors will not be investigated in this study because of these technical limitations. However, it is concluded that functional variation for these traits could provide significant insight into the biochemical control of α-1,6-linkage hydrolysis, and potentially provide a practical approach to increasing the yield of fermentable sugars from malt extract.

### 1.4.6 α-Glucosidase

α-Glucosidase (EC 3.2.1.20) is the least well characterised of the starch degrading enzymes in germinating barley. α-Glucosidase catalyses the release of glucose from maltose and other small dextrins, and is important during germination for the production of glucose which can be assimilated and metabolised by the growing embryo. However, yeasts rapidly metabolise maltose, and therefore do not require hydrolysis to glucose for fermentation.

α-glucosidase is found in the mature grain, and after the initiation of germination there is further synthesis of the enzyme (Jorgensen 1965, MacGregor and Lenoir 1987). Two

α-glucosidase isoenzymes have been purified from malt and designated as G1 (high pI) and G2 (low pI) (Sissons and MacGregor 1994).

It has been suggested that α-glucosidase may increase the effectiveness of α-amylase and ß-amylase during mashing by removing maltose, which is a possible competitive inhibitor (Sun and Henson 1991, MacGregor 1996). However recent results have shown maltose does not significantly inhibit ß-amylase at typical mashing concentrations (Ma *et al.* 2000). There is also a synergistic effect between G1 and α-amylases in the hydrolysis of intact starch granules, while G2 and α-amylase exhibit additive effects (Sissons and MacGregor 1994).

There is not strong evidence to associate the level of α-glucosidase activity with improved malt quality. More work is required to characterise and quantify any effects that α-glucosidase may have on the malting and brewing processes. Whether advances in malt quality can be made through altering the levels or properties of α-glucosidase will be determined with a more detailed understanding of the role of this enzyme during germination, and in malting and brewing. This research will not be pursued in this investigation.

### 1.4.7 ß-Amylase

There are two forms of ß-amylase expressed in barley, delineated by their spatial and temporal expression. One is endosperm specific, accumulating in the starchy endosperm of developing seeds where it comprises a major component of the total protein present in the mature grain. The other form is described as ubiquitous, exhibiting only a transient presence in the developing kernel, and is found in other seedling tissues including leaves and roots (Shewry *et al.* 1988, Daussant *et al.* 1994). This discussion provides a review of the

biochemistry of germination and malting, and is therefore focussed on the endosperm specific ß-amylase.

### 1.4.7.1 Biochemistry of ß-amylase

ß-Amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) is a key enzyme in the hydrolysis of starch in germinating barley grains. ß-amylase catalyses the liberation of ß-maltose from the non-reducing ends of starch and related 1,4-α-glucans. ß-Amylase is synthesised during the development of the barley grain, and responds positively to nitrogen nutrition in parallel to the hordein storage proteins (Giese and Hopp 1984, Giese and Hejgaard 1984). ß-amylase is one of the major proteins present in mature grains accounting for 1-2% of total protein in the starchy endosperm (Hejgaard and Boisen 1980). The very low level of ß-amylase in the mutant barley Riso 1508 suggests that little ß-amylase is required for successful germination and seedling growth under standard conditions (Kreis *et al.* 1987).

ß-amylase is present in three fractions in the mature grain. One fraction, the active or 'free' enzyme is water soluble, the 'bound' form can be extracted in the presence of reducing agents or proteolytic enzymes (Sallans and Anderson 1940, Sandegrene and Klang 1950, Bendelow 1964), and the 'latent' fraction can be extracted with detergent in combination with reducing agents (Evans *et al.* 1997). Immunocytochemical data has localised the enzyme to the endosperm and mid-region aleurone, as major and minor sites respectively (Shen-Miller *et al.* 1991), and biochemical analysis has shown the periphery of starch granules to be the main site of bound ß-amylase deposition (Hara-Nishimura *et al.* 1986).

The literature contains numerous investigations demonstrating a high degree of polymorphism in ß-amylase with respect to size and charge. Chromatofocusing and isoelectric focusing have been used to resolve up to eight ß-amylase isoforms with isoelectric

points ranging from pH 4.2 to pH 6.4 (LaBerge and Marchylo 1983, Lundgard and Svensson 1987, Shewry *et al.* 1988). A size range from $M_r$ 40,000 to 400,000 has been demonstrated by gel filtration, SDS-PAGE and ultracentrifugation (Visuri and Nummi 1972, Niku-Paavola *et al.* 1973, Bilderback 1974). This heterogeneity may result at least partly from the formation of polymers with itself and other proteins, including heterodimers with protein Z (Hejgaard 1976, Hejgaard and Carlsen 1977, LaBerge and Marchylo 1983). The presence of thiol groups which can participate in intermolecular disulphide bond formation are likely to be involved in the formation of such aggregates (Visuri and Nummi 1972).

Developmental changes in the isoforms of ß-amylase also occur during grain maturation and germination. In mature grain the major isoform is a single polypeptide chain of $M_r$ 59,700, which is converted during germination to an isoform of $M_r$ 56,000 via two intermediate forms (Lundgard and Svensson 1987, Shewry *et al.* 1988). The reduction in molecular weight is accompanied by a basic shift in the isoelectric point of the protein (Evans *et al.* 1997). The conversion of isoforms is mediated by limited proteolysis in the carboxy-terminal region of ß-amylase by malt endopeptidase activity (Lundgard and Svensson 1986, Guerin *et al.* 1992).

The primary structure of barley endosperm ß-amylase has been deduced from the nucleotide sequences of full length cDNA clones (Kreis *et al.* 1987, Yoshigi *et al.* 1994). The carboxy-terminal region contains four glycine rich repeats and a cysteine residue at position 503 which may be involved in interactions with protein Z and other cellular components (Kreis *et al.* 1987, Lundgard and Svensson 1987). The removal of these structures from the C-terminal region of the protein is presumably the key to converting the bound form of the enzyme into the free form. The role of the carboxy-terminal region of the ß-amylase enzyme was investigated using site directed mutagenesis to delete 54 amino acids from the C-terminus of the protein (Yoshigi *et al.* 1995). The normal recombinant enzyme showed

heterogeneity on isoelectric focusing, separating into six distinct bands (Yoshigi *et al.* 1994), whereas the mutant ß-amylase gave a single protein band of pI 6.85 (Yoshigi *et al.* 1995). In addition, the mutant enzyme was significantly more heat labile, suggesting a significant role for the carboxy-terminal region in the stability of the protein (Yoshigi *et al.* 1995).

The bound form of ß-amylase has been referred to as inactive (Bendelow 1964, Hejgaard 1976), however it does possess limited activity against soluble starch (Hara-Nishimura *et al.* 1986, Sopanen and Lauriere 1989). The activation of bound ß-amylase during germination has been the subject of conflicting reports in the literature. Studies of purified ß-amylase have shown the enzyme to have the same specific activity before and after proteolytic cleavage of the C-terminus (Lungard and Svensson 1987), leading to speculation that the reduced activity of bound ß-amylase is due to steric hindrances (Sopanen and Lauriere 1989). The activity of the latent fraction has not been determined due to the denaturing conditions used to isolate it (Evans *et al.* 1997).

### 1.4.7.2 ß-amylase in Malting and Brewing

ß-Amylase is acknowledged as a key enzyme in malting and brewing because it is the only hydrolytic enzyme showing a consistent positive correlation with diastatic power (Delcour and Verschaeve 1987, Lin and Yu 1990, Gibson and Solah 1995). Path coefficient analysis has shown ß-amylase to be the most important enzyme in the degradation of boiled soluble starch (Sun and Henson 1991). Furthermore, the presence of linear maltodextrins in wort and beer indicates incomplete hydrolysis of starch dextrins by ß-amylase, and as much as 25% of degraded starch is in the form of unfermentable dextrins (Enevoldsen and Schmidt 1974). Consistent with this, a strong association has been shown between ß-amylase activity and wort attenuation in all-malt mashes (Hamilton and Lewis 1974). The level of wort

fermentability sharply decreases at temperatures greater than 62.5°C, coincident with thermal inactivation of ß-amylase (Hamilton and Lewis 1974). These results suggest that ß-amylase may be a rate-limiting enzyme in the breakdown of starch during mashing, and the practice of adding starch-containing adjuncts to the mash could be expected to amplify the importance of the ß-amylase enzyme.

The heightened importance of ß-amylase in malt quality is largely due to the thermolabile nature of the enzyme. Under relatively standard malting conditions between 10-50% of the ß-amylase activity in green malt is destroyed during kilning (Runkel 1975, Bamforth 1986, Sjoholm *et al.* 1995, Evans *et al.* 1997b). The hydrolysis of starch to reducing sugars during mashing normally proceeds at temperatures of at least 65°C to facilitate the gelatinisation of starch granules, however these conditions are sufficient to rapidly reduce ß-amylase activity (Narziss *et al.* 1973). Examination of the thermostability of ß-amylase in isothermal mashes has shown at least 50% of activity survives 1 hour at 62.5°C, whereas 50% of activity remains after only 30 minutes at 65°C (Sjoholm *et al.* 1995).

In an attempt to compensate for the losses of enzyme activity during kilning and mashing, brewers using starch based adjuncts demand malt high in ß-amylase activity. Allelic variation for ß-amylase that offered increased thermostability or increased expression levels could be expected to significantly impact upon wort fermentability and diastatic power. An alternative approach has been to manipulate the thermostability of ß-amylase through mutagenesis. A recombinant enzyme with 7°C increase in thermostability (as measured by $T_{50}$) has now been produced (Okada *et al.* 1995).

### 1.4.7.3 Genetics of ß-amylase

Two loci encoding barley ß-amylase have been identified. The *Bmy*2 locus on chromosome 2H encodes the ubiquitous form of the enzyme (Kreis *et al.* 1988). The endosperm specific form of ß-amylase is encoded by the *Bmy*1 locus on the long arm of chromosome 4H (Powling *et al.* 1981, Nielsen *et al.* 1985).

The control of ß-amylase expression is poorly understood, however two mutant genes on chromosome 7H dramatically affect the deposition of ß-amylase in the endosperm. The Hiproly high-lysine barley variety contains a mutation in the *lys1* gene and exhibits substantially higher levels of ß-amylase in the mature grain (Hejgaard and Boisen 1980). Riso mutant 1508 contains an altered *lys3* gene, described as *lys3a* (Ingverson *et al.* 1973), which contains very low levels of ß-amylase (Allison 1978). This mutation also interferes with the transcription of the B- and C-hordeins whose levels are severely reduced due to hypermethylation of their promoters (Sorensen *et al.* 1996).

Two *Bmy1* alleles have been identified in *H. vulgare* using an agarose based electrophoresis system analogous to native PAGE, and have been termed Sd1 and Sd2 (Starch Degrading 1 and 2, Allison 1973). Both forms of the enzyme have the same $M_r$ of 59,000 Da and exhibit the same complex banding pattern when subjected to IEF, however the Sd2 pattern is shifted to a more basic position. The characteristic basic shift of the Sd2 type is evident in both the mature barley grain (Allison and Ellis 1973) and in the proteolytically cleaved forms of ß-amylase produced during germination (Allison 1973, Allison and Swanston 1974). Segregation analysis of ß-amylase band patterns in mature and germinating grains demonstrated that the phenotype is controlled by co-dominant alleles at a single locus (Allison 1973). In a more recent investigation 29 cultivars were analysed using isoelectric focusing (IEF) and four different banding patterns were described and designated A,B,C, and

D (Thompson *et al.* 1990). The 29 cultivars were then examined in another report by the same group, however only two alleles were identified and they were designated *ß-amy-1a* and *ß-amy-1b* (Forster *et al.* 1991). In research towards barley varietal identification 66 varieties were examined and two ß-amylase banding patterns were detected and referred to as Ar and Br types (Nielsen and Johansen 1986). No correlations were made with the original investigations into ß-amylase banding patterns reported by Allison (1973) and Allison and Ellis (1973). There has been no published report of investigation into the biochemical differences between these two alleles, so it is unknown if either confers any advantage in malting quality. These investigations do suggest malting barley varieties are a mixture of the two genotypes.

The proportion of bound ß-amylase co-segregates with the Sd1 and Sd2 alleles. An examination of 46 cultivars showed those with Sd1 type had less than 50% of ß-amylase in the 'free' form, whereas Sd2 types had greater than 50% in the 'free' form (Allison and Swanston 1974). Segregation analysis had previously demonstrated that the free/bound ratio is determined by a single locus with incomplete dominance (Bendelow 1964). It has not been determined whether the free/bound ratios are a function the ß-amylase allele, or if they are controlled by a closely linked locus.

The gene sequence encoding barley ß-amylase has been determined using full length cDNA's from two varieties, Hiproly (Kreis *et al.* 1987) and Haruna nijo (Yoshigi *et al.* 1994). Both cultivars exhibit the Sd2 ß-amylase band pattern. The two sequences are very similar, however there are three amino acid differences as follows; Ala-233, Ser-347, Met-527 (Haruna nijo), and Val-233, Met-347, Ile-527 (Hiproly). This variation corresponds to a difference of only 36 Da, and since no charged amino acids have been substituted there is no difference in apparent isoelectric point. A major deficiency in the literature is the lack of an

Sd1 gene sequence that would allow some comparison between the two alleles, and potentially identify the biochemical basis for the different isoelectric point.

ß-Amylase banding patterns in mature grains of *H. spontaneum* have been examined using isoelectric focusing (Chalmers *et al.* 1992). This study reported 7 ß-amylase banding types from 135 accessions of wild barley collected from ecologically diverse sites in Israel. The banding patterns were used to assess variability in *H. spontaneum* and to show grain isozyme distribution is correlated with ecogeographical factors, in particular annual rainfall (Chalmers *et al.* 1992). Whether the banding patterns are allelic, whether these variants have an effect on malting quality, or if the variation in seed ß-amylase results in alternative isoforms after germination and the resulting proteolytic cleavage was not determined.

ß-Amylase enzyme activity has been assessed in germinated grains of *H. spontaneum* (Ahokas and Naskali 1990a and 1990b). Levels of enzyme activity exhibited wide variation and were usually significantly higher in wild barley than the Danish malting variety Bomi, with some accessions containing up to 6 times more ß-amylase activity than the reference variety (Ahokas and Naskali 1990a). In subsequent work *H. spontaneum* lines were backcrossed with cv. Adorra as the recurrent parent, and agronomic traits were the basis for selection (Ahokas and Erkkila 1992). Activity assays on 5 day germinated grains of the progeny identified lines containing up to 1.7 times more ß-amylase activity than cv. Adorra, corrected for both protein and grain mass (Ahokas and Erkkila 1992). The source of the increased activity was not determined, and may not be the result of variant ß-amylase alleles such as those described by Chalmers *et al.* (1992).

ß-Amylase will be a focus of this research due to its influence on malting quality through diastatic power and fermentable sugar production. ß-amylase has been the subject of inconsistent and conflicting publications, and there would be significant benefit in a detailed characterisation of this enzyme. This study aims to assess the genetic variation for ß-amylase

within both domesticated and wild barley, and to provide a structural and functional characterisation of the alternative alleles. The characterisation of the alternative alleles may provide barley breeders the opportunity to improve malting quality by selecting for a particular ß-amylase allele.

## 1.5 *Hordeum vulgare* ssp. *spontaneum* as a source of genetic variation

*Hordeum vulgare* ssp. *spontaneum* is recognised as the sole progenitor of cultivated barley (Zohary 1969) and crosses readily with *Hordeum vulgare* resulting in fully fertile progeny (Kobyljanskij 1967). *H. spontaneum* is a diploid annual cereal widespread in the Mediterranean region from the Euphrates Basin to the Jordan Rift Valley (Harlan and Zohary 1966). It occupies a wide diversity of habitats ranging from mesic Mediterranean to desert and is equally variable in morphology, consisting of robust plants with large spikes in the mesic and warm environments to smaller forms on the arid steppes (Nevo *et al.* 1979a). The plant is typically profusely tillering, semierect in growth habit, asynchronously flowering within the plant, and has the brittle rachis trait (Corke and Atsmon 1990).

The genetic diversity of *H. spontaneum* has been extensively examined from the standpoint of population genetics using a range of approaches. Diversity has been assessed using isozyme analyses (Brown *et al.* 1978, Nevo *et al.* 1979a, Nevo *et al.* 1979b, Nevo *et al.* 1986, Jana and Pietrzak 1988), chloroplast DNA (Clegg *et al.* 1984, Holwerda *et al.* 1986), mitochondrial DNA (Holwerda *et al.* 1986), ribosomal DNA (Saghai Maroof *et al.* 1990, Chalmers *et al.* 1992, Ramamoorthy, *et al.* 1994) and RFLP analysis of genomic DNA (Peterson *et al.* 1994). The general conclusion from these studies is that *H. spontaneum* contains significantly more genetic variability than cultivated barley, and much of this variation is correlated with environmental factors suggesting adaptive significance. Therefore

the wild barley, *Hordeum spontaneum*, is an important genetic resource available for exploitation in barley breeding.

Analysis of the *H. spontaneum* gene pool has yielded genes conferring resistance to powdery mildew (Jahoor and Fischbeck 1987), leaf rust (Feuerstein *et al.* 1990) and leaf scald (Abbott *et al.* 1992). Wild barley has also been used for protein improvement in feed barley (Corke and Atsmon 1990, Corke 1992). The broad aim of this investigation is to screen this natural variation to identify alternative alleles to the key malt quality enzymes, and to determine if these alleles confer advantages in malting quality that could be exploited in barley breeding.

## 1.6 Conclusions

The malting and brewing processes rely heavily on the hydrolytic enzymes of barley. Increasing the effectiveness of the key malt enzymes would provide a significant advantage in developing new barley varieties to meet malt quality specifications. The biochemistry of the key barley proteins was considered in examining the potential opportunities for improving malt quality by exploiting the natural variation present in the cultivated and wild barley genepools. These opportunities are as follows:

- Improvements in the thermostability or level of activity of ß-glucanase could lead to more rapid modification during malting and increased filtration efficiency during brewing.
- The physiological function of protein Z has not been determined, but its presence in high levels in beer and its association with beer quality make it a significant protein. Identification of genetic variation within this character may provide insights into the role of this protein and opportunities to improve beer quality.

- Proteases are of obvious importance in the degradation of the endosperm, however more fundamental information on the specific enzymes involved and their direct impacts on malt quality are required.
- Increasing the levels of α-amylase activity in malt may not lead to significant improvements in malt quality because this enzyme is not typically rate limiting in the hydrolysis of starch. However, brewing systems using very high levels of starch adjuncts may benefit from increased α-amylase activity.
- Improving the effectiveness of ß-amylase during mashing may lead to significant improvements in the hydrolysis of starch, particularly in brewing systems using unmalted cereal adjunct.
- Increasing the level of limit dextrinase activity could potentially yield improvements to malt quality, although this may be better achieved by decreasing the amount of the LD inhibitor. More work is required to determine the significance of the limit dextrinase inhibitor under mashing conditions.
- The importance of α-glucosidase during malting and mashing needs clarification before it can become of practical interest in barley breeding.

*Hordeum spontaneum* is a source of genetic variation that can be exploited in barley breeding for malt quality. This thesis examines the opportunities for achieving this by screening an extensive collection of both domestic and wild barleys for variation in ß-glucanase, protein Z, and ß-amylase. In addition to identifying genetic variation for protein Z, the genetics of protein Z is investigated, facilitating the molecular mapping of the protein $Z_7$ locus. The basic biochemical properties of the variant forms of the protein are characterised.

The genetic variation for ß-amylase is mapped to the *Bmy*1 locus on chromosome 4H, demonstrating that the observed variation arises from alternative alleles at this locus, and not post-translational modification of the protein. The enzyme activity and thermostability of the variant ß-amylase alleles are examined using a combination of $F_3$ pooling and protein purification. The effect of alternative ß-amylase alleles on the key economic quality parameter, wort fermentability, is determined. A subset of the ß-amylase enzymes are subjected to detailed biochemical characterisation employing a range of approaches including protein purification, peptide mapping, mass spectrometry and protein sequencing. A range of marker systems are developed and validated for the selection of specific ß-amylase alleles, and marker assisted selection is applied to introgress a novel thermostable ß-amylase from wild barley into elite breeding germplasm. The success of this approach is used to discuss broader opportunities to utilise wild relatives for plant breeding and crop improvement.

# Chapter 2: Genetic Variation in Barley Serpins

## 2.1 Introduction

Barley serpins Z4 and Z7 (BSZ4 and BSZ7, previously described as protein Z4 and protein Z7) are members of the serine proteinase inhibitor superfamily. Serpins are sacrificial inhibitors that bind the target proteinase enzyme resulting in the non-dissociating cleavage of the site reactive loop (Bjork *et al.* 1982, Dahl *et al.* 1996). The physiological role of serpins in mature and germinating barley is not well defined, and the high levels present in beer haze (Hejgaard and Kaersgaard 1983) and beer foam (Hejgaard 1977, Evans and Sheehan 2002) has resulted in significant interest in terms of both plant physiology and the brewing industry.

BSZ4 is encoded by a multigene locus designated *Paz1* on the short arm of chromosome 4H (Nielsen *et al.*. 1983, Rasmussen *et al.*. 1984, Evans *et al.* 1994), and BSZ7 has been shown to be encoded on chromosome 5H by immunological analysis of wheat/barley addition lines (Hejgaard 1984). A gene for a third serpin, protein Zx, has been isolated and sequenced however its expression has not been detected (Rasmussen 1993).

BSZ4 exhibits charge heterogeneity resulting in complex band patterns on IEF gels, and three discrete band patterns have been detected in grain extracts from *Hordeum vulgare* (Evans *et al.* 1995). The typical serpin IEF pattern consists of three major bands with isoelectric points of 4.95, 5.2 and 5.4, interspersed with a series of minor bands. The first variant originates from the *Lys 3a* mutant Riso1508, which appears similar to the standard IEF band pattern, with three bands showing enhanced intensity. The second variant band pattern was identified in the variety Grimmett and is characterised by the absence of the most acidic band. Additional variation in IEF band pattern was reported in the variety Tallon, however this was found to result from modification of serpins during or after the extraction

process. The Finnish barley variety Pirrka contains no immunologically detectable BSZ4 (Hejgaard 1982), and is a putative *Paz*1 null (Brandt *et al.*. 1990). Allelic variation for BSZ7 has not been reported, however quantitative differences in the level of BSZ7 in mature barley grain have been identified in a survey of barley varieties (Evans and Hejgaard 1999a).

Genetic variation for barley serpins provides a useful tool to examine the function of these proteins in barley, and their impact on malt and beer quality. The *Paz*1 null phenotype has been used to investigate the association of bound ß-amylase with BSZ4 (Evans *et al.* 1994) and the potential role of BSZ4 in beer foam stability (Gibson *et al.* 1996, Evans *et al.* 1999b). The wild barley *Hordeum vulgare* ssp. *spontaneum* has been shown to comprise significantly higher genetic diversity than cultivated barley (Nevo 1991, Brown 1992). Within the current study, the level of serpin polymorphism within the wild barley *H. spontaneum* is determined, and the genetic basis for this variation, and the basic biochemical characteristics of the alternative forms of BSZ4 and BSZ7 are assessed. These analyses are expected to identify novel genetic variation for barley serpins, and this may subsequently provide insight into the physiological role of these proteins in germinating barley.

## 2.2 Materials and Methods

### 2.2.1 Plant material

A total of 155 accessions of *Hordeum vulgare* ssp *spontaneum* were provided by Dr Tony Brown, CSIRO Division of Plant Industry, Canberra. The material included 19 accessions collected from Iran, 11 accessions from Turkey, one sample from Morocco, and 124 accessions from 24 sites in Israel, for which the specific geographic locations and climatic conditions have been described (Nevo *et al.* 1979). Passport details of individual accessions and their respective *Bmy*1 genotypes are listed in Appendix I.

*H. spontaneum* accessions were grown and crossed in glasshouses and the progeny grown in controlled environment rooms from 1994 to 1996, all material was manually threshed and grain stored sealed at 4°C. Reference lines of *H. vulgare* comprising the varieties Clipper, Schooner, Sloop, Alexis, Franklin, Pirrka and Harrington, were obtained from the South Australian Barley Improvement Program.

### 2.2.2 Isoelectric focusing (IEF)

IEF was performed on an LKB Multiphor II electrophoresis system using ultrathin (0.4 mm) polyacrylamide gels containing 5.5% (v/v) carrier ampholytes (Pharmacia, Uppsala, Sweden) cast onto Gelbond PAG support (FMC Products, Rockland, Maine, U.S.A.). Individual components of the IEF gel were; 1.8 mL glycerol, 3.3 mL 30% acrylamide, 0.33 mL pH 4-6.5 ampholytes, 0.66 mL pH 4.5-5.4 ampholytes, 25 μL TEMED, 50 μL 10% ammonium persulphate, 11.8 mL distilled water. Prior to addition of the catalysts the solution was degassed under vacuum in a sonicator bath. The catholyte was 0.1M NaOH and the anolyte was 0.04 M glutamic acid, and electrode wicks were soaked in buffer and blotted free of excess solution prior to application. Light paraffin oil was used to provide contact between the gel and the cooling plate. IEF was performed at 10°C with prefocusing for 20 min at 5 W constant power. Separation was performed for 3500 volt hours at 10 W constant power with upper limits of 2200 V and 50 mA.

Extracts were prepared from 10 mg barley flour or half grains extracted with 200μL 1% glycine and 143 mM 2-mercaptoethanol, then diluted 1:2 in 1% glycine and 5μL loaded at the cathode. Following IEF, proteins were transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany) by passive transfer. Apparent pI values were estimated relative to native IEF markers (Bio-Rad, Richmond California).

### 2.2.3 SDS-PAGE

Vertical slab SDS-PAGE was performed using 1 mm separating gel (T 12.5 %, C 2.7 %) with stacking gel (T 5 %, C 2.7 %). The sample buffer used for extraction and dilution consisted of 0.05 M Tris-HCl pH 6.8, 4 % SDS, 5 M urea and 143 mM ß-mercaptoethanol. Separated proteins were transferred to nitrocellulose membranes (Sartorius, Göttingen, Germany) by electroblotting. The immunoblot was developed as described in section 2.2.4. Apparent molecular masses were estimated relative to pre-stained markers (Novex, San Diego, California).

### 2.2.4 Immunoblotting

Immunoblots were developed as described in Chapter 2 using a polyclonal antibody specific for barley BSZ4 (Evans and Hejgaard 1999a) diluted 1 in 2000 and detected with horse radish peroxidase (HRP) labelled goat anti-rabbit antibodies (Bio-Rad) diluted 1 in 5000, using 4-chloro-1-napthol (Bio-Rad) as the substrate. The anti-BSZ4 antibody exhibits some cross reactivity with BSZ7 in immunoblotting (Evans and Hejgaard 1999a).

### 2.2.5 Genetic Analysis

A population of 91 doubled haploids (DH) derived from the $F_1$ of a cross between cv. Alexis and cv. Sloop (WI2875-22) and a population of 114 recombinant inbred lines (RIL) from a cv. Alexis X WI2875-1 cross were provided by the National Barley Molecular Marker Program. The two WI-2875 selections exhibit the type A band pattern and cv. Alexis exhibits the type B pattern (Figure 3.3). The progeny from the two crosses were screened for polymorphism in the acidic serpin bands by IEF and immunoblotting. Data from the mapping populations was applied to a linkage map using MapManager QT software (Manley and Elliot 1993) utilising the Kosambi map unit function (Kosambi 1944).

*H. spontaneum* accessions exhibiting variant serpin IEF band patterns were crossed with *H. vulgare* cv. Clipper, which carries the type A band pattern. The *H. spontaneum* X *H. vulgare* hybrids were selfed and segregation analysis performed by IEF of grain extracts prepared from single $F_2$ grains from each cross. Segregation ratios were tested for conformity with Mendelian expectations for single loci using chi-square tests.

## 2.3 Results

### 2.3.1 Variation in barley serpins

Polymorphism for barley serpins was determined in extracts of mature grain from 154 accessions of the wild barley *H. spontaneum* by IEF and immunoblotting. A total of seven alternative serpin band patterns were identified and are described as type A, B, C, D, E, F and G, as shown in Figure 2.1. Each IEF band pattern contains numerous minor bands. The type A band pattern consists of three major bands with approximate pI values of 4.95, 5.2 and 5.4, and corresponds to the typical pattern observed in cultivated barley. The type B band pattern exhibits similar bands with pI values of 5.2 and 5.5, but is characterised by the absence of the acidic band at pH 4.95 and the presence of a minor double band at pH 5.0. This is consistent with the variant band pattern reported in the barley variety Grimmett (Evans *et al.* 1995). An additional band of pI 4.6 was also observed in some samples, however this appears to result from modification of serpins during the extraction process because it could be induced by prolonged extraction (data not shown). The type B banding pattern was also observed in the barley variety Alexis (Fig 2.3). The variant band pattern described as type C also includes major bands at pH 5.2 and 5.5, but in addition it contains an acidic band at pH 5.05. The type D band pattern consists of three major bands at pH 5.05, 5.6 and 5.8. The type E band pattern exhibits at least four major bands including a doublet at pH 5.5 and bands at pH 5.6, 5.7 and

6.0, and a minor acidic band at pH 4.7. The type F band pattern is characterised by three major bands with pI values of 5.5, 5.7 and 5.8. The type G band pattern consists of three bands with pI values of approximately 5.85, 6.0 and 6.1. None of the seven alternative band patterns correspond to the pattern reported for the barley line Riso 1508. The band pattern of each *H. spontaneum* accession analysed is listed in Appendix I.

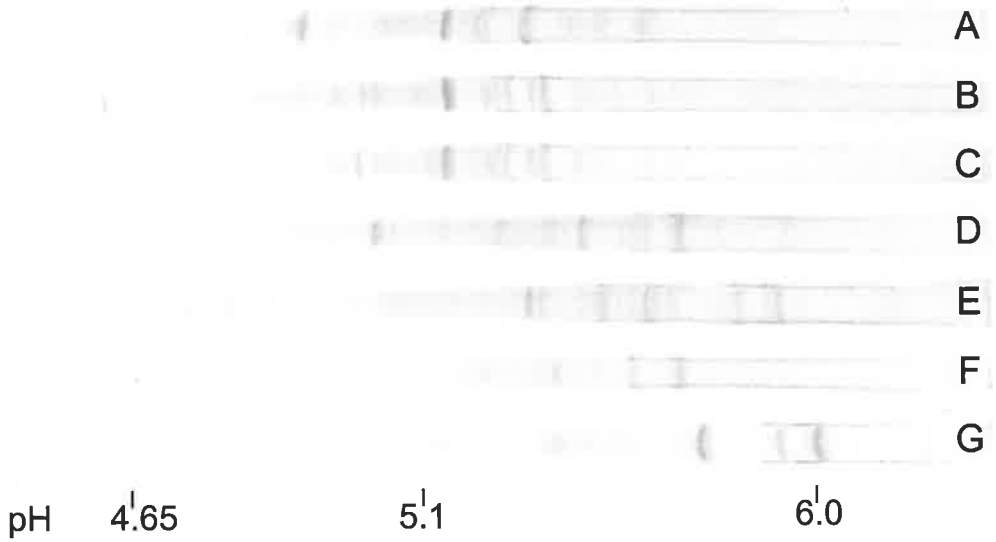



**Figure 2.1:** Immunoblot of barley extracts separated by IEF and probed with anti-BSZ4 antibodies. The seven alternative band patterns are labelled A through to G.

### 2.3.2 Variation in apparent $M_r$ for the alternative forms of barley serpins

Analysis of the seven alternative serpins for variation in apparent molecular mass by SDS-PAGE and immunoblotting of barley grain extracts is shown in Figure 2.2. Each variant exhibits a major band of approximately 48 kDa corresponding to the uncleaved BSZ4. This compares to the molecular masses determined for *in vitro* translation products of 44 - 46 kDa (Rasmussen *et al.* 1984), and of 43128 Da deduced from a genomic *Paz*1 clone (Brandt *et al.* 1990). A second band of approximately 46 kDa corresponding to the cleaved form of BSZ4

(Evans and Hejgaard 1999) is also present in Figure 2.2. The cleaved serpins exhibit polymorphism, with this band not present in the type D variant, and exhibiting an increased apparent molecular mass in the type E variant.

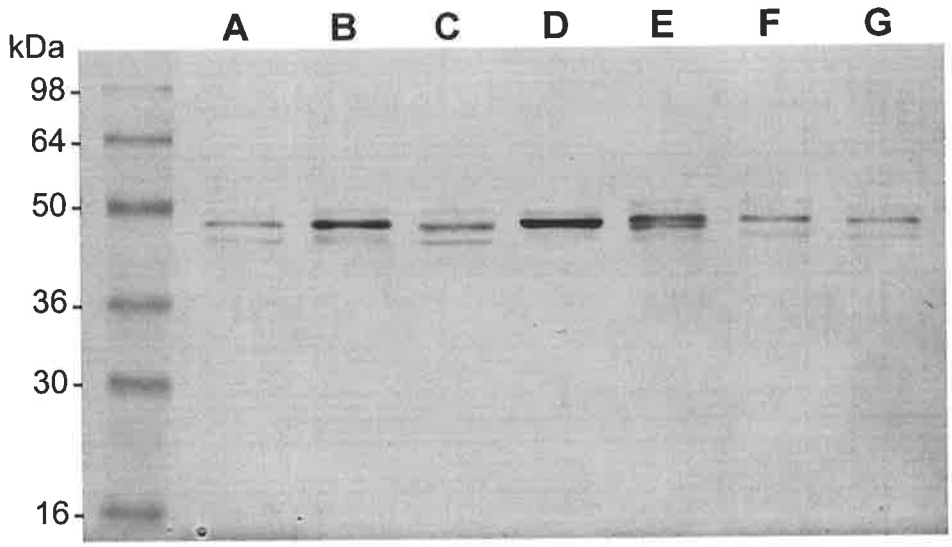



**Figure 2.2:** Immunoblot of grain extracts separated by SDS-PAGE and probed with anti-BSZ4 antibodies. The seven alternative band patterns detected by IEF are labelled A through to G.

### 2.3.3 Molecular mapping of the low pI serpin bands

The polymorphism in the low pI serpin bands was mapped in two populations of 91 doubled haploids and 114 recombinant inbred lines segregating for the type A and type B band patterns (Figure 2.3). The segregation of individuals was 43 type A: 48 B in the DH population, and 65 type A: 49 type B in the RIL population. The $X^2$ values ($X^2$=0.27 and 2.25, respectively) indicate the segregation fits the expected 1:1 ratio for a single gene encoding the acidic serpin.

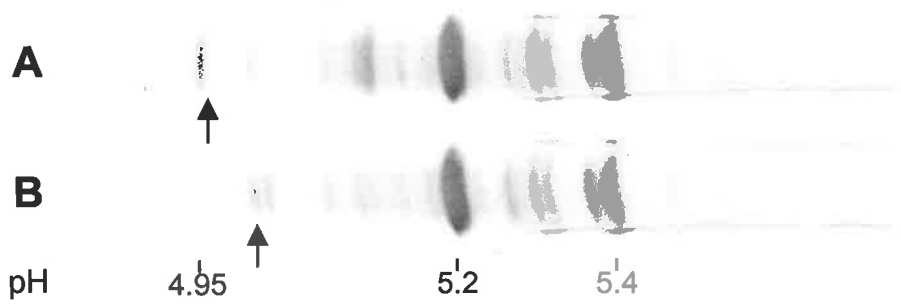
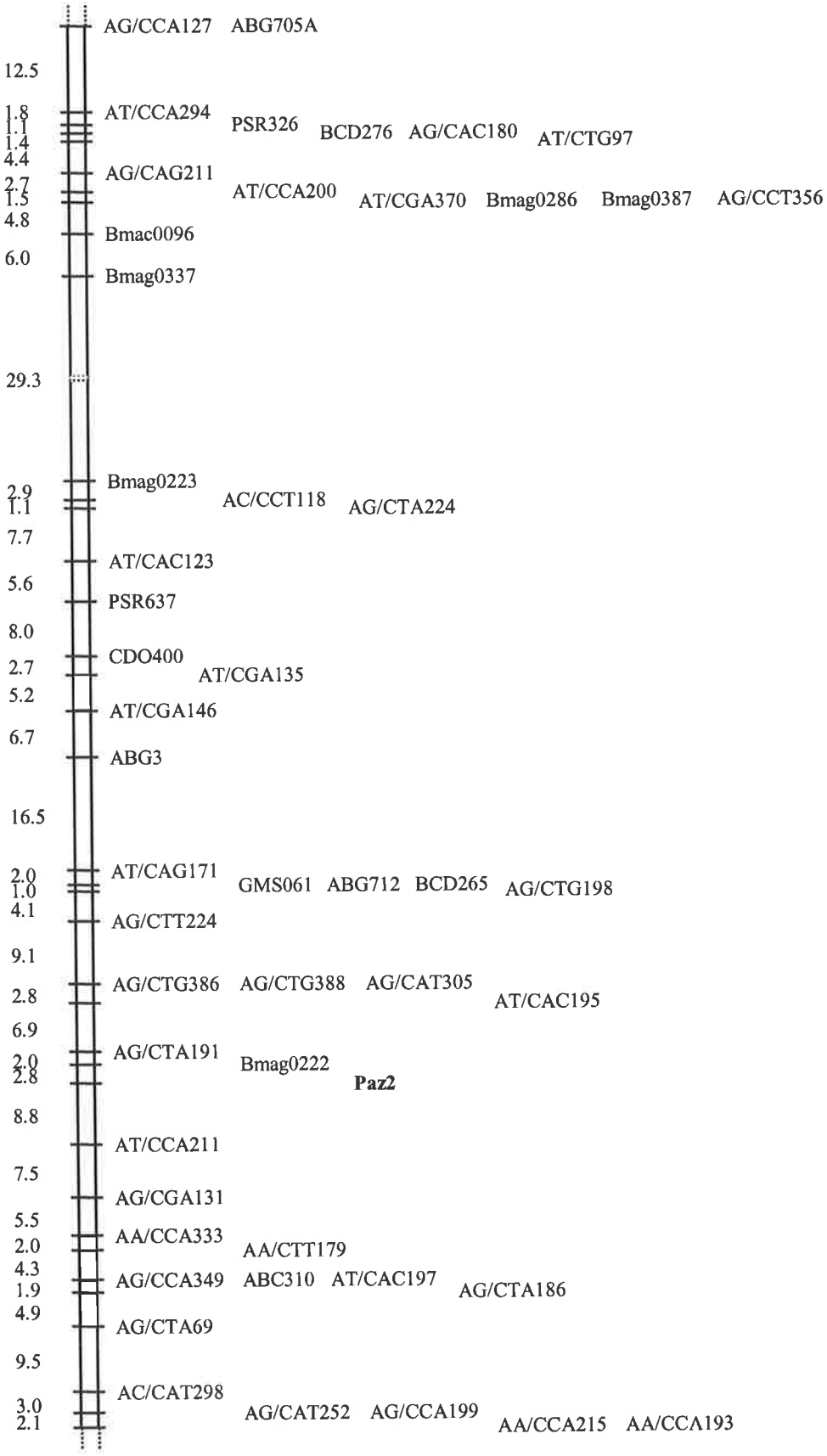



**Figure 2.3**: Immunoblot of barley grain extracts separated by IEF from varieties Sloop (type A) and Alexis (type B) used as parents for mapping the acidic serpin bands. Arrows denote the polymorphic bands.

The variation in the acidic serpin band was mapped to the long arm of chromosome 5H in the doubled haploid population, closely linked to the microsatellite Bmag0222 (2.8 cM) and flanked on the distal side by the AFLP AT/CCA211 (9.0 cM) (Figure 2.4). The relative position and map distances were conserved in the RIL population (not shown).

The gene encoding BSZ7 has been localized to chromosome 5H by immunological analysis of wheat/barley addition lines (Hejgaard 1984), and the isoelectric points of the polymorphic serpin bands are consistent with values obtained for purified BSZ7 (Evans and Hejgaard 1999). The locus identified in the present study will be referred to as *Paz*2, putatively encoding BSZ7.

**Figure 2.4**: The map location of the *Paz*2 locus on chromosome 5H (P = 0.001).



### 2.3.4 Genetic basis for the alternative serpin band patterns in *H. spontaneum*

Serpins exhibit multiple molecular forms due to the formation complexes with proteinases and the release of the cleaved serpin (Dahl *et al.* 1996). BSZ4 has also been reported to form dimers and/or heterodimers (Hejgaard and Carlsen 1977) which would also result in complex charge heterogeneity. The variation in IEF band patterns from *H. spontaneum* shown in Figure 2.1 may therefore be the result of either differences in post-translational modifications or discrete *Paz*1 or *Paz*2 alleles. The genetic basis of the alternative serpin band patterns was determined by segregation analysis of $F_2$ populations segregating for the type A band pattern from *H. vulgare* cv. Clipper and each of the type D,E,F and G band patterns from *H. spontaneum*. Segregation analysis was not performed on the type C band pattern. The type B and C band patterns exhibit variation in BSZ7, but are monomorphic for BSZ4. The detailed examination of the genetic relationships between the band patterns follows below.

#### 3.3.4.1 Segregation analysis of type A and type D serpin band patterns

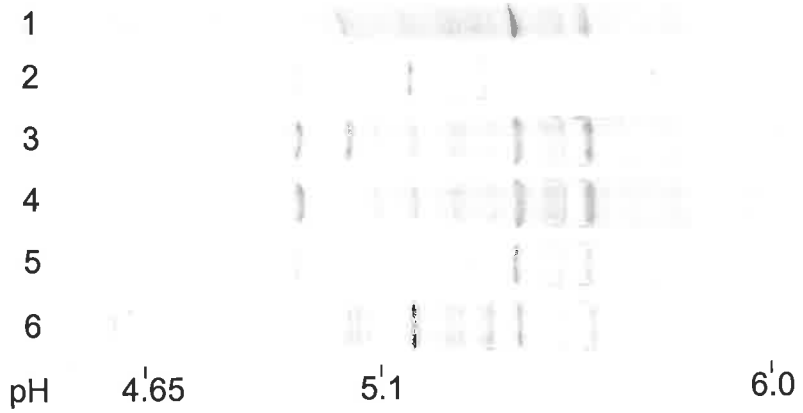



**Figure 2.5:** IEF immunoblot based segregation analysis of the type A and type D serpin band patterns in a Clipper X *H. spontaneum* CPI 77147/5 cross. Lane 1: type D parent, 2: type A parent, 3: $F_1$ progeny, 4-6: three additional phenotypes observed in the $F_2$ progeny.

Segregation analysis of the type A and D serpins was performed by IEF and immunoblotting analysis of the $F_2$ progeny of a Clipper X *H. spontaneum* (CPI 77147/5) cross. The serpin band patterns of the parental lines and representative progeny are shown in Figure 2.5. The $F_1$ progeny exhibit a combination of all the bands present in the parental lines. The acidic BSZ7 bands at pI 4.95 in type A and 5.05 in type D segregate independently of the BSZ4 bands, as demonstrated by the BSZ7 band from type A present both with the type A BSZ4 pattern (lane 2) and the type D pattern (lane 5). The BSZ7 band from type D is present in heterozygous individuals (lane 3), but was not observed in homozygous form in the $F_2$ progeny. An additional band pattern was detected in the $F_2$ progeny (lane 6), which is heterozygous for the major BSZ4 bands but exhibits novel acidic bands with pI values of 4.6 and approximately 5.05. Although the acidic BSZ4 bands exhibit independent assortment, they do not segregate according to Mendelian expectations. The observed frequency was 30 type A: 14 heterozygotes: 7 $F_2$ progeny exhibiting the novel acidic bands.

The BSZ4 bands of pI 5.2 and 5.4 for type A and pI 5.6 and 5.8 for type D segregate as discrete units. The observed frequency was 15 type A: 50 heterozygotes: 16 type D progeny, consistent with the action of two co-dominant alleles ($X^2 = 4.49$, $p = 0.09$).

### 2.3.4.2 Segregation analysis of type A and type E serpin band patterns

Segregation analysis of the type A and E band patterns was performed in the $F_2$ progeny of a Clipper X *H. spontaneum* (CPI 77146/32) cross by IEF and immunoblotting. The serpin band patterns of the parental lines and representative progeny are shown in Figure 2.6. The $F_1$ progeny exhibit a combination of all the bands present in the parental lines (Figure 2.6, lane 3).

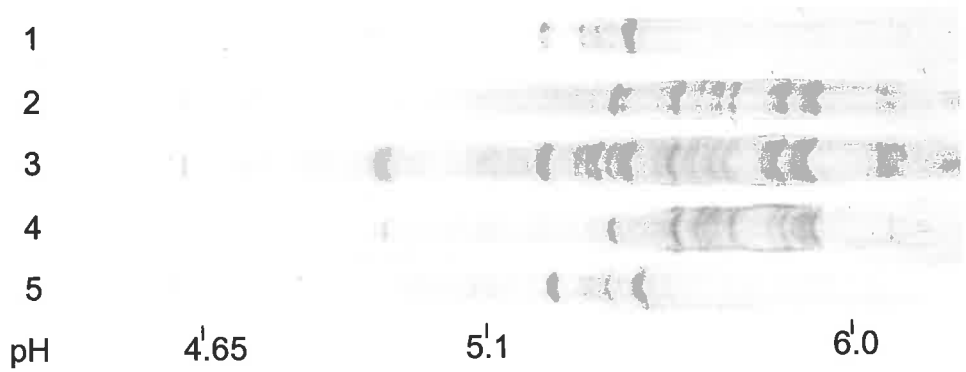



**Figure 2.6:** IEF immunoblot based segregation analysis of the type A and type E serpin band patterns in a Clipper X *H. spontaneum* CPI 77146/32 cross. Lane 1: type A parent, 2: type E parent, 3: $F_1$ progeny, 4 and 5: two additional phenotypes observed in the $F_2$ progeny.

The BSZ7 bands at pI 4.95 in type A and pI 4.6 in type E segregate independently of the BSZ4 bands. An individual with the type A acidic band and the type E BSZ4 bands is shown in lane 4 of Figure 2.5, and the reciprocal is shown in lane 5. $F_2$ progeny heterozygous for the BSZ7 bands were also identified, demonstrating codominant expression of the type A and type E forms of BSZ7.

The BSZ4 bands at pI 5.2 and 5.4 for type A and pI 5.5, 5.6, 5.7 and 5,8 for type E segregate as discrete units. The observed ratio in the $F_2$ generation was 20 type A: 31 heterozygotes: 23 type E progeny, consistent with the action of two co-dominant alleles ($X^2$ = 1.2 , p = 0.53).

### 2.3.4.3 Segregation analysis of type A and type F serpin band patterns

Segregation analysis of the type A and type F serpins was performed by IEF and immunoblotting analysis. The $F_2$ populations from two Clipper X *H. spontaneum* crosses using the lines CPI 77143/19 and CPI 77135/10 were analysed. Both wild barley lines exhibit the type F band pattern. The serpin band patterns of the parental lines and $F_1$ progeny are

shown in Figure 2.7. The $F_1$ progeny exhibit a combination of all bands present in the two parental lines.

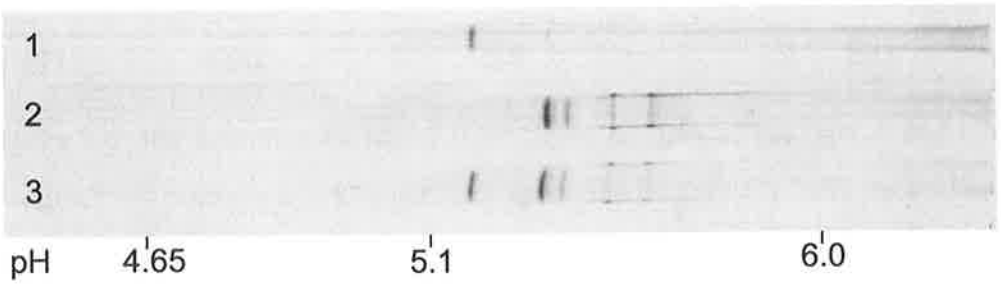



**Figure 2.7:** IEF immunoblot based segregation analysis of the type A and type F BSZ4 band patterns observed in two Clipper X *H. spontaneum* crosses. Lane 1: type A parent, 2: type F parent, 3: $F_1$ progeny.

All bands from the type F band pattern segregate as a discrete unit, therefore this phentoype does not appear to have a band complementary to the acidic type A band. The acidic band from the type A BSZ4 was faintly stained and difficult to score in these two populations, and therefore segregation ratios are not presented. The major bands in the two forms of BSZ4 segregate as discrete units, and the type A and F forms of BSZ4 exhibit codominance, with heterozygote progeny containing a combination of both parental types. The $F_2$ progeny significantly depart from the expected ratio (1:2:1) for single locus segregation in both crosses, with a combined ratio of 13 type A: 26 heterozygotes: 27 type F progeny ($X^2 = 6.98$, $p = 0.03$).

### 2.3.4.4 Segregation analysis of type A and type G serpin band patterns

Segregation analysis of the type A and G band patterns was performed in the $F_2$ progeny of a Clipper X *H. spontaneum* (CPI 77129/28) cross by IEF and immunoblotting as shown in Figure 2.8. The $F_1$ progeny exhibit a combination of all the bands observed in the parental lines (lane 3). The BSZ7 bands from the type A pattern segregate independently of the type A BSZ4 bands, with $F_2$ progeny exhibiting the type G BSZ4 bands in addition to the BSZ7 bands from the type A pattern shown in Figure 3.7 (lane 4).

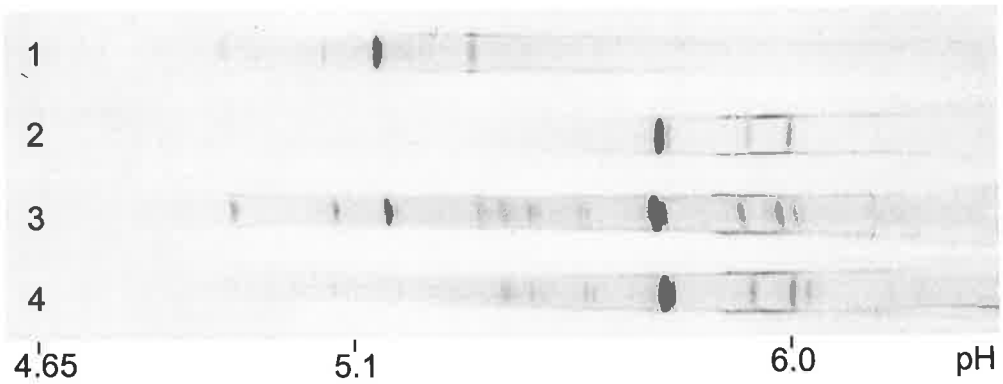



**Figure 2.8:** IEF immunoblot based segregation analysis of the type A and type G serpin band patterns in a Clipper X *H. spontaneum* CPI 77129/28 cross. Lane 1: type A parent, 2: type G parent, 3: $F_1$ progeny, 4: $F_2$ progeny exhibiting the major BSZ4 bands from the type G parent and the acidic bands from the type A parent.

The type G band pattern does not exhibit bands homologous to BSZ7 in the type A band pattern. The BSZ4 bands at pI 5.2 and 5.4 for type A and pI 5.85, 6.0 and 6.1 for type G segregate as discrete units. The observed ratio in the $F_2$ generation was 7 type A: 17 heterozygotes: 11 type G progeny ($X^2 = 0.41$ , $p = 0.8$), consistent with the action of two co-dominant alleles.

### 2.3.5 Eco-geographical Distribution of Serpin Variation

Studies of natural populations of *H. spontaneum* have suggested significant correlations between genetic diversity and environmental parameters (Nevo *et al.* 1979, Chalmers *et al.* 1992). Detailed measures of genetic variability and geographic associations have been used to suggest adaptive significance of genetic variation in wild populations (Nevo *et al.* 1986). While the material used in this study does not comprise complete populations, it does permit analysis of the eco-geographic distribution of the observed variation in BSZ4.

The relative frequency of each serpin band pattern is shown with respect to the original source of the *H. spontaneum* accessions in Table 3.1. The alternative band patterns range in frequency from 43.9% for type C, to single accessions exhibiting the type D and G patterns. The type A and B patterns found within *H. vulgare* were observed in only 32.9% of the *H. spontaneum* accessions.

The level of variation in barley serpins exhibits sharp differentiation between the five sources of *H. spontaneum*. The material originating from Iran, Turkey and Morocco was restricted to the type A, B, and C band patterns, whereas the accessions from Israel were significantly more diverse. All seven alternative band patterns were observed in the material from Israel, but they were not evenly distributed across environments. In particular the relatively common type F pattern was not observed in accessions from the Negev Desert, and the type B pattern did not occur in material from the Jordan Valley.

**Table 3.1:** Eco-geographic distribution of barley serpin variation in 154 accessions of *Hordeum vulgare spp spontaneum*.

| Band Pattern | IEF band pattern frequency | | | | | |
|---|---|---|---|---|---|---|
| | Israel | | | | Iran, Turkey & Morocco | Total |
| | Mountains | Jordan Valley | Coastal | Desert | | |
| A | | | 0.080 | 0.033 | 0.065 | 0.032 |
| B | 0.370 | | 0.280 | 0.667 | 0.065 | 0.297 |
| C | 0.391 | 0.739 | 0.040 | 0.167 | 0.870 | 0.439 |
| D | 0.022 | | | 0.100 | | 0.026 |
| E | | | | 0.033 | | 0.006 |
| F | 0.217 | 0.261 | 0.560 | | | 0.194 |
| G | | | 0.040 | | | 0.006 |
| | n = 46 | n = 23 | n = 25 | n = 30 | n = 31 | n = 155 |
| $H_e$ | 0.663 | 0.386 | 0.598 | 0.515 | 0.235 | 0.680 |
| $n_e$ | 3.0 | 1.6 | 2.5 | 2.1 | 1.3 | 3.1 |
| $n_a$ | 4 | 2 | 5 | 5 | 3 | 7 |

$H_e$ = probability that two random gametes are dissimilar.
$N_e = (1- H_e)^{-1}$ = effective number of alleles.
$N_a$ = actual number of phenotypes.

The genetic variation in barley serpins is summarised in Table 3.1 in terms of three diversity statistics. The statistic $H_e$ is Nei's genic diversity, and is calculated as one minus the sum of squares of the frequency of each band pattern. The "effective number of alleles", $N_e$, describes the number of equally frequent band patterns required to yield the observed value for $H_e$. The actual number of observed phenotypes is denoted $N_a$. A comparison of these three measures of genetic diversity across the five environments shows *H. spontaneum* accessions from the Coastal and Mountain regions of Israel exhibit the highest levels of genetic diversity for serpins, followed by the Negev Desert and then the Jordan Valley regions. The composite

collection of wild barley from Iran, Turkey and Morocco displayed the lowest level of variation in barley serpins, despite the wide range of original environments.

## 3.4 Discussion

Analysis of 154 accessions of the wild barley *Hordeum vulgare* ssp. *spontaneum* revealed seven different serpin band patterns, two of which (types A and B) were also observed in cultivated barley. Each barley serpin exhibits an apparent molecular mass of 48 kDa, however some polymorphism was observed in the cleaved form. The type E variant exhibited a band presumed to be the cleaved BSZ4 of 47.6 kDa and the cleaved form was not observed in the type D variant. The remaining five BSZ4 variants exhibited a cleaved form of 46 kDa. This evidence suggests the seven alternative forms of BSZ4 do not result from major structural differences, but are more likely the result of amino acid substitutions or small deletion/insertion events that are readily resolved by analytical IEF but not SDS-PAGE. The molecular masses estimated by SDS-PAGE are slightly higher than those of 44-46 kDa determined for *in vitro* translation products (Rasmussen *et al.* 1984), and 43.1 kDa deduced from a genomic *Paz*1 clone (Brandt *et al.* 1990).

The population genetic structure of *H. spontaneum* is characterised by high variability, sharp genetic differentiation over short geographical distances, and a relatively high proportion of unique alleles (Nevo *et al.* 1986). The polymorphism observed in barley serpins is consistent with this general structure of *H. spontaneum* populations, exhibiting a high level of genetic diversity ($H_e$ = 0.68), and two of the seven phenotypes were restricted to single ecogeographical regions. In addition, the relatively common type B phenotype (29.7%) was not observed in accessions from the Jordan Valley, and the type F phenotype (19.4%) was not

observed in individuals from the Negev Desert of Israel or the composite collection from Iran, Turkey and Morocco.

The level of genetic diversity within populations of *H. spontaneum* from Israel has been shown to be significantly higher than for populations from Iran and Turkey, and this has been linked to the increased ecological diversity in the range of wild barley in Israel (Nevo *et al.* 1986). The ecogeographical distribution of BSZ4 variation is generally consistent with the spatial pattern of distribution described for *H. spontaneum*, with material from Israel exhibiting much higher diversity ($H_e = 0.704$) than material from Iran, Turkey and Morocco ($H_e = 0.235$).

The type A and type B serpin band patterns differ only in the isoelectric point of the most acidic bands. The genetic basis of this polymorphism was mapped and shown to be a locus on the long arm of chromosome 5H, and not associated with the BSZ4 gene on chromosome 4H. A gene encoding BSZ7 has previously been localized to chromosome 5H by immunological analysis of wheat/barley addition lines (Hejgaard 1984), and the isoelectric points of the polymorphic serpin bands are consistent with values obtained for purified BSZ7 (Evans and Hejgaard 1999). It is therefore concluded that the anti-BSZ4 antibodies exhibit some cross-reactivity with BSZ7 under the conditions used in this study, consistent with a previously reported cross-reaction (Evans and Hejgaard 1999). The locus on the long arm of chromosome 5H is the first reported map position for the *Paz*2 locus encoding BSZ7. The two forms of barley serpins are known to share a high degree of immunological identity, since BSZ7 was initially identified through antibody cross-reactivity (Hejgaard 1984).

Five alternative forms of BSZ7 were detected in *H. spontaneum*. The allelic forms of the protein exhibited pI values of 4.95 in the type A, close to pI 5.0 in the type B, C and D forms of BSZ4, and at pI 4.7 in the type E form. The accessions exhibiting type F and G barley serpins did not exhibit BSZ7 bands, however they cannot be considered null for the

acidic barley serpin. Further analysis of these genotypes with specific anti-BSZ7 antibodies is required to determine whether they contain null *Paz*2 alleles. Segregation analysis of populations polymorphic for BSZ7 demonstrated codominant expression of the protein, however significant departures from Mendelian expectations were also observed. Novel putative BSZ7 bands were detected in one population segregating for type A and D band patterns, however the results were not consistent with the protein adopting a dimeric structure.

The type A, B and C band patterns only differed in their complement of acidic BSZ7 bands, therefore these three band patterns represent the same *Paz*1 allele. The IEF band patterns described as types D, E and G were shown to result from alternative *Paz*1 alleles exhibiting co-dominant expression, and no evidence of BSZ4 adopting a dimeric structure was observed. The type F BSZ4 was also shown to exhibit co-dominant expression, however a significant departure from Mendelian expectations for a single locus trait was observed in two separate segregating populations. Distorted segregation ratios are often observed in progeny of both inter- and intraspecific hybrids, and may be due to transmission ratio distortion. This may either result from gametophytic selection through pollen competition (Mulcahy and Mulcahy 1983) or from the selective loss of some genotypes through abortion of the gamete or zygote (Faris *et al.* 1998), rather than the involvement of independent loci. Segregation analysis of the type F band pattern in alternative crosses and reciprocal crosses, or the generation of an appropriate mapping population, are possible methods of resolving the genetic basis for the type F BSZ4 band pattern that was detected in 19.4% of the *H. spontaneum* accessions analysed.

There is currently no method for measuring the activity of endogenous barley serpins, and the application of ELISA to accurately quantitate serpin levels would require each alternative form of BSZ4 and BSZ7 to be purified to standardise the immunoaffinity of the

antibodies. Therefore no attempt has been made to associate the variant serpins with quantitative differences or functional changes.

Mammalian serpins are involved in the regulation of complex physiological processes such as blood coagulation and fibrinolysis, and typically inhibit specific endogenous proteinases (Schulze *et al.* 1994). The role of serpins in barley is not well defined, although BSZ4 is a quantitatively significant protein, accounting for up to 5% of the albumen fraction in mature barley (Hejgaard 1982). The serpins are also important in the industrial processing of barley, being present in significant levels in beer and beer foam (Hejgaard 1982). Furthermore, the level of BSZ4 is negatively correlated with Kolbach Index (Evans *et al.* 1999) suggesting a potential role for serpins in modulating the mobilisation of endosperm components during germination. As such, variation in BSZ4 may potentially have significantly influence on malt quality. Exploiting the genetic variation in BSZ4 identified in this study may provide a useful approach to further examine the physiological function of serpins in barley, and the impact of these proteins in malt and beer quality. The development of isogenic lines containing alternative *Paz*1 or *Paz*2 alleles would be a significant tool in further barley serpin research.

# Chapter 3: Identification and Characterisation of Allelic Variation for ß-amylase within *Hordeum vulgare.*

## 3.1 Introduction

Cultivated barley (*Hordeum vulgare* L.) is restricted to two alternative ß-amylase band patterns, encoded by codominant alleles designated *Bmy*1-Sd1 and *Bmy*1-Sd2 (Allison 1973, Allison and Swanston 1974). The two alternative forms of ß-amylase can be resolved in both the mature grain (Allison and Ellis 1973) and the proteolytically cleaved forms of ß-amylase produced after germination (Allison 1973, Allison and Swanston 1974). In work towards developing varietal identification methods, 66 barley cultivars were examined and two ß-amylase band patterns were identified and referred to as Ar and Br types (Nielsen and Johansen 1986). In a more recent investigation 29 cultivars were analysed using isoelectric focusing (IEF) and four discrete band patterns were designated types A, B, C and D (Thompson *et al.* 1990). The same 29 cultivars were subsequently examined in another report by the same group, however only two band patterns were identified and these were described as *ß-amy-1a* and *ß-amy-1b* (Forster *et al.* 1991). An additional form of ß-amylase has been reported in a number of Algerian barley varieties including cv. Zenit and Asse, and has been designated *Bmy*1A1 (Netsvetaev 1993). In this study the band patterns were analysed only in extracts from germinated barley using native electrophoresis in agarose.

A diverse range of electrophoretic procedures have been used in the seven investigations of ß-amylase polymorphism, and no comparisons were made in any of these studies with the original work of Allison 1973 and Allison and Ellis 1973. Although these early studies attempted to analyse the impact of *Bmy*1 alleles on ß-amylase expression levels, none of the subsequent reports have tested for functional differences between the alternative forms of barley ß-amylase. This study aims to clarify the genetic variation for ß-amylase

present in the *H. vulgare* gene pool, and to provide the first analysis of thermostability variation between the alternative forms of barley ß-amylase.

## 3.2 Materials and Methods

### 3.2.1 Plant Material

The 150 barley varieties analysed for variation in ß-amylase band patterns were either obtained from the Australian Winter Cereals Collection (AWCC, Tamworth NSW), or from the SA Barley Improvement Program in the case of Australian and Japanese barley varieties. Barley varieties used for micromalting and thermostability assays were grown in the 1996/97 season at Weetulta, South Australia.

Micromalting and standard malt quality analyses were performed by the Waite Malting Quality Evaluation Laboratory. Samples of 100 g of the barley cultivars were micromalted in duplicate in an automated micromalting unit (Phoenix Systems, Adelaide, Australia). The malting schedule consisted of steep and air rest 7:8:9:6:0.5 hours (wet:dry:wet:dry:wet) at 15°C, germination for 88.5 hours at 15°C, and kilning at 30-40°C 9 hours, 40-60°C 4 hours, 60-70°C 2 hours and 70-80°C 4.5 hours. Haruna Nijo malt was supplied by Mr Ken Fukuda, Sapporo Breweries Ltd, Japan.

### 3.2.2 Isoelectric Focusing (IEF)

IEF was performed as described in section 2.2.2. Extracts were prepared from 20 mg barley or malt flour extracted for 30 min with 1.0 mL 1 % glycine containing 143 mM 2-mercaptoethanol. Extracts were centrifuged at 10,000g for 5 minutes and 5 µL of the supernatant loaded 3 cm from the anode. Amylase bands were detected by starch staining

(Guerin *et al.* 1992), and apparent pI values were estimated relative to native IEF markers (Bio-Rad).

### 3.2.3 ß-amylase Activity

Seed and malt extracts were prepared by incubating 100 mg samples with 1.0 mL extraction buffer containing 100 mM maleic acid, 1 mM di-sodium EDTA, 0.02% sodium azide and 1mg/mL BSA (Sigma, St Louis USA) for 2 hours at room temperature. Extracts were centrifuged at 10,000g for 10 min and the supernatant retained. Enzyme activity was determined using the substrate *p*-nitrophenyl maltopentaoside (PNPG5, Megazyme, Ireland). One unit of ß-amylase activity is defined as the amount of enzyme required to release one µmol of *p*-nitrophenol per min in the presence of excess α-glucosidase under the defined assay conditions (McLeary and Codd 1989).

### 3.2.4 Thermal Inactivation

Barley and malt extracts were divided into aliquots for initial assay and heat treatments. After heating, samples were chilled on ice and centrifuged at 10,000g for 5 min at 4°C. The rate of enzyme inactivation was monitored both by incubating samples for 5 min at temperatures from 40 to 65°C, and also by incubating samples over a time course at 60°C. Relative thermostability of ß-amylase in malt extracts was determined using a two point assay where initial activity and residual activity after 10 min incubation at 60°C were measured.

### 3.2.5 Genetic Analysis

A population of 95 doubled haploid derived lines from the cross Galleon X Haruna Nijo (generated by Dr S. Logue) were analysed in duplicate for ß-amylase thermostability using the two-point assay.

Data from the mapping population was applied to a linkage map of barley (Langridge *et al.* 1995) using MapManager QT (Version 8.0) software (Manley and Elliot 1993) and the Kosambi mapping function (Kosambi 1944). Associations between molecular markers and QTL for ß-amylase thermostability were tested using interval analysis (Lander and Botstein 1989). A graphical display of the QTL associations was generated using QGENE (Nelson 1997).

## 3.3 Results

### 3.3.1 ß-amylase variation within *H. vulgare*

The variation for ß-amylase within *H. vulgare* was assessed by IEF of barley extracts from 68 cultivars. While analytical IEF has previously been used to investigate polymorphism in ß-amylase, in this study all components of the electrophoresis system were optimised for maximum resolution and reproducibility. Barley ß-amylase exhibits complex charge heterogeneity, however only two distinct band patterns were observed (Figure 3.1), which correspond to the Sd1 and Sd2 forms of ß-amylase originally described by Allison (1973). The Sd1 band pattern consists of bands of pI 6.0, 5.5, 5.2 and a double band of pI 5.0. The Sd2 ß-amylase exhibits a similar band pattern shifted to a more basic pI, with bands of pI 6.2, 5.8, 5.6 and a double band of pI 5.2 (Figure 3.1).

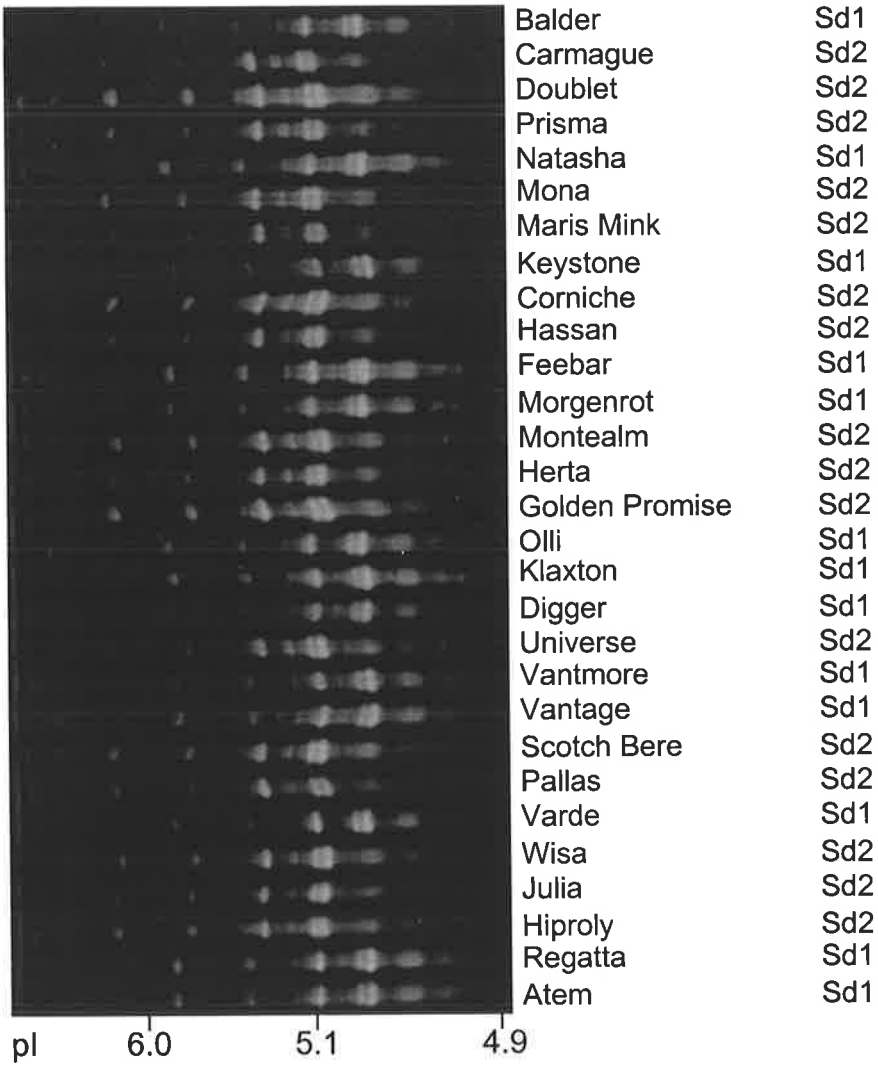



**Figure 3.1:** Isoelectric focusing gel of barley extracts starch stained to show ß-amylase bands. The ß-amylase band pattern of each barley variety is shown.

The assignment of variation for ß-amylase has had at least three different systems of nomenclature, despite barley varieties being distributed similarly regardless of the electrophoretic procedure used (Table 3.1). The original nomenclature used by Allison (1973) is adopted in the current study, with the two forms of ß-amylase described as Sd1 and Sd2. The two forms of the enzyme are encoded by codominant alleles on chromosome 4HL (Allison 1973, Powling *et al.* 1981), and the respective alleles are referred to as *Bmy*1-Sd1

and *Bmy*1-Sd2. Table 3.1 shows the Sd1 IEF band pattern is equivalent to the Sd1 malt pattern (Allison 1973, Allison and Swanston 1974), the Bmy1 Ar band pattern (Nielsen and Johansen 1986) and the *ß-amy-1a* band pattern (Forster *et al.* 1991) with only five conflicts in assignment from 29 varieties analysed. The Sd2 IEF band pattern is equivalent to the Sd2 malt pattern (Allison 1973, Allison and Swanston 1974), the Bmy1 Br band pattern (Nielsen and Johansen 1986) and the *ß-amy-1b* band pattern (Forster *et al.* 1991) with five conflicts in assignment from 39 varieties analysed.

**Table 3.1:** Barley ß-amylase IEF band patterns compared with published reports for ß-amylase variation. * Denotes conflict in assignment between IEF banding pattern and those published, - denotes not determined.

| Variety | IEF band pattern | Allison (1973) and Allison & Swanston (1974) | Nielsen & Johansen (1986) | Forster *et al.* (1991) |
|---|---|---|---|---|
| Source | Barley | Malt | Barley | Barley |
| Alexis | Sd1 | - | - | β-amy 1b |
| Aramir | Sd1 | - | Bmyl1 Ar | - |
| Atem | Sd1 | - | - | β-amy 1b |
| Balder | Sd1 | Sd1 | - | - |
| Bente | Sd1 | - | Bmyl1 Ar | - |
| Blenheim | Sd1 | - | - | β-amy 1b |
| Carina | Sd1 | - | Bmyl1 Ar | - |
| Charlottetown 80 | Sd1 | Sd1 | - | - |
| Dandy | Sd1* | - | - | β-amy 1a* |
| Digger | Sd1 | - | - | β-amy 1b |
| Feebar | Sd1 | Sd1 | - | - |
| Georgie | Sd1 | - | Bmyl1 Ar | - |
| Gerda | Sd1 | Sd1 | - | - |
| Golf | Sd1 | - | - | β-amy 1b |
| Keystone | Sd1 | Sd1 | - | - |
| Lami | Sd1 | - | Bmyl1 Ar | - |
| Magnum | Sd1 | - | Bmyl1 Ar | - |
| Morgenrot | Sd1* | Sd2* | - | - |
| Natasha | Sd1 | - | - | β-amy 1b |
| Olli | Sd1 | Sd1 | - | - |
| Pirkka | Sd1 | Sd1 | - | - |
| Plumage Archer | Sd1* | Sd2* | - | - |
| Regatta | Sd1* | - | - | β-amy 1a* |
| Triumph | Sd1 | - | - | β-amy 1b |
| Vada | Sd1 | Sd1 | - | - |
| Vantage | Sd1 | Sd1 | - | - |
| Vantmore | Sd1 | Sd1 | - | - |
| Vega | Sd1* | - | Bmyl1 Br* | - |
| Zephyr | Sd1 | Sd1 | - | - |

**Table 3.1:** (continued)

| | | | | |
|---|---|---|---|---|
| | | | | - |
| Akka | Sd2 | Sd2 | - | - |
| Birka | Sd2 | - | Bmyl1 Br | - |
| Betzes | Sd2 | Sd2 | - | β-amy 1a |
| Boreham Warrior | Sd2 | Sd2 | - | |
| Bussell | Sd2 | Sd2 | - | - |
| Camague | Sd2 | - | - | β-amy 1a |
| Cambrinus | Sd2 | Sd2 | - | - |
| Carlsberg II | Sd2 | Sd2 | - | - |
| Conquest | Sd2 | Sd2 | - | - |
| Corniche | Sd2 | - | - | β-amy 1a |
| Doublet | Sd2 | - | - | β-amy 1a |
| Emir | Sd2 | - | Bmyl1 Br | - |
| Gerbel | Sd2 | - | Bmyl1 Br | - |
| Glacier AC38 | Sd2* | Sd1* | - | - |
| Golden Promise | Sd2 | Sd2 | - | β-amy 1a |
| Harry | Sd2 | - | Bmyl1 Br | - |
| Hassan | Sd2 | Sd2 | - | - |
| Herta | Sd2 | Sd2 | - | - |
| Hi Proly | Sd2 | Sd2 | - | - |
| Ingrid | Sd2* | Sd1 | - | - |
| Julia | Sd2 | Sd2 | - | - |
| Koru | Sd2 | - | Bmyl1 Br | - |
| Kristina | Sd2 | Sd2 | - | - |
| Maris Baldric | Sd2 | Sd2 | - | β-amy 1b* |
| Maris Mink | Sd2 | Sd2 | - | - |
| Midas | Sd2 | Sd2 | - | - |
| Mona | Sd2 | Sd2 | - | - |
| Montcalm | Sd2 | Sd2 | - | - |
| Pallas | Sd2 | Sd2 | - | - |
| Prisma | Sd2 | - | - | β-amy 1a |
| Scotch Bere | Sd2* | Sd1* | - | - |
| Sherpa | Sd2 | - | - | β-amy 1a |
| Sonja | Sd2 | - | Bmyl1 Br | - |
| Sultan | Sd2 | Sd2 | - | - |
| Tyne | Sd2 | - | - | β-amy 1a |
| Tyra | Sd2 | - | Bmyl1 Br | - |
| Universe | Sd2* | Sd1* | - | - |
| Wisa | Sd2* | Sd1* | - | - |
| Ymer | Sd2 | Sd2 | - | - |

A further 75 barley varieties were screened for ß-amylase polymorphism to investigate the occurrence of the *Bmy*1A1 band pattern (Netsvetaev 1993). The additional material comprised a diverse range of germplasm, including the Algerian cultivars Zenit and Asse which were reported to exhibit the variant ß-amylase band pattern. Only the two band patterns shown in Figure 3.1 were observed in the additional barley varieties tested, including Asse and Zenit which exhibited the Sd1 and Sd2 band patterns respectively (Table 3.2).

**Table 3.2:** The 74 additional barley (*H. vulgare*) varieties analysed for ß-amylase polymorphism by IEF of grain extracts.

| Sd1 IEF Band Pattern | Sd2 IEF Band Pattern | |
|---|---|---|
| Asse | 2EBYT 23 | Ketch |
| Bearpaw | Abyssinia 403046 | KMBR 52 |
| Bomi | Abyssinia 403047 | Lara |
| Caminant | Arapiles | Malebo |
| Cheri | Bandulla | Mazurka |
| Dawn | Barque | Moondyne |
| Dera | Cantala | Morrell |
| Ellice | Chebec | Namoi |
| Fitzgerald | Chieften | O'Conner |
| Franklin | Clipper | Parwan |
| Gairdner | CM 72 | Piroline |
| Galaxy | CYMMYT 42002 | Pomo |
| Gula | Dampier | Proctor |
| Harrington | Ethiopia 183 | Rubin |
| Kaputar | Fergie | Sahara (3771/1) |
| Kiltra | Forrest | Schooner |
| Kustaa | Fuji Nijo | Skiff |
| Kymppi | Galleon | Steptoe |
| Maltine | Gilbert | Stirling |
| Manley | Gimpel | Tweed |
| Monarch | Halcyon | Weeah |
| Morex | Haruna Nijo | WB185 |
| Pokko | HE 3631 | WI 2827 |
| Shannon | Igri | Zenit |
| Sloop | | |
| Tallon | | |
| TR306 | | |

The *Bmy*1A1 band pattern was reported in extracts of germinated barley (Netsvetaev 1993), which primarily contains the proteolytically cleaved ß-amylase (Lundgaard and Svensson 1986). To determine if removal of the 4 kDa peptide from the carboxy-terminus of ß-amylase could reveal previously unidentified surface charge variation, extracts from germinated barley were examined by IEF. The proteolytic cleavage of ß-amylase yields new isoforms with increased pI, and the analysis of band patterns is complicated by the presence of α-amylase. ß-amylase and α-amylase bands were distinguished on the basis of both thermostability and immunological identity (data not shown). Figure 3.2 shows the ß-amylase band patterns exhibited in germinated extracts by Asse and Zenit are identical to the varieties Franklin (Sd1) and Schooner (Sd2) respectively, consistent with the phenotyping results from mature grain for the two Algerian varieties.

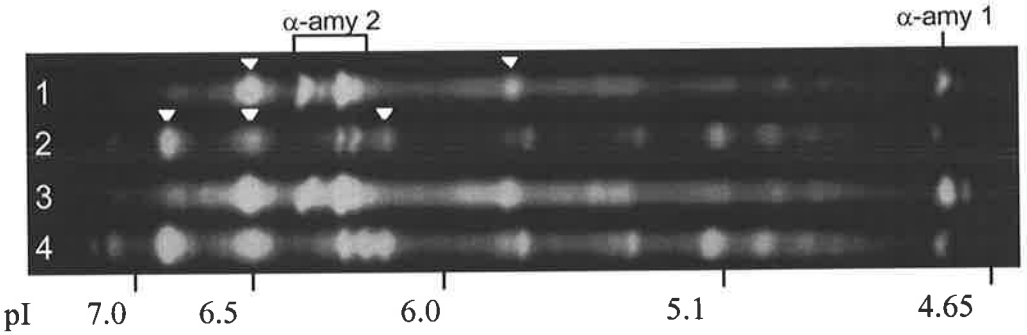



**Figure 3.2:** Extracts from 5 day germinated barley separated by IEF and activity stained to show amylase bands. Lane 1: Franklin (Sd1), 2: Schooner (Sd2), 3: Asse (Sd1), 4: Zenit (Sd2). α-Amylase bands are labelled and arrowheads indicate the major ß-amylase bands generated after germination.

### 3.3.2 Identification of Variation in ß-amylase Thermostability

Significant variation in the relative thermostability of barley ß-amylase was observed, and this variation was only partially consistent with IEF band patterns. The rates of irreversible thermal inactivation of the Sd1 and Sd2 forms of ß-amylase in barley extracts is shown in Figure 3.3. Varieties with the *Bmy*1-Sd1 allele were characterised as a discrete group, exhibiting an intermediate level of thermal stability. The *Bmy*1-Sd2 varieties were divided into discrete groups, with high (Sd2H) and low (Sd2L) relative thermostabilities. In addition, the varieties Forrest and Morrell were found to exhibit an intermediate level of stability (data not shown).

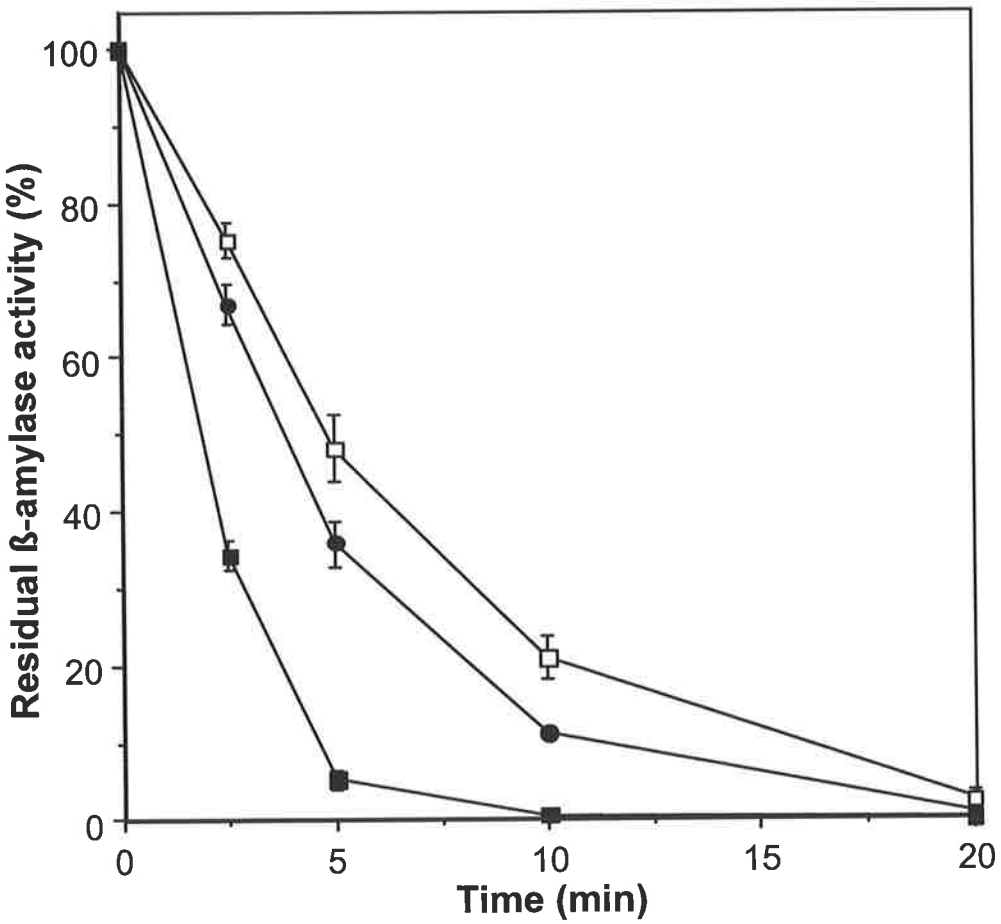



**Figure 3.3:** Irreversible thermal inactivation of ß-amylase in barley extracts incubated at 60°C. —■— Sd2L, —●— Sd1, —□— Sd2H. Values are the mean of five barley varieties and the standard error of the mean is shown.

### 3.3.3 Variation in thermostability persists through germination and the associated COOH-terminal proteolysis of ß-amylase

Barley ß-amylase undergoes proteolytic cleavage after germination, with between approximately 38 to 42 amino acids removed from the C-terminus of the enzyme (Lundgaard and Svensson 1986). To determine if the variation in enzyme thermostability persists into the cleaved forms of ß-amylase, ten barley varieties representing the range of stability within the *Bmy*1-Sd1 and Sd2 types were micromalted, and the level of residual enzyme activity after thermal inactivation determined.

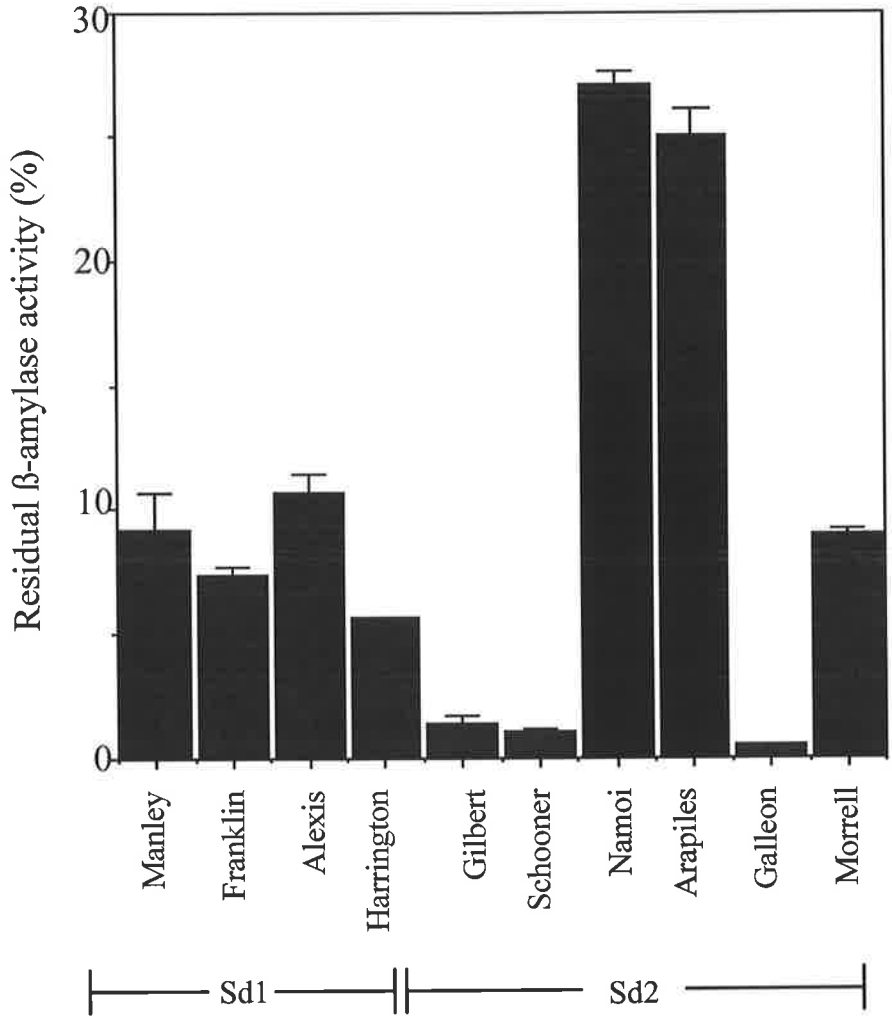



**Figure 3.4:** Residual ß-amylase activity in malt extracts after incubation at 60°C for 10 minutes. Activity is expressed as a percentage of initial activity and the standard deviation of 4 independent determinations is shown.

Figure 3.4 shows significant variation in ß-amylase thermostability in extracts of micromalted barley, and this variation is consistent with the rates of thermal inactivation determined with extracts from mature barley grain. The *Bmy*1-Sd1 varieties fell into a discrete group with an intermediate level of thermostability. The *Bmy*1-Sd2 barley varieties separated into the same high (Sd2H) and low (Sd2L) stability groups observed in barley extracts, with the level of residual activity ranging from approximately 25% for the high stability lines, to less than 2% in the low stability lines. An intermediate level of residual ß-amylase was also found in malt extracts of the Sd2 variety Morrell, consistent with barley grain extracts

### 3.3.4 QTL Mapping of Variation in ß-amylase Thermostability.

The relative thermostability of ß-amylase was mapped to determine if the difference in the levels of residual ß-amylase activity were due to an intrinsic characteristic of the enzyme, or secondary factors modulating enzyme stability. The mapping population consisted of 95 doubled haploid derived individuals from a Galleon X Haruna Nijo cross. Both parental lines carry the *Bmy*1-Sd2 form of the enzyme, however Galleon exhibits low ß-amylase thermostability (Sd2-L), and Haruna Nijo high stability (Sd2-H), allowing the genetics of ß-amylase thermostability to be examined.

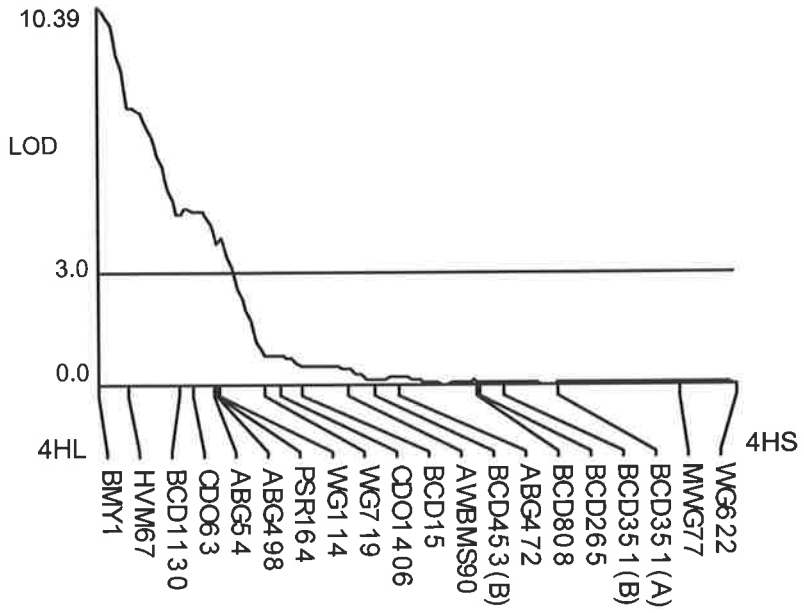



**Figure 3.5:** Log-likelihood (LOD) values for the association of ß-amylase thermostability and molecular markers on chromosome 4H.

The double haploid population fell into two non-overlapping classes of ß-amylase thermostability. At the confidence level of P = 0.0001, only one significant association between molecular markers and enzyme thermostability was detected (Figure 3.5), with Haruna Nijo contributing the high thermostability phenotype. This QTL corresponds to the *Bmy*1 locus on chromosome 4H, and no other genomic regions influencing ß-amylase thermostability were detected.

## 3.4 Discussion

IEF of barley extracts was used to examine ß-amylase polymorphism within cultivated barley (*H. vulgare*). Only two electrophoretically distinct forms of ß-amylase were observed in a survey of 142 barley varieties. The two alternative band patterns correspond to the previously described Sd1 and Sd2 forms of ß-amylase (Allison 1973, Allison and Swanston 1974), the Bmy1 Ar and Br band patterns (Nielsen and Johansen 1986) and the *ß-amy-1a* and

*β-amy-1b* band patterns (Forster *et al.* 1991). A total of 10 conflicts in assignment were found in the 68 varieties previously examined, however these differences can probably be explained by varietal heterozygosity, outcrossing or miss-labelling that has occurred in the intervening time period.

A third electrophoretically distinct form of ß-amylase has been reported in a range of Algerian barley varieties (Netsvetaev 1993). This study was performed using native agarose electrophoresis of germinated extracts stained for amylolytic activity. In the present study, ß-amylase band patterns from barley grain extracts of the Algerian varieties Asse and Zenit were found to conform to the Sd1 and Sd2 band patterns (respectively), and were subsequently examined in germinated extracts. Germination results in proteolytic cleavage of ß-amylase which yields at least six new ß-amylase bands that are significantly more basic than the isoforms from mature grain, some of which are obscured by α-amylase (α-amy2) bands (Evans *et al.* 1997). ß-amylase and α-amylase bands were distinguished both by thermal inactivation and immunological identity, and the varieties Asse and Zenit were shown to exhibit Sd1 and Sd2 malt ß-amylase band patterns respectively. The discrepancy in assignment could arise from differences in the barley samples used in the respective studies, or alternatively from the incorrect designation of α-amylase polymorphism, which is possible due to the presence of at least three alternative α-amy2 alleles (Brown and Jacobsen 1982, Takano and Takeda 1985).

Significant variation in the rates of thermal inactivation of the alternative forms of ß-amylase was observed in barley extracts. The Sd1 form of ß-amylase behaves as a discrete allele with respect to the thermostability trait, consistently exhibiting an intermediate level of irreversible thermal inactivation. The Sd2 varieties were separated into three separate groups based on relative thermostability, exhibiting low (Sd2L), intermediate and high (Sd2H) levels

of residual activity after heat treatment. These results have now been repeated in a survey of Japanese and international barley varieties (Kihara *et al.* 1998), however in this subset of germplasm the Sd2H ß-amylase was only observed in Japanese barley varieties.

The variation in thermostability was shown to persist after proteolytic cleavage of the enzyme after germination, with relatively small differences detected between the relative thermostability of the seed and malt forms of ß-amylase. However the level of observed levels of thermostability may be significantly influenced by the composition of the extract. The level of ß-amylase activity after incubation of extracts at elevated temperatures can be modulated by a number of factors. Protein content varies between barley varieties and the level of soluble protein (Ray *et al.* 1994) and carbohydrate (DeCordt *et al.* 1994) in the extract can have a significant impact on enzyme stability. More specific protein/protein interactions may also affect stability, and ß-amylase has been reported to form dimers and heterodimers with other barley proteins (Hejgaard 1976). Testing enzyme thermostability in crude extracts is also complicated by the presence of proteolytic enzymes in the barley grain and malt (Zhang and Jones 1995).

Molecular mapping of variation in relative thermostability of ß-amylase between an Sd2H and an Sd2L line revealed a single QTL at the *Bmy1* locus on chromosome 4H. This indicates that variation observed in enzyme stability in crude extracts is due to differences in the ß-amylase enzyme, and is not significantly affected by other loci. This further suggests that the high and low levels of stability observed within the Sd2 ß-amylase are the result of discrete *Bmy*1 alleles, even though these forms of the enzyme cannot be differentiated by electrophoresis. The genetic basis of the intermediate level of thermal stability observed within the Sd2 phenotype was not investigated, however similar observations in a survey of Japanese breeding lines (Kihara *et al.* 1998), suggests that this may result from an additional allele, rather than heterogenous samples.

The observation of variation in ß-amylase thermostability in barley and malt extracts, the mapping of this trait to the *Bmy*1 locus, and the identification of the Sd2L form of ß-amylase in a significant proportion of barley varieties suggests potential for improving malting quality. However, the unequivocal assignment of allelic variation requires analysis of purified enzymes demonstrating properties consistent with the observations made on crude extracts. In addition, although the thermolabile nature of ß-amylase has been the discussed in the literature, the practical significance of differences in thermostability between the alternative forms of ß-amylase requires empirical determination.

# Chapter 4: Identification and Characterisation of Allelic Variation for ß-amylase in *Hordeum vulgare* ssp. *spontaneum.*

## 4.1 Introduction

Barley ß-amylase is expressed as a 59.6 kDa protein during grain development and is present in three fractions in mature barley grain. The active or free enzyme is water soluble, while the bound fraction can be extracted in the presence of reducing agents or proteolytic enzymes (Sallans and Anderson 1940, Sandegren and Klang 1950). The recently described latent fraction has only been extracted with detergent and reducing agent (Evans *et al.* 1997b). During germination ß-amylase undergoes carboxypeptidase mediated proteolysis to generate a 55.5 kDa form of the enzyme (Lundgaard and Svennson 1987, Guerin *et al.* 1992), concomitant with conversion of the latent and bound fractions to free enzyme (Evans *et al.* 1997).

*H. vulgare* contains three allelic forms of ß-amylase, described as Sd1, Sd2L and Sd2H, with only the Sd1 enzyme being electrophoretically distinct (see Chapter 3). The Sd1 ß-amylase is also associated with a different proportion of free/bound enzyme in mature barley grain, with *Bmy*1-Sd1 varieties containing more than 55 % of the enzyme bound, while *Bmy*1-Sd2 varieties have less than 45 % of ß-amylase bound (Allison and Swanston 1974, Evans *et al.* 1997).

ß-amylase expression responds positively to nitrogen nutrition (Giese and Hejgaard 1984, Giese and Hopp 1984), and barley ß-amylase levels are significantly correlated with grain nitrogen (Arends *et al.* 1995). The Sd1 and Sd2 ß-amylase band patterns have both been associated with increased enzyme activity, depending on the particular cross examined (Swanston 1980 and 1983). The inconsistency of these results is due to the presence of the two alternative forms of ß-amylase which both exhibit the Sd2 band pattern (Chapter 3). The *Bmy*1-Sd2L allele is associated with low levels of enzyme activity, while the *Bmy*1-Sd1 and *Bmy*1-Sd2H alleles are associated with increased ß-amylase activity. The mechanism

responsible for these differences has recently been suggested to be intron based regulation of gene expression, due to the association of an insertion/deletion event in intron III of the *Bmy*1 gene with alternative expression levels (Erkkila *et al.* 1998).

Although only three *Bmy*1 alleles have been identified in cultivated barley, additional alleles may be present in the wild barley (*Hordeum vulgare* ssp. *spontaneum*) gene pool. The potential of wild barley as a genetic resource for crop improvement is well established, and much of this potential lies in the increased genetic variation observed in *H. spontaneum* compared to cultivated barley (Brown 1992, Nevo 1992). An assessment of ß-amylase variation in *H. spontaneum* reported three phenotypes restricted to wild barley. However the genetic relationships and functional properties of these putative allozymes were not examined (Chalmers *et al.* 1992). ß-amylase enzyme activity has also been assessed in germinated grains of *H. spontaneum* and shown to exhibit wide variation, with some accessions containing up to six times more activity than the barley variety Bomi (Ahokas and Naskali 1990a and 1990b). Lines with high ß-amylase activity were recovered from unselected backcrosses with the variety Adorra (Ahokas and Errkila 1992). However it was subsequently shown that the low activity lines had inherited the *Bmy*1-Sd2L allele from Adorra and the high activity lines carried a novel gene from *H. spontaneum* which was identical to the *Bmy*1-Sd2H allele except for a 39 bp deletion in intron III (Erkkila *et al.* 1998).

Analysing the influence of alternative alleles on enzyme expression levels may be performed using a backcrossing program, which aims to produce near isogenic lines differing only at the locus of interest. Typically $BC_4$ would be considered acceptable, although the number of generations required may be reduced by marker assisted selection for the recurrent genetic background. The method tested in this study is an adaptation of bulked segregant analysis developed for molecular marker generation (Michelmore *et al.* 1991). This approach involves screening a segregating population to generate two groups homozygous for the gene of interest, which then consist of random but equivalent genetic backgrounds, and comparing the trait of interest between the two groups. The reduced time required to generate appropriate populations is a significant advantage of this method compared to either conventional or marker assisted backcrossing to produce near isogenic lines.

The identification of alternative ß-amylase alleles for introgression into elite cultivars is one possible approach to breeding barley for improved malt quality. An allele may be preferred on the basis of either increased expression levels or enhanced properties of the corresponding enzyme. This study includes an assessment of ß-amylase variation within *H. spontaneum*, genetic analysis of this variation, and evaluation of genetic diversity within wild barley at the *Bmy*1 locus. The influence of the alternative alleles on ß-amylase expression levels is also investigated. The allelic forms of ß-amylase are characterised in terms of isoelectric point, molecular mass, and thermostability both before and after post-germination proteolytic processing.

## 4.2 Materials and Methods

### 4.2.1 Plant Material

A total of 155 accessions of *Hordeum vulgare* ssp *spontaneum* as described in section 2.2.1 were assessed. Details of individual accessions and their respective *Bmy*1 genotypes are listed in appendix I.

*H. spontaneum* accessions were grown and crossed in glasshouses and the progeny grown in controlled environment rooms from 1994 to 1996, all material was manually threshed and grain stored sealed at 4°C. Barley was germinated for enzyme analysis was surface sterilisied in 0.2% $AgNO_3$, washed in distilled water, steeped for 1 hr and germinated on moist filter paper in sealed petri dishes for 5 days in the dark. Shoots and roots were removed, and the samples freeze-dried and stored at -20°C.

### 4.2.2 Total Nitrogen Determination

Total grain nitrogen was determined by the Dumas combustion method (Dumas 1826). Analyses were performed in duplicate with reference to oatmeal AR2026 and alfalfa AR2018 standards (Alpha Resources Inc., Stevensville, Michigan) on a Model 1500 Automatic Nitrogen Analyzer (Carlo Erba, Rodano, Italy).

### 4.2.3 Isoelectric Focusing (IEF)

IEF was performed as described in Section 3.2.2 using a non-linear gradient of pH 4-6.5 for seed extracts and pH 4.5-10.5 for extracts from germinated barley. Amylase bands were detected by starch staining (Guerin *et al.* 1992) and immunoblotting. Separated proteins were transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany) by passive transfer. The immunoblot was developed using rabbit anti-ß-amylase serum (Evans *et al.* 1997a) detected with HRP labelled goat anti-rabbit antibodies (Bio-Rad) using 4-chloro-1-napthol (Bio-Rad) as the substrate. Apparent pI values were estimated relative to native IEF markers (Bio-Rad).

### 4.2.4 SDS-PAGE and Immunoblotting

SDS-PAGE was performed as described in Section 2.2.3 and immunoblotting was performed as described in Section 4.2.3.

### 4.2.5 Genetic Analysis

*H. spontaneum* accessions exhibiting variant ß-amylase IEF band patterns were crossed with *H. vulgare* cv. Clipper. The *H. spontaneum* accessions were CPI 77146/33, CPI 77146/32 and CPI 71283/20 for the *Bmy*1-Sd3, Sd4 and Sd5 alleles respectively. The *H. vulgare* X *H. spontaneum* hybrids were selfed and segregation analysis performed by IEF of extracts prepared from at least 139 half-grains from each cross. Segregation ratios were tested for conformity with Mendelian expectations for single loci using chi-square tests.

The relative contribution of the *Bmy*1-Sd4 and -Sd5 alleles to ß-amylase activity was analysed using a form of bulked segregant analysis adapted from Michelmore *et al.* (1991). Half-grains from each $F_2$ population were screened by IEF and homozygous individuals were grown and selfed to generate two pairs of $F_3$ populations. Each pair of population's consisted of at least 14 individuals homozygous for the variant ß-amylase and at least 14 individuals homozygous for the *Bmy*1-Sd2L allele from Clipper. $F_2$ material from CPI 77146/33 x Clipper crosses did not germinate, therefore the *Bmy*1-Sd3 allele was not included in the bulked segregant analysis. For a gene segregating in an $F_2$ generation, the probability of a

bulk of individuals having the gene and a second bulk of the same size not having the gene will be: $2(1-[^1/_4]^n)(^1/_4)^n$ when the locus is unlinked to the target gene. Therefore with $n = 14$, $p = 7.45 \times 10^{-9}$. Each pair of $F_3$ populations therefore consisted of random and equivalent genetic backgrounds, but contained different *Bmy*1 alleles. Differences between the levels of enzyme activity in each pair of populations was analysed by ANOVA least significant difference test using JMP version 3.0.2 software (SAS Institute Inc, Cary, NC).

### 4.2.6 ß-amylase Activity

ß-amylase activity was determined as described in section 3.2.3. The level of combined (free plus bound) ß-amylase was determined using extraction buffer containing 143 mM 2-mercaptoethanol. Free ß-amylase was determined in extracts prepared in the absence of reducing agent.

### 4.2.7 Thermal Inactivation

The relative thermostability of ß-amylase was determined as described in section 3.2.4.

### 4.2.8 PCR Analysis of ß-amylase Intron III

PCR was performed by Mr Stuart Coventry, University of Adelaide, as described by Erkkila *et al.* (1998). Genomic DNA was extracted from 10 cm leaf segments by grinding under liquid $N_2$ and homogenising with 600 µL 1% sarkosyl, 2% PVP, 100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.5. Samples were extracted twice with phenol/chloroform/iso-amyl alcohol (25:24:1), and the DNA precipitated with 60 µL 3M Na acetate pH 4.8 and 600 µL isopropanol. The pellet was washed in 1 mL 70% ethanol (55°C) and the DNA resuspended and stored in 50 µL 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 containing 40 µg $mL^{-1}$ RNAse A (Sigma).

*Bmy*1 intron III specific sequences were amplified from genomic DNA with the primer pair 5'-GATGGTCGTTCCCAGGCATC-3' and 5'-AGGGAACCGCACGTGTGGGGTCAATGA-3'. The reaction mixture contained 0.1 µg of

template DNA, 10 pmol of each PCR primer, 0.2 mM deoxyribonucleotide triphosphate, 1.2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X100, and 0.2 units of *Taq* polymerase (Promega) in a total reaction volume of 25 μL. PCR was reacted for 2 min at 95°C, 1 min at 94°C, 45 sec at 58°C, and 1.5 min at 72°C for 35 cycles, followed by 10 min at 72°C. The PCR fragments were separated in 2% agarose gels with ethidium bromide.

## 4.3 Results

### 4.3.1 Variation in ß-amylase within *H. spontaneum*

The ß-amylase IEF band patterns observed in 154 *H. spontaneum* accessions are represented by the five alternative patterns shown in Figure 4.1. The Sd1 and Sd2 IEF band patterns detected in *H. vulgare* (Chapter 3) were also present in wild barley, and were found in 46.5 and 48.4 % of the accessions, respectively. Three additional band patterns were observed and described as Sd3, Sd4, and Sd5. The Sd3 band pattern consists of similar complex banding to the Sd1 and Sd2 forms of ß-amylase, shifted to a more basic pI, with major bands at pH 6.1, 6.05, 5.8 and a double band at pH 5.5. The Sd3 ß-amylase was restricted to a single accession of *H. spontaneum* from Southern Israel (CPI #77146/33). The Sd4 ß-amylase exhibits a major band at pH 5.5 and minor bands at pH 5.15, and was also restricted to one accession of *H. spontaneum* from Southern Israel (CPI #77146/32). The Sd5 ß-amylase exhibits a major band at pH 4.95 and a diffuse band at pH 4.85. The Sd5 band pattern was identified in six accessions of *H. spontaneum* from a single site in Central Israel (CPI #71283/8, /10, /20, /25, /42, and /48).

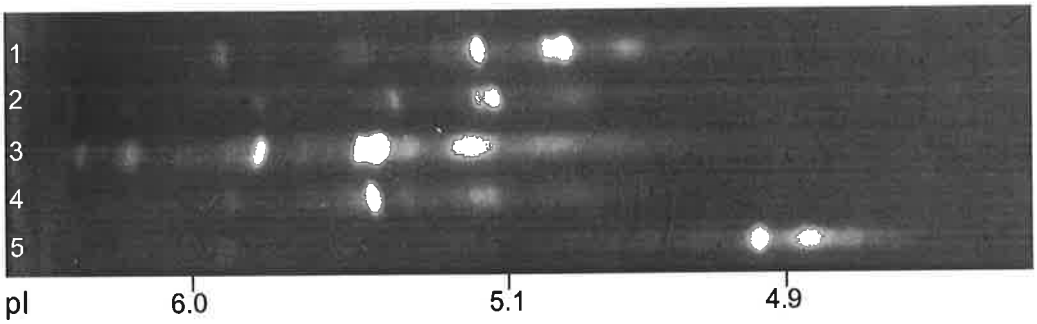



**Figure 4.1:** Isoelectric focusing of barley extracts starch stained to show ß-amylase activity. Lane 1: Sd1, 2: Sd2, 3: Sd3, 4: Sd4, 5: Sd5.

### 4.3.2 Genetic Basis for Alternative ß-amylase Band Patterns

The Sd1 and Sd2 band patterns found in cultivated barley are the result of two codominant alleles (Allison 1973), and this polymorphism has been mapped to the *Bmy*1 locus on chromosome 4H (Li 1998). ß-amylase exhibits multiple molecular forms (Figure 4.1), which may be the result of post-translational modifications or expression of a gene family. Segregation analysis between the Sd2 band pattern and each of the variant band patterns was performed to determine whether the variant band patterns detected in *H. spontaneum* result from differences in post-translational modifications or from alternative *Bmy*1 alleles.

*H. spontaneum* accessions containing the variant forms of ß-amylase were crossed with the variety Clipper which carries the *Bmy*1-Sd2L allele (Chapter 3). $F_1$ individuals were selfed and the resulting $F_2$ grains screened by IEF. The band patterns each segregate as discrete units, with heterozygote individuals exhibiting a combination of all bands observed in the parents. The variant Sd3, Sd4 and Sd5 band patterns from *H. spontaneum* each segregate in a 1:2:1 ratio with respect to the *Bmy*1-Sd2L allele (Table 4.1), indicating they are the result of alternative codominant *Bmy*1 alleles.

**Table 4.1:** Segregation of the Sd3, Sd4 and Sd5 ß-amylase band patterns with respect to the *Bmy*1-Sd2L allele from cv. Clipper.

| Cross | No. of samples | IEF Band Pattern | | | | |
|---|---|---|---|---|---|---|
| | | Variant | Heterozygote | Sd2-L | $\chi^2$ | p |
| Sd3 x Clipper | 139 | 36 | 72 | 31 | 0.91 | 0.19 |
| Sd4 x Clipper | 276 | 66 | 143 | 67 | 0.95 | 0.11 |
| Sd5 x Clipper | 164 | 42 | 84 | 38 | 0.96 | 0.08 |

### 4.3.3 Ecogeographical Distribution of ß-amylase Variation

The frequency of each ß-amylase band pattern with respect to the collection site of the *H. spontaneum* accessions is shown in Table 4.2. The alternative band patterns range in frequency from 82.6% for the Sd2 band pattern in accessions from the Jordan Valley, to single accessions exhibiting the Sd3 and Sd4 band patterns. However, it is significant that the Sd2 band pattern represents two alternative *Bmy*1 alleles (*Bmy*1-SdL and Sd2H) that cannot be distinguished by isoelectric focusing (Chapter 3). Analysis of the wild barley accessions exhibiting the Sd2 band pattern for ß-amylase thermostability could be used to discriminate between the Sd2H and Sd2L individuals, however this has not been performed in the present study.

**Table 4.2:** Ecogeographical distribution and diversity estimates of ß-amylase variation in *Hordeum vulgare* ssp. *spontaneum*.

| Band Pattern | **IEF banding frequency** | | | | | |
|---|---|---|---|---|---|---|
| | **Israel** | | | | **Iran, Turkey & Morocco** | **Total** |
| | Mountains | Jordan Valley | Coastal | Desert | | |
| Sd1 | 0.109 | 0.174 | 0.520 | 0.667 | 0.742 | 0.419 |
| Sd2 | 0.761 | 0.826 | 0.480 | 0.267 | 0.258 | 0.529 |
| Sd3 | | | | 0.033 | | 0.006 |
| Sd4 | | | | 0.033 | | 0.006 |
| Sd5 | 0.130 | | | | | 0.039 |
| | n = 46 | n = 23 | n = 25 | n = 30 | n = 31 | n = 155 |
| $H_e$ | 0.392 | 0.287 | 0.499 | 0.482 | 0.383 | 0.543 |
| $n_e$ | 1.6 | 1.4 | 2 | 1.9 | 1.6 | 2.2 |
| $n_a$ | 3 | 2 | 2 | 4 | 2 | 5 |

$H_e$ = probability that two random gametes are dissimilar.
$N_e = (1- H_e)^{-1}$ = effective number of alleles.
$N_a$ = actual number of phenotypes.

The level of ß-amylase polymorphism exhibits significant variation between the five sources of *H. spontaneum*. In addition to the restricted distribution of the rare Sd3, Sd4 and Sd5 alleles, the relative proportion of the Sd1 and Sd2 band patterns is variable across environments. The Sd1 band pattern ranges in frequency from 0.109 in the Israeli Mountains to 0.667 in the Negev Desert and 0.742 in the composite collection from Iran, Turkey and Morocco.

A comparison of three measures of genetic diversity across the five environments shows the *H. spontaneum* germplasm from the Jordan Valley and the composite collection exhibit the lowest levels of ß-amylase polymorphism, and accessions from the Negev Desert exhibit the highest levels of genetic diversity (Table 4.2). Despite the detection of only two phenotypes, the coastal region of Israel exhibits relatively high $H_e$ due to the similar frequency of the Sd1 and Sd2 band patterns. All three measures of genetic diversity are

underestimated due to the occurrence of three distinct alleles each exhibiting the Sd2 band pattern (Chapter 3).

### 4.3.4 Physical Characterisation of Variant ß-amylase Enzymes

Immunoblotting of ß-amylase from barley extracts separated by SDS-PAGE is shown in Figure 4.2. The Sd1 and Sd2 forms of ß-amylase exhibit an apparent molecular mass of approximately 60 kDa in mature grain and approximately 56 kDa after germination, consistent with the previous reports of 59.6 kDa (Kreis *et al.* 1987) and 55.5 kDa (Lundgaard and Svennson 1986). The Sd3 and Sd5 forms of ß-amylase from wild barley both exhibit an apparent molecular mass of 60 kDa in mature grain and 56 kDa after germination, identical to the Sd1 and Sd2 enzymes. Significantly, as much as 50% of ß-amylase still remains in the uncleaved form 5 days after the initiation of germination. In contrast, the Sd4 ß-amylase has a molecular mass of 56 kDa in the mature grain and does not appear to undergo significant size reduction during germination (Figure 4.2).

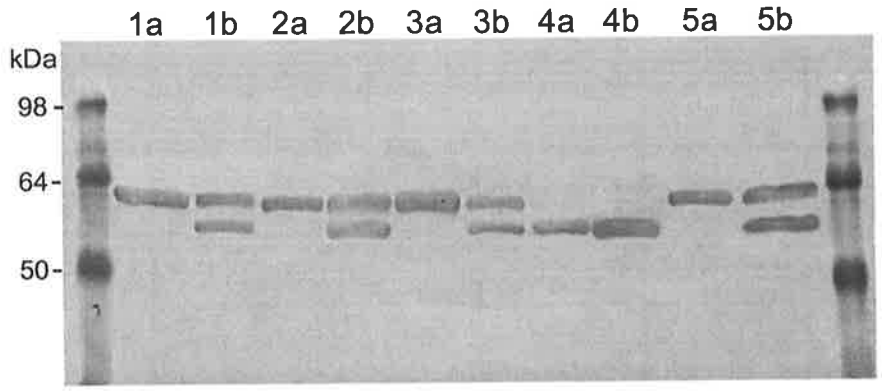



**Figure 4.2:** SDS-PAGE immunoblot of ß-amylase from extracts of (a) mature barley grain, and (b) 5 day germinated barley. Lane 1: Sd1, 2: Sd2, 3: Sd3, 4: Sd4, 5: Sd5.

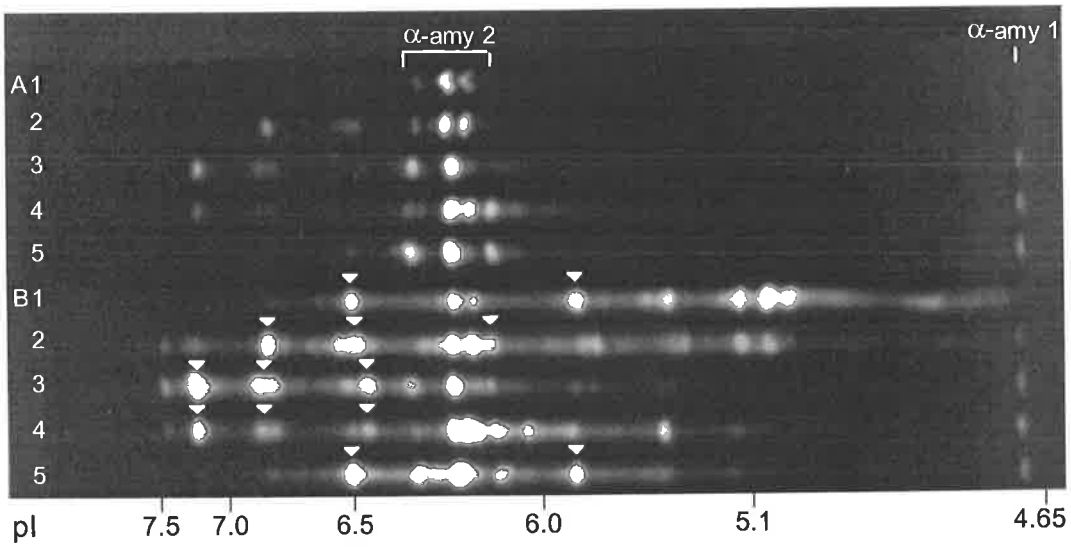



**Figure 4.3:** Isoelectric focusing of extracts from 5 day germinated barley stained to show amylase bands. A: Extracts heat treated at 65 °C for 10 min. B: Extracts prior to heat treatment. Lane 1: Sd1, 2: Sd2, 3: Sd3, 4: Sd4, 5: Sd5. α-amylase bands are labelled. Arrowheads indicate the major ß-amylase bands generated after germination.

The IEF band patterns of the five alternative forms of ß-amylase were examined after the proteolytic cleavage that occurs during germination (Figure 4.3). To verify that banding patterns were not the result of sample heterogeneity, single grains were halved and the proximal end germinated, and extracts from the two halves were compared by IEF. α-Amylase and ß-amylase bands were distinguished on the basis of thermostability (Figure 4.3) and immunological identity (data not shown). Figure 4.3 shows the five germinated barley samples also exhibit variation in α-amylase 2 band patterns, consistent with previous analysis of α-amylase polymorphism in these accessions of *H. spontaneum* (Brown and Jacobsen 1982).

The appearance of new ß-amylase bands after germination in each of the five alternative forms of ß-amylase is shown in Figure 4.3. The cleaved forms of the Sd1 and Sd2 enzymes are differentiated by IEF consistent with previous analyses (Evans *et al.* 1997). The Sd3 ß-amylase is converted to four major isoforms (pI 6.4, 6.8, 6.9, and 7.3) exhibiting a

similar band pattern to the Sd2 ß-amylase but shifted to a more basic pI. This parallels the comparison of the band patterns of the two 59.6 kDa forms of the enzyme in Figure 4.1.

The appearance of new bands in the Sd4 ß-amylase suggests it also undergoes proteolytic cleavage, despite not showing visible reduction in apparent molecular mass as determined by SDS-PAGE. The Sd4 ß-amylase exhibits the same IEF band pattern as the Sd3 enzyme after germination, suggesting the two cleaved forms of the enzyme are closely related. Similarly, proteolytic cleavage of the Sd5 enzyme yields the same band pattern as the cleaved Sd1 ß-amylase (Figure 4.3).

### 4.3.5 Free/bound ß-amylase Ratios

The free/bound levels of ß-amylase were determined for the Sd3, Sd4 and Sd5 forms of ß-amylase both in accessions of wild barley and homozygous hybrid progeny, and compared with free/bound ratios of cultivated barley carrying *Bmy*1-Sd1 or *Bmy*1-Sd2 alleles. The Sd3 enzyme was associated with approximately 32% bound ß-amylase, similar to the Sd2L enzyme in Clipper (25%) and Schooner (37%), and the Sd2H enzyme in Haruna Nijo (22%). Both the Sd4 and Sd5 forms of ß-amylase exhibited high levels of enzyme in the bound form. Lines containing the Sd4 ß-amylase had approximately 61% of the enzyme in bound form and Sd5 lines contained approximately 75% of ß-amylase bound, similar to the Sd1 varieties Pirrka (62%) and Harrington (69%).

### 4.3.6 Thermostability of Variant ß-amylase Enzymes

The rate of irreversible thermal inactivation of the alternative forms of ß-amylase in extracts from barley grain is shown in Figure 4.4. The Sd2L ß-amylase is the most sensitive to thermal inactivation, the Sd1 enzyme exhibits an intermediate level of stability and Sd3 ß-amylase is the most thermostable form of the enzyme. The Sd4 and Sd5 forms of ß-amylase from mature barley grain exhibit identical rates of thermal inactivation, and the rate of decay for the two enzymes represents an intermediate level of thermostability equivalent to the Sd1 ß-amylase.

The impact of genetic background on observed thermostability in barley extracts was determined by comparing *Bmy*1-Sd2L, -Sd4 and -Sd5 homozygote $F_2$ individuals with parental lines. In each case the rate of thermal inactivation was identical in the respective parent and progeny (data not shown), suggesting wide genetic backgrounds do not significantly affect observed levels of ß-amylase thermostability as determined in crude extracts.

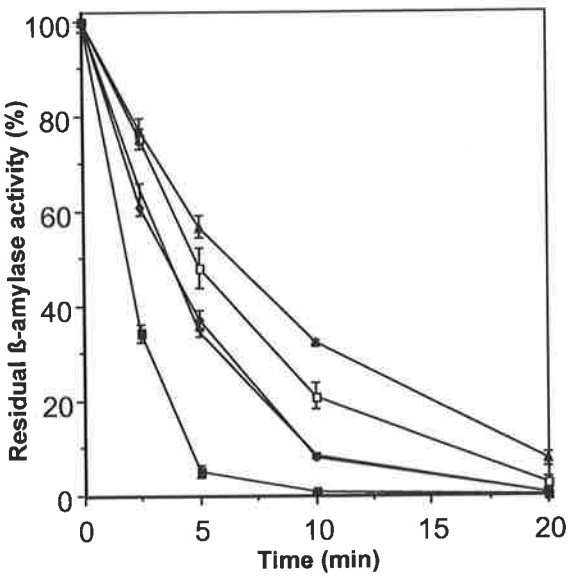



**Figure 4.4:** Irreversible thermal inactivation of ß-amylase in barley extracts incubated at 60°C. Symbol ■ : Sd2L, □ : Sd2H, ▲ : Sd3, ◆ : Sd4, ✕ : Sd5. Values are the mean of five barley samples and the standard error of the means are shown.

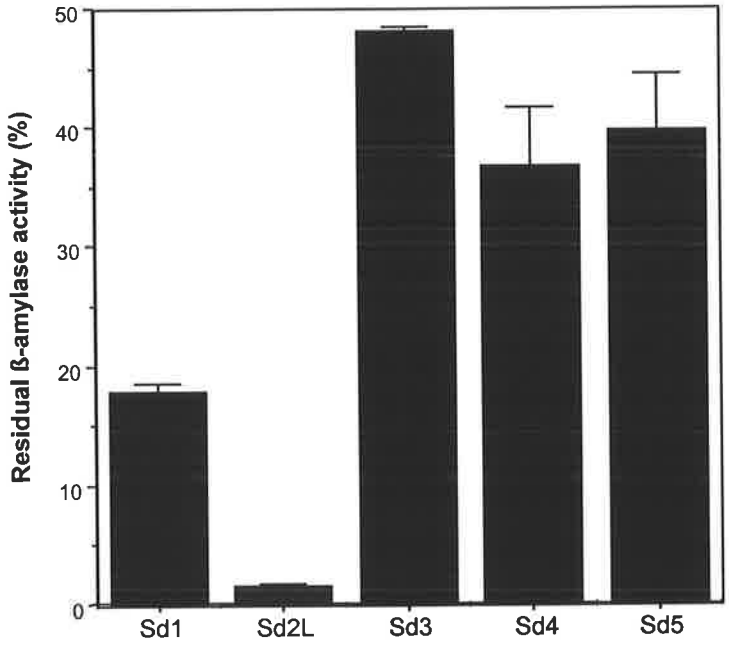



**Figure 4.5:** Irreversible thermal inactivation of ß-amylase in extracts of five day germinated barley incubated for 10 min at 60°C. Values are the mean of two germinated barley samples analysed in duplicate and the standard deviation is shown.

The relative thermostability of the five alternative forms of barley ß-amylase after 5 days germination is shown in Figure 4.5. The Sd1, Sd2L and Sd3 forms of the enzyme exhibit intermediate, low and high levels of residual ß-amylase activity respectively, consistent with the results from grain extracts. The total level of residual ß-amylase activity is slightly higher than observed in extracts from mature grain after 10 minutes incubation at 60°C (Figure 4.4). After germination the Sd4 and Sd5 enzymes exhibit residual ß-amylase levels significantly higher than the Sd1 enzyme, similar to the Sd2H enzyme (not shown), but lower than the levels observed for the Sd3 ß-amylase (Figure 4.5). This is in contrast to the results from grain extracts that show the Sd4 and Sd5 enzymes have the same relative thermostability as the Sd1 ß-amylase prior to proteolytic cleavage.

### 4.3.7 Contribution of alternative *Bmy*1 alleles to ß-amylase activity

#### 4.3.7.1 Analysis of Insertion/Deletion Events in *Bmy*1 Intron III

The expression level of the *Bmy*1 gene is influenced by a 126 base pair (bp) palindromic insertion/deletion event in intron III (Errkila *et al.* 1998). The presence of the 126 bp insertion is associated with reduced expression levels (Errkila *et al.* 1998). The PCR primers developed by Errkila *et al.* (1998) were used to amplify the intron III region from genomic DNA of a range of barley cultivars to confirm which *Bmy*1 alleles contain the intron insertion. The *H. spontaneum* accessions carrying the *Bmy*1-Sd3, -Sd4 and -Sd5 alleles were also examined to determine which alleles can be associated with high expression levels.

The *Bmy*1-Sd1 and -Sd2H alleles exhibit a 516 bp PCR product (Figure 4.6), consistent with the expected size of intron III. The *Bmy*1-Sd3 and -Sd5 alleles from *H. spontaneum* also exhibit the standard length intron III. A 643 bp PCR product was observed for the *Bmy*1-Sd2L and *Bmy*1-Sd4 alleles (Figure 4.9), consistent with a 126 bp insertion in intron III that is associated with reduced expression levels. The 39 bp deletion reported in a ß-amylase gene from *H. spontaneum* by Errkila *et al.* (1998) was not observed in these alleles.

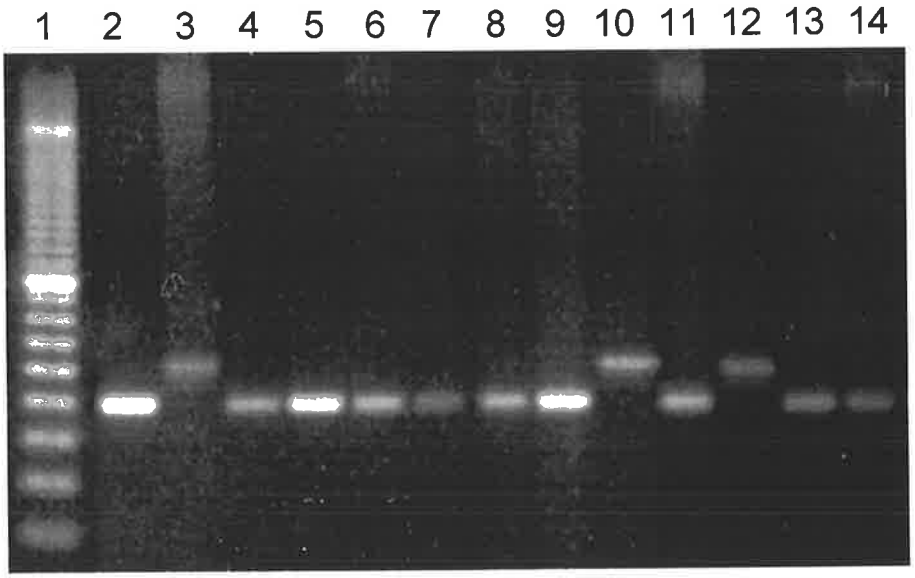



**Figure 4.6:** PCR analysis of intron III of six alternative *Bmy*1 alleles. Lane 1: 100 bp markers, 2: Arapiles (Sd2H), 3: Barque (Sd2L), 4: Bearpaw (Sd1), 5: Blenheim (Sd1), 6: Bowman (Sd1), 7: Chariot (Sd1), 8: Fitzgerald (Sd1), 9: Franklin (Sd1), 10: Clipper (Sd2L), 11: CPI 77146/33 (Sd3), 12: CPI 77146/32 (Sd4), 13: CPI 71283/20 (Sd5), 14: Haruna Nijo (Sd2H).

#### 4.3.7.2 Effect of *Bmy*1 alleles on enzyme activity in bulked segregant populations

A total of 139 $F_2$ individuals from an Sd3 x Sd2 cross were screened by IEF, but no homozygous $F_3$ plants were successfully regenerated from the remaining half grains, therefore the contribution of the *Bmy*1-Sd3 allele could not be estimated by an $F_3$ derived bulked segregant strategy. However the very high level of ß-amylase activity in the wild parent suggests the *Bmy*1-Sd3 allele is not incompatible with high levels of enzyme activity, with glasshouse grown material exhibiting a mean ß-amylase level of 3085 U/g, almost 3 times higher than the reference variety Clipper.

The relative contribution of the *Bmy1*-Sd4 and -Sd5 alleles to ß-amylase activity was assessed using bulked segregant analysis. The bulked segregant populations for both the Sd4 and Sd5 alleles exhibited transgressive segregation, with individuals ranging from 30% to 175% of the ß-amylase activity of the parental lines (Table 4.2). Despite the large range in enzyme activity the Sd2L and Sd4 populations exhibit similar mean ß-amylase levels, and ß-amylase activity is not significantly different ($p=0.05$) between the two populations. This is consistent with the results from the analysis of *Bmy*1-Sd4 intron III.

Total grain nitrogen was determined for each individual in the bulked segregant populations. The two pairs of populations exhibited transgressive segregation and continuous variation for grain nitrogen, ranging from 1.4% to 3.5% of grain mass, and total nitrogen was significantly correlated with ß-amylase activity in both pairs of populations ($r=0.83$ $p<0.001$). The expression of ß-amylase as function of total nitrogen yields similar mean values for the Sd4 and corresponding Sd2L populations (Table 4.2), and analysis of variance shows the two populations are not significantly different.

**Table 4.2:** Relative contribution of *Bmy*1-Sd4 and -Sd5 alleles to grain ß-amylase activity compared to the *Bmy*1-Sd2L allele from cv Clipper.

| Allele tested | Line | ß-amylase (U/g) | | ß-amylase (U/%N) | |
|---|---|---|---|---|---|
| | | Mean | Range | Mean | Range |
| *Bmy*1-Sd4 | CPI 77146/32 | 832 | | 296 | |
| | Sd4 population | 900 | 459-1667 | 407 | 238-505 |
| | Sd2 population | 855 | 427-1838 | 401 | 249-605 |
| | Clipper | 1087 | | 487 | |
| *Bmy*1-Sd5 | CPI 71283/20 | 1847 | | 564 | |
| | Sd5 population | 1225 | 493-1962 | 473 | 284-635 |
| | Sd2 population | 1133 | 601-2039 | 399 | 290-548 |
| | Clipper | 1401 | | 661 | |

The two bulked segregant populations for the *Bmy*1-Sd5 allele also exhibit transgressive segregation, with ß-amylase levels ranging from 27% to 146% of the parental lines (Table 4.2). However in this cross the mean ß-amylase activity of the Sd5 population is 8% higher than the Sd2L population, and when expressed as a function of total nitrogen the Sd5 individuals average an 18% increase over the Sd2L population. A comparison of the distribution of ß-amylase activity and total grain nitrogen is shown in Figure 4.7. The Sd5 and Sd2L populations exhibit linear correlations with total grain nitrogen ($r = 0.93$, $p<0.001$ and $r = 0.88$, $p<0.001$ respectively), with the Sd5 individuals typically exhibiting increased ß-amylase activity at any given level of grain nitrogen, although there is some overlap between the two populations. Despite these observed differences, the wide range in ß-amylase activity results in no statistically significant difference between the two populations as determined by analysis of variance.

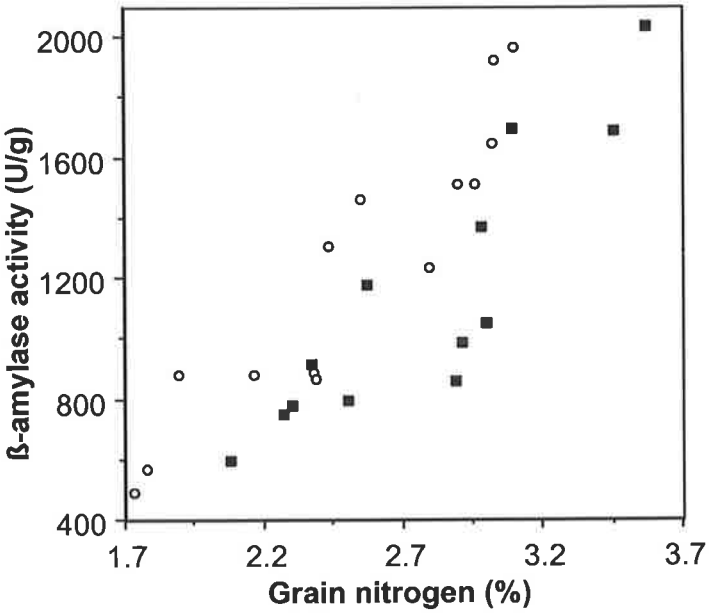



**Figure 4.7:** Distribution of ß-amylase activity and total grain nitrogen in Sd5 (○) and Sd2L (■) $F_3$ bulked segregant populations.

### 4.3.8 Influence of Genetic Background on ß-amylase Activity

A form of bulked segregant analysis has previously been employed to analyse the association between ß-amylase activity and the Sd1 and Sd2 band patterns (Swanston 1980). Population sizes of 9 to 15 homozygous $F_3$ bulks were analysed and shown to yield statistically significant differences in ß-amylase activity between the Sd1 and Sd2 populations derived the crosses Akka x Universe and Conquest x Maris Mink. In the present study, the $F_3$ populations were derived from *H. spontaneum* and Clipper, which exhibit significant differences in plant type as illustrated in Figure 4.8. The *H. spontaneum* accessions employed as parental lines exhibit traits characteristic of wild barley including prostrate growth habit, the brittle rachis trait, and asynchronous flowering within the plant. In contrast, the Australian malting variety Clipper is an erect, 2-row, spring type barley cultivar (Figure 4.8).


**Figure 4.8:** *H. spontaneum* accession CPI 71283-20 and cv. Clipper used as parental lines to generate F3 bulked segregant populations exhibit significantly different plant types.

Genetic variability within 96 barley cultivars and breeding lines, including five accessions of *H. spontaneum* from the current study, has been evaluated by RFLP analysis (Chalmers *et al.* 2001). Cluster analysis based on genetic distance values revealed pedigree relatedness among the germplasm as shown in Figure 4.9. The hierarchy of clusters shows good agreement with the origins of these lines, and the five accessions of *H. spontaneum* form a separate grouping from the barley varieties and landraces. The mean genetic distance estimate, calculated as the number of polymorphisms as a proportion of total RFLPs scored, between *H. spontaneum* and Clipper was very high (0.32). The relatedness of the barley varieties was characterised by much lower levels of polymorphism. The genetic distances of 0.16 between Clipper and Sloop, and 0.18 from Clipper to Barque, are half of the genetic distance between Clipper and any *H. spontaneum* accession.

The level of ß-amylase expression is influenced by the structure of intron III (Errkila *et al.* 1998), however it is also subject to significant epistatic effects (Hayes *et al.* 1993, Mather *et al.* 1997). The extent of transgressive segregation for ß-amylase activity in the bulked segregant populations may be a function of the genetic distance between the parents. The failure of the bulked segregant analysis to conclusively associate the *Bmy*1-Sd5 allele with higher enzyme activity than the *Bmy*1-Sd2L allele, despite the intron analysis indicating it should, suggests an alternative population structure would be more appropriate. $F_2$ derived bulks may be suitable for the generation of bulked segregant populations for molecular marker development (Michelmore *et al.* 1991), but biochemical traits influenced by genetic background may require lower levels of genetic diversity than in *H. vulgare* x *H. spontaneum* crosses.

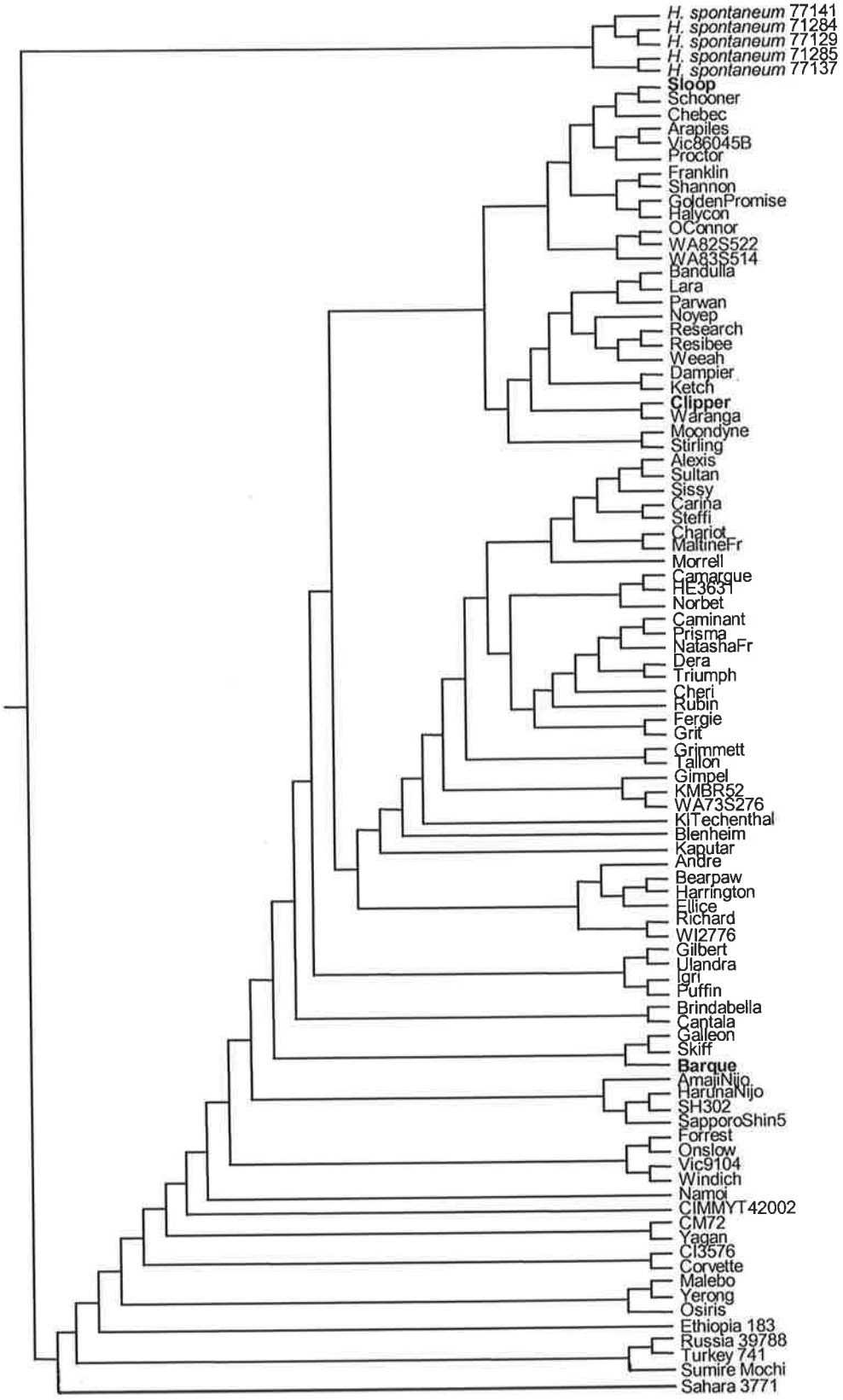



**Figure 4.9:** Cluster analysis performed on RFLP polymorphism between 96 barley varieties including five accessions of *H. spontaneum* from the current study (Chalmers *et al.* 2001).

## 4.4 Discussion

Analysis of 155 accessions of *Hordeum vulgare* ssp. *spontaneum* revealed five distinct ß-amylase banding patterns, including the Sd1 and Sd2 forms of the enzyme previously identified in *Hordeum vulgare* (Chapter 3). A previous study using a similar collection of wild barley also reported three novel ß-amylase phenotypes, and found significant relationships between ß-amylase variation and ecogeographic parameters, with 78% of the phenotypic variation explained by the number of rainy days per year and mean temperature in January (Chalmers *et al.* 1992). The distribution of ß-amylase polymorphism in the present study exhibits significant variation across environments, consistent with the analysis of Chalmers *et al.* (1992). Specific ecogeographical correlations were not tested due to the non-random structure of the *H. spontaneum* collection. ß-amylase variation was also characterised by a high proportion of rare band patterns, with the Sd3, Sd4, and Sd5 forms of ß-amylase each observed at single collection sites. The polymorphism observed in ß-amylase is therefore consistent with the general structure of *H. spontaneum* populations, exhibiting high variability, genetic differentiation across environments, and a relatively high proportion of unique alleles (Nevo *et al.* 1986).

The identification of novel *Bmy*1 alleles in single accessions of wild barley offers the possibility that further alleles could be identified in a more exhaustive survey. But a completely exhaustive analysis of wild barley would not be feasible. Analysis of the genetic structure of ß-amylase variation in wild barley indicates accessions isolated from particular ecogeographic regions are more likely to contain genetic variation. Targeted allele discovery

would be most efficient if focussed upon accessions from within Israel, and more specifically within very low rainfall regions (Table 4.2).

Significantly, the use of IEF in both the present study and that of Chalmers *et al.* (1992) underestimates the level of ß-amylase polymorphism due to the presence of two alternative alleles (*Bmy*1-Sd2L and -Sd2H) which both exhibit the Sd2 band pattern (Chapter 3). Native IEF is a high resolution and high throughput screening method but it cannot resolve proteins containing amino acid substitutions that do not alter the net surface charge. The development of a rapid method for screening ß-amylase thermostability would provide an opportunity to perform a more detailed analysis of ß-amylase variation within *H. spontaneum*.

The three banding patterns unique to wild barley result from three alternative *Bmy*1 alleles and are described as *Bmy*1-Sd3, -Sd4 and -Sd5. The corresponding Sd3 and Sd5 forms of the enzyme are present as 60 kDa proteins in mature grain and undergo proteolytic cleavage during germination to generate 56 kDa enzymes, consistent with the previously characterised Sd1 and Sd2 enzymes (Evans *et al.* 1997). In contrast, the Sd4 ß-amylase is present in mature grain as a 56 kDa enzyme and does not undergo significant reduction in apparent molecular mass after germination.

The carboxypeptidase mediated proteolysis of ß-amylase also alters the IEF band pattern, with the generation of new isoforms with increased isoelectric points. The cleaved forms of ß-amylase are not completely differentiated by IEF, with the Sd3 and Sd4 enzymes exhibiting identical band patterns, indicating that the Sd4 enzyme does undergo some proteolytic cleavage despite not exhibiting a significantly reduced molecular mass after germination. The proteolytically cleaved form of the Sd5 enzyme exhibits the same IEF band pattern as the cleaved Sd1 enzyme. The variation responsible for differences in the isoelectric

points of the uncleaved enzymes are therefore likely to occur within the regions of the enzyme removed during proteolytic processing.

A further important characteristic of ß-amylase is the ratio of free to bound forms of the enzyme. Free/bound levels of ß-amylase have been found to occur in either high or low levels in domesticated barley, with Sd1 varieties having greater than 50% of the enzyme in bound form and Sd2 varieties having less than 50% of ß-amylase in bound form (Allison and Swanston 1974). The three alternative forms of ß-amylase identified in wild barley were found to exhibit consistent free/bound levels in a range of genetic backgrounds. The Sd3 ß-amylase was associated with low levels of bound enzyme, similar to the Sd2L and Sd2H forms of ß-amylase. Both the Sd4 and Sd5 forms of ß-amylase exhibited high levels of enzyme in the bound fraction, similar to the Sd1 phenotype.

The level of free/bound ß-amylase is independent of environmental effects (Bendelow 1964), co-segregates with ß-amylase banding pattern, is not influenced by genetic background, and may be considered to result from intrinsic properties of the ß-amylase enzyme. The bound ß-amylase can be extracted with reducing agents or limited carboxy-terminal proteolysis. Therefore the cysteine residue at position 503 is presumed to be involved in the binding mechanism. However the occurrence of high levels of bound enzyme with the 56 kDa Sd4 ß-amylase is not consistent with a major role for the C-terminus in determining the level of bound enzyme. Sequence data for three ß-amylase clones have been published (Kreis *et al.* 1987, Lundgaard and Svensson 1987, Yoshigi *et al.* 1994). However they have been shown to correspond to the Sd2L and Sd2H forms of ß-amylase that exhibit the same levels of bound enzyme (Chapter 3). The molecular basis for the different free to bound ratios is yet to be resolved and any physiological significance of the free and bound fractions has not been determined.

The alternative forms of the ß-amylase enzyme exhibit significant differences in relative thermostability in barley extracts. The Sd2L enzyme is the most thermolabile, the Sd1, Sd4 and Sd5 enzymes exhibit intermediate levels of thermostability, the Sd2H enzyme exhibits high thermostability, and the Sd3 ß-amylase is the most resistant to thermal inactivation. The relative thermostability of the Sd1, Sd2L, Sd2H and Sd3 enzymes is maintained after germination and the associated carboxy-terminal processing. The Sd4 and Sd5 enzymes exhibit higher thermostability than the Sd1 enzyme after carboxy-terminal proteolysis. Significantly, the 5 day germinated barley samples contain a mixture of the mature ß-amylase enzyme and the proteolytically cleaved form, therefore the relative thermostability of the Sd4 and Sd5 enzymes shown in Figure 4.5 may be underestimated due to the presence of the more labile uncleaved forms of ß-amylase. The difference in thermostability between the 59.6 kDa and 55.5 kDa forms of the Sd5 enzyme suggests a role for the carboxy-terminal region in the thermal inactivation of barley ß-amylase. Determining the extent of proteolysis of the Sd4 ß-amylase may be even more informative. Analysis of the sequence divergence between the alternative forms of ß-amylase may suggest a mechanism of thermal inactivation and identify key regions in stabilising barley ß-amylase.

The structure of intron III in the Bmy1 gene influences the level of expression (Errkila *et al.* 1998). Analysis of *Bmy*1 intron III in a range of barley cultivars revealed the *Bmy*1-Sd1 and -Sd2H alleles contain the standard length intron, while the *Bmy*1-Sd2L allele exhibits the 126 bp insertion reported by Errkila *et al.* (1998). The association of the Sd2L allele with lower expression levels has subsequently been confirmed (Coventry *et al.* 1999). This is consistent with the absence of the *Bmy*1-Sd2L allele in barley varieties known to exhibit high levels of ß-amylase activity (Chapter 3). The *Bmy*1-Sd4 allele from *H. spontaneum* also contains the 126 bp insertion in intron III, and could therefore be expected to confer similar

activity levels to the *Bmy*1-Sd2L allele. The *Bmy*1-Sd3 and *Bmy*1-Sd5 alleles from *H. spontaneum* contain the standard length intron III, suggesting they would confer high expression levels similar to the *Bmy*1-Sd1 and Sd2H alleles.

Bulked segregant populations were developed to test the contribution of the *Bmy*1-Sd4 and –Sd5 alleles to ß-amylase activity. As expected, there was no difference between the *Bmy*1-Sd2L and Sd4 alleles, and there was an apparent increase in activity conferred by the *Bmy*1-Sd5 allele, however the difference was not statistically significant. The variation in enzyme activity within populations was too large relative to variation between populations to unequivocally identify any difference in activity conferred by the *Bmy*1-Sd5 allele. Wide crosses are more likely to yield transgressive segregation due to the possibility of both parents contributing favourable and unfavourable alleles to a pleitropic trait. Therefore the application of more closely related parents (as used by Swanston, 1980) could be expected to increase the resolution of bulked segregant analysis for quantitative traits. Due to the genetic distances between cultivated and wild barley it may be beneficial to perform bulked segregant analysis on lines derived from at least a $BC_1$ generation, analogous to the recently described advanced backross QTL strategy (Tanksley and Nelson 1996).

The transgressive segregation observed for ß-amylase activity and the significant variation in activity at any given level of grain nitrogen for each *Bmy*1 allele (Figure 4.6) indicates ß-amylase expression is subject to pleitropic effects. Therefore the differences in intron III only explain a percentage of the variation in barley ß-amylase activity. This notion is supported by results from QTL mapping, which show several genomic regions are typically associated with ß-amylase activity, and only the QTL on chromosome 1H is related to grain protein (Hayes *et al.* 1993, Mather *et al.* 1997). Therefore the ability of genotypic selection to

replace phenotypic selection for barley ß-amylase activity will be limited unless the genetic control of ß-amylase expression is further characterised.

The alternative forms of barley ß-amylase reported in this study are valuable resource for further investigation into the fundamental properties of barley ß-amylase including the biochemical basis for variation in free/bound fractions and differences in thermostability. The identification of the Sd3 ß-amylase with increased thermostability and potential for high expression levels may provide an opportunity to improve malt quality through conventional barley breeding.

# Chapter 5: Purification and Characterisation of Allelic Forms of Barley ß-amylase

## 5.1 Introduction

Barley ß-amylase is a key enzyme in the hydrolysis of starch for brewing and distilling. As previously discussed the thermolabile nature of ß-amylase increases the relative importance of the enzyme in malt quality (Chapter 1). The results presented in Chapter 4 provide strong evidence for the presence of allelic forms of ß-amylase that exhibit differences in thermostability. These conclusions are based on varietal comparisons and QTL mapping conducted on ß-amylase in crude extracts. Reproducing the thermostability variation with purified ß-amylase enzymes is required to demonstrate that these differences result from intrinsic properties of the enzymes.

ß-amylase is subject to carboxy-terminal proteolysis during germination. The cysteine endopeptidases have been shown to mediate the proteolytic release of the bound fraction of ß-amylase (Guerin *et al.* 1992), and these enzymes are synthesised *de novo* during germination (Hardie 1975). However, the structure of cleaved ß-amylase has only been examined in preparations from mature grain that were degraded by proteolytic enzymes present in mature barley grain during extraction (Lundgard and Svensson 1987). Analysis of the cleavage sites of ß-amylase purified from germinated barley is presented, and the proteolytic processing is compared between the Sd1 and Sd2L ß-amylase enzymes.

This Chapter also aims to provide sequence characterisation of the alternative enzymes. Barley ß-amylase cDNA sequences have previously been determined from cv. Hiproly (Kreis *et al.* 1987) and cv. Haruna nijo (Yoshigi *et al.* 1994). The sequences contain nine nucleotide differences resulting in three amino acid substitutions: Val233Ala, Leu347Ser, and Ile527Met. The authors present sequence homology with ß-amylase from

soybean and sweet potato as evidence that the sequence from cv. Haruna nijo is correct, but do not discuss genetic variation between cultivars as a possible source of sequence differences. More recently, three additional cDNA clones have been isolated, with the sequences from *H.v.*ssp. *spontaneum* PI296897 and the experimental line 86-H2-64 identical to cv. Haruna nijo, and the sequence from cv. Adorra consistent with that of cv. Hiproly (Erkkilä *et al.* 1998). The results presented in Chapter 3 show the Hiproly, Haruna nijo and Adorra cultivars all exhibit the Sd2 IEF band pattern, which is consistent with the amino acid substitutions not involving charged residues. Analysis of ß-amylase thermostability in crude extracts from these varieties show Hiproly and Adorra contain the low thermostability enzyme whereas ß-amylase from Haruna nijo is significantly more thermostable, suggesting the discrepancies in the sequence data may be responsible for the Sd2L and Sd2H phenotypes.

The *Bmy*1-Sd1 allele is present in a significant proportion of malting barley varieties (Tables 3.1 and 3.2). The amino acid sequence of the Sd1 ß-amylase was determined in this study, and the sequence of the Sd2L enzyme was confirmed. The thermostable Sd3 ß-amylase identified in an accession of *H.v.* ssp. *spontaneum* (Chapter 4) was purified from mature barley grain, and its amino acid sequence determined. The sequence differences between the four alternative forms of barley ß-amylase are discussed in terms of the functional characteristics of the respective enzymes and their evolutionary relationships.

A comparison of the primary structures of ß-amylases from higher plants shows the amino acid sequences exhibit more than 60% similarity (Svensson 1988). The three-dimensional structure of soybean ß-amylase has been determined at high resolution (Mikami *et al.* 1993, Mikami *et al.* 1994), and more recently the crystal structure of a mutant barley ß-amylase has been reported (Mikami *et al.* 1999). The two enzymes show a high level of structural homology, with an r.m.s. distance of 0.62Å (Mikami *et al.* 1999). ß-amylase forms a single domain comprising an $(\alpha/\beta)_8$ barrel core that has three long loops forming a deep

pocket close to the centre of the ß-barrel containing the active site. One wall of the pocket is formed by a flexible loop (L3) that closes upon substrate binding, shielding the catalytic groups and the reaction centre from the solvent. The same molecular architecture has been described for the monomeric form of sweet potato ß-amylase (Cheong *et al.* 1995). The soybean and mutant barley ß-amylase structures were used as the basis for the construction of a homology based model of barley ß-amylase. The sequence differences between the four forms of barley ß-amylase were localised within the structure and the role of amino acid substitutions on enzyme structure and stability were analysed. The amino acid differences between the four allelic forms of barley ß-amylase are discussed with respect to the functional differences between the enzymes.

## 5.2 Materials and Methods

### 5.2.1 ß-amylase Purification

Sd1 and Sd2L ß-amylase enzymes were purified from mature grain of *H. vulgare* cultivars Franklin and Schooner respectively (Evans *et al.* 1997). The proteolytically cleaved Sd1 and Sd2L enzymes were purified from commercially produced green malt of cultivars Franklin and Schooner (Joe Whites Maltings, Port Adelaide) (Evans *et al.* 1997). The Sd3 ß-amylase enzyme was purified from mature grain of *H.v.*ssp. *spontaneum* CPI 77146-33.

Barley grain was milled in a Glen Creston 10-800 grinder (Stanmore, England) and extracted with 0.1 M monothioglycerol (MTG), 10 mM EDTA, pH 5.0 for 2 h at 4°C with constant stirring. After settling, the supernatant was removed and the extraction repeated. The combined extracts were fractionated at 20-60% saturation of $(NH_4)_2SO_4$, dialysed against 15 mM Tris, 50 mM MTG, pH 7.0, and applied to a DEAE-cellulose DE-52 column (Whatman, Maidstone, England). ß-amylase activity was eluted isocratically with 15 mM Tris, 50 mM

MTG, 0.5 M NaCl, pH 7.0. ß-amylase preparations were concentrated and adjusted to pH 8.5 in an Amicon ultrafiltration cell using PM-30 membranes (Amicon, Massachusetts, USA) and applied to a DEAE-cellulose DE-52 column equilibrated in 15 mM Tris, 50 mM MTG, pH 8.5, and eluted with a 0.15 M NaCl gradient at 0.05% $min^{-1}$. Fractions containing ß-amylase activity were pooled and buffer exchanged into 50 mM acetate, 10 mM dithiothreitol (DTT), 0.1M NaCl, pH 5.0 and applied to a Bio-gel P60 column (Bio-Rad) equilibrated in the same buffer. The purified Sd2L enzymes from grain and malt were stored at -20°C in 10 mM acetate pH 5.0 containing 3 mM DTT after flushing with $N_2$. To avoid precipitation during freezing the purified Sd1 and Sd3 enzymes were stored at -20°C in 75 mM sodium carbonate pH 8.0, 5 mM DTT, 5% glycerol after flushing with $N_2$.

### 5.2.2 Electrophoresis

IEF samples of chromatographic fractions and purified ß-amylase were diluted in 1% glycine and 0.5 µg loaded per lane. IEF was performed as described in section 3.2.2. SDS-PAGE and immunoblotting were performed as described in section 4.2.4.

### 5.2.3 ß-amylase activity

Enzyme activity was determined using the Betamyl® assay (Megazyme, Ireland). Units of activity are defined as the amount of enzyme required, in the presence of excess α-glucosidase, to release one µmole of *p*-nitrophenol from *p*-nitrophenyl maltopentaoside $min^{-1}$ under the defined assay conditions (McLeary and Codd 1989).

### 5.2.4 Thermal inactivation

Purified enzymes were heat treated in 100 mM maleic acid, 1 mM EDTA, pH 6.2 containing 2.8 mg $mL^{-1}$ BSA. ß-amylase was added at a rate of 100µg $mL^{-1}$. The final protein concentration of 2.9 mg $mL^{-1}$ was the mean protein concentration of crude extracts used to analyse ß-amylase thermostability (Chapter 4) as determined by Bradford Coomassie Blue assay (Bio-Rad), using BSA as the standard. The rate of enzyme inactivation was monitored both by incubating samples for 5 min at temperatures from 40 to 65°C, and during a time course at 60°C. After heating, samples were chilled on ice and centrifuged at 10,000 g for 5 min at 4°C.

### 5.2.5 Protein sequencing

Prior to sequencing, enzyme preparations were further purified by reversed phase-HPLC on a 1090 LC (Hewlett-Packard, Rockville, IL, USA) with a 4.6 x 250 mm C-18 protein and peptide column, 300Å pore size (Vydac, Hesperia, CA, USA). Eluent A was aqueous 0.05% triflouroacetic acid (TFA), eluent B was 0.045% TFA in acetonitrile, the flow rate was maintained at 0.6 mL $min^{-1}$ and the protein eluted with a gradient of 2.5% B $min^{-1}$.

N-terminal protein sequencing was performed on a Hewlett-Packard G1000A sequencer with an on-line 1090 LC for PTH-amino acid analysis. C-terminal protein sequencing was performed by the Biomolecular Resource Facility, Australian National University, on an Applied Biosystems Procise Sequencer with online analysis of ATH-amino acid derivatives.

### 5.2.6 Amino acid analysis

Amino acid analysis was performed by Mr J. Lahnstein, University of Adelaide. ß-amylase concentrations and compositions were determined on an AminoQuant Series II® amino acid analyser (Hewlett-Packard) with reference to norvaline and sarcosine internal standards and with Pierce H amino acid standards (Pierce, Rockford, IL, USA) used as external standards. Sample derivatisation was performed with *o*-phthalaldehyde, 3-mercaptopropionic acid and 9-fluorenylmethylchloroformate by an automated precolumn method (Schuster 1988).

### 5.2.7 Peptide mapping

60 µg ß-amylase was reduced and alkylated in 5 mM DTT, 6 M guanidine-HCl, 100 mM Tris-HCl, pH 8.5. Samples were heated at 65°C for 30 min and cooled to room temperature followed by the addition of iodoacetic acid (40 µL, 108 mM in 1 M Tris-HCl, pH 8.5) prepared immediately before use. Alkylation proceeded for 20 min in the dark at room temperature. Excess iodoacetic acid was quenched by the addition of 60 µL 50 mM DTT. Alkylation mixtures were buffer exchanged into protease digestion buffer with 3K cutoff Nanosep™ centrifugal concentrators (Filtron, Northborough, MA, USA).

EndoLysC (Promega, Madison, WI, USA) digestions were performed in 6 M urea, 25 mM Tris-HCl, pH 7.0 at an enzyme to substrate ratio of 1:50. Hydrolysis was allowed to proceed overnight at room temperature and the digestion stopped by the addition of 0.5% TFA. LysC peptides were prepared for tryptic digestion by resuspending dried RP-HPLC fractions in 20 µL 4 M guanidine-HCl and adding 80 µL 50 mM $NH_4HCO_3$, pH 7.8. Modified sequencing grade trypsin (Promega) was added at an enzyme to substrate ratio of

1:25 and hydrolysis proceeded overnight at room temperature. Digestion was stopped by addition of 0.1% TFA.

Peptides were separated by reversed phase HPLC on a Hewlett-Packard 1090 LC with a 4.6 x 250 mm C-18 protein and peptide column (Vydac). Eluent A was 0.05% TFA, eluent B was 0.045% TFA in acetonitrile, and the flow rate was 0.6 mL $min^{-1}$ using a gradient of 0.6% B $min^{-1}$. Peptides eluted from the column were monitored by absorbance at 280 and 214 nm.

### 5.2.8 Mass spectrometry

Electrospray ionisation (ESI) mass spectrometry of peptides was performed on a Sciex API-3000 MS (PerkinElmer, Wellesley, MA, USA), calibrated with polypropylene glycol and operated in the positive ion mode. Peptides collected from RP-HPLC were dried and dissolved in 10μL 2.5% acetic acid, 50% acetonitrile in water. A volume of 5μL was loaded into an HPLC loop for flow injection with an eluent mixture of 2.5% acetic acid and 50% acetonitrile. The sample was loaded into the MS at a rate of 20 μL $min^{-1}$. Spectra were collected from m/z 500 to 2200 at 0.1 mass unit steps.

Purified protein and peptides that did not ionise under ESI-MS conditions were analysed by matrix assisted laser desorption ionisation (MALDI) mass spectrometry (Voyager, PerSeptive Biosystems, Framingham, MA, USA). Samples purified by RP-HPLC were concentrated and analysed in positive ion mode and using a sinapinic acid matrix.

### 5.2.9 Analysis of modified lysine

The elution positions of the PTH (phenylthiohydantoin) derivatives of Nε-methyl-lysine.HCl (Sigma, St Louis, MO, USA), Nε,Nε-dimethyl-lysine.HCl (Bachem, Bubendorf, Switzerland), Nε,Nε,Nε-trimethyl-lysine.HCl (Sigma), Nε-acetyl-lysine (Bachem), and δ-

hydroxy-lysine.HCl (Sigma) were determined on a Hewlett Packard model G1000A protein sequencer. Manual derivatisation was modified from that of Bidlingmeyer *et al.* (1984). 400 nmol of each amino acid was dissolved in 9 µL diisopropylethylamine/water/1-propanol (10:30:60). 21 µL 12% PITC in ethanol was added and reacted for 20 min at room temperature.

Samples were dried and redissolved in $CH_3CN$ to 10 pmol $\mu L^{-1}$. 100 pmol was loaded into the sequencer conversion flask and a standard conversion cycle run. Due to high signal strength only 60 pmol monomethyllysine was loaded. Chromatographic conditions were as specified by Hewlett Packard method PTH_3B.M. Manual derivatisation was also performed on mixtures of amino acid standards (Hewlett Packard) to demonstrate all PTH-derivatives were generated and eluted correctly.

### 5.2.10 Construction of three dimensional models

Homology based models of the three-dimensional structures of barley ß-amylase enzymes were generated using Swiss-Model software (Peitsch 1995, Peitsch 1996) based on the 2.5Å structure of a mutant barley ß-amylase (PDB identifier 1B1Y, Mikami *et al.* 1999), and energy minimised using Gromos96 (Biomolecular Software, Zurich, Switzerland). Structures were compared with the 2.2Å crystal structure of soybean ß-amylase (PDB identifier 1BYA, Mikami *et al.* 1993) and model quality was evaluated using What-If (Vriend 1990) and What-Check (Hooft *et al.* 1996) software. Molecular visualisation and analysis was performed using MolMol (Koradi *et al.* 1996) and Swiss-Pdb Viewer 3.5 (Guex and Peitsch 1997) software.

## 5.3 Results

### 5.3.1 Characterisation of the Cleaved Sd1 and Sd2L ß-amylase Enzymes From Germinated Barley

The differences between the Sd1 and Sd2L enzymes in isoelectric point and thermostability persist through proteolytic processing (Chapter 3). The differences in primary structure responsible for these characteristics must therefore be retained within the cleaved enzyme. The cleaved enzyme is approximately 4 kDa shorter than the full length form, potentially simplifying the determination of sequence differences by peptide mapping in addition to facilitating analysis of the proteolytic cleavage sites.

#### 5.3.1.1 Primary Structure Analysis

Protein sequencing of purified ß-amylase from germinated barley identified the proteolytic removal of methionine and glutamic acid from the $NH_2$-terminus of both the Sd1 and Sd2L enzymes (Figure 5.1). Direct sequencing of the first 104 amino acids failed to identify any variation between the Sd1 and Sd2L enzymes, with the amino acid sequence identical to published cDNA sequence for the Sd2L enzyme.

Carboxy-terminal sequencing identified three cleavage sites present in both the Sd1 and Sd2L enzymes. The major species terminated at G489 and two additional termination sites were identified at H483 and G496, at levels approaching the lower limits of detection, as shown in Figure 5.1. The cleavage at G489 delimits the precise end of the glycine rich repeats at the C-terminus of barley ß-amylase. The sequence data was characterised by high levels of background signal that was found in two independent preparations of each Sd1 and Sd2L ß-amylase enzymes. The high level of background indicates the presence of ragged-ends, consistent with incomplete proteolysis of the ß-amylase enzyme.

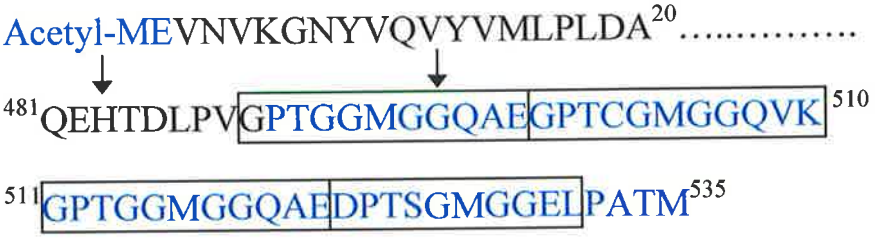



**Figure 5.1**: Representation of the proteolytic processing of barley ß-amylase during germination. Sequence in blue indicates peptides removed during processing, arrows indicate minor C-terminal termination sites. Boxed regions delimit the four glycine rich repeats.

### 5.3.1.2 Peptide Mapping of ß-amylase from Germinated Barley

Sd1 and Sd2L ß-amylase enzymes purified from germinated barley were analysed by peptide mapping. Digest conditions for both trypsin and endo Lys C were optimised for proteinase level, time, pH (Figure 5.2), chaotropic agents and cysteine derivatisation. Proteolysis was incomplete under all tested conditions, preventing baseline separation of peptides.

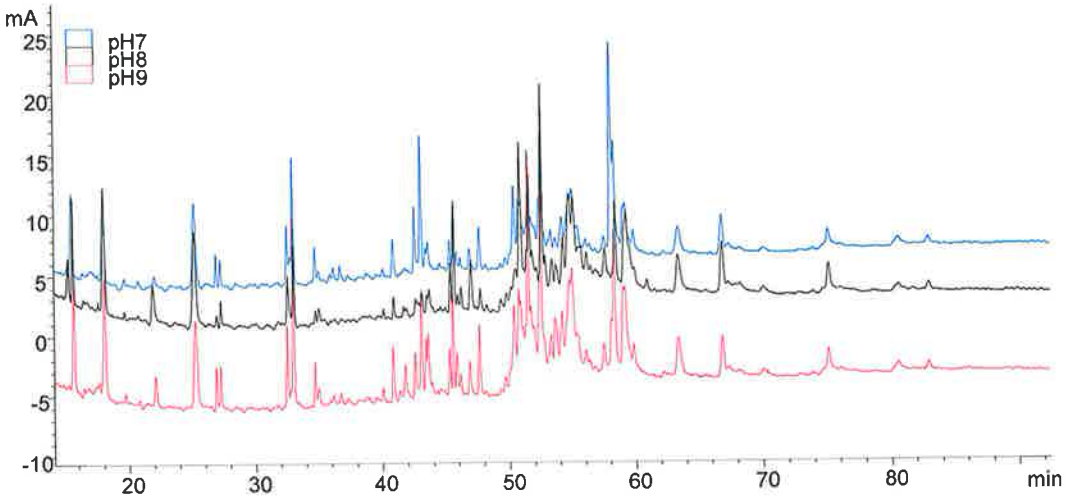



**Figure 5.2**: Reversed phase-HPLC separation of peptides resulting from endo-LysC digests of Sd1 malt ß-amylase at three pH levels.

Sequence analysis of isolated peptides revealed a non-standard amino acid within two different peptides corresponding to amino acid residues 270 and 433 in the ß-amylase sequence. Lysine is expected at these sites based on the cDNA sequence from cv. Hiproly (Kreis *et al.* 1987). The retention time of the novel PTH derivative was 13.20 minutes as shown in Figure 5.3. The peptides were not cleaved by endo Lys-C at the modified residue, as indicated by sequencing read-through. No secondary sequences were detected subsequent to the modified amino acid to suggest lysine cross-linking (data not shown).

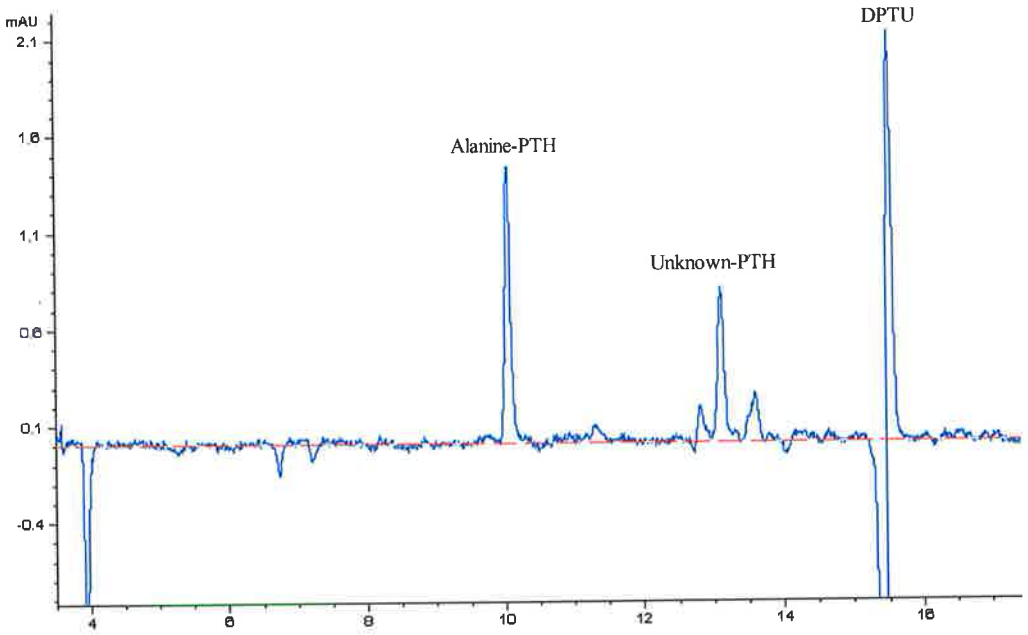



**Figure 5.3**: Retention time of the novel phenylthiohydantoin (PTH) derivative with respect to PTH-alanine and diphenylthiourea (DPTU).

Table 5.1 details amino acid analysis performed on the two purified enzymes compared to the expected amino acid composition, based on the published cDNA sequence cleaved at the sites determined by primary sequence analysis (as shown in Fig 5.1). The compositions of the two forms of ß-amylase show good agreement with each other and with the cDNA, with the exception of an overestimation of arginine in the Sd2L enzyme. Significantly, the levels of lysine detected in the two purified proteins are 45% lower than expected, consistent with the presence of post-translational modifications to lysine.

**Table 5.1:** Amino acid analysis of Sd1 and Sd2L ß-amylases purified from malt compared to the expected amino acid composition based on cDNA sequence. Values are the mean of three independent determinations. The observed and expected numbers of lysine are shown in bold.

| Amino Acid | Sd2L | Sd1 | cDNA[1] |
|---|---|---|---|
| ASX[2] | 60.5 | 62.1 | 56 |
| GLX[3] | 52.9 | 56.5 | 53 |
| SER | 22.0 | 24.4 | 21 |
| HIS | 12.4 | 13.9 | 14 |
| GLY | 36.4 | 37.2 | 40 |
| THR | 16.9 | 17 | 16 |
| CYS | 4.2 | 5.3 | 4 |
| ALA | 38.6 | 41.3 | 38 |
| ARG | 47.0 | 27.2 | 27 |
| TYR | 21.4 | 26.1 | 24 |
| VAL | 35.6 | 36.1 | 36 |
| MET | 11.2 | 14.6 | 14 |
| PHE | 22.6 | 24.2 | 23 |
| ILE | 20.2 | 21.1 | 22 |
| LEU | 38.8 | 40.8 | 40 |
| **LYS** | **13.7** | **13.1** | **21** |
| PRO | 31.2 | 30.3 | 31 |

1: cDNA sequence (Kreis *et al.* 1987) cleaved at the sites determined by N- and C-terminal protein sequencing.
2: Combined value for asparagine and aspartic acid, typically 10% higher than actual values.
3: Combined value for glutamine and glutamic acid.

### 5.3.1.3 Identification of Post-translational Modifications to Lysine

The epsilon amino group of lysine is highly reactive and subsequently a number of post-translational modifications of lysine have been characterised in native proteins. PTH derivatives for five of the most common lysine modifications were synthesised and their retention times are shown with respect to amino acid standards in Figures 5.4 and 5.5. The retention times of the PTH derivatives of monomethyl-, dimethyl-, and trimethyllysine were 17.10, 12.15 and 11.60 minutes respectively. The retention times of the PTH derivatives of acetyl- and hydroxylysine were 10.20 and 7.10 minutes respectively. The elution times of the

PTH derivatives of the modified lysine residues were not consistent with the retention time of 13.20 minutes observed for the modified form of lysine identified in ß-amylase from germinated barley (Figure 5.3).

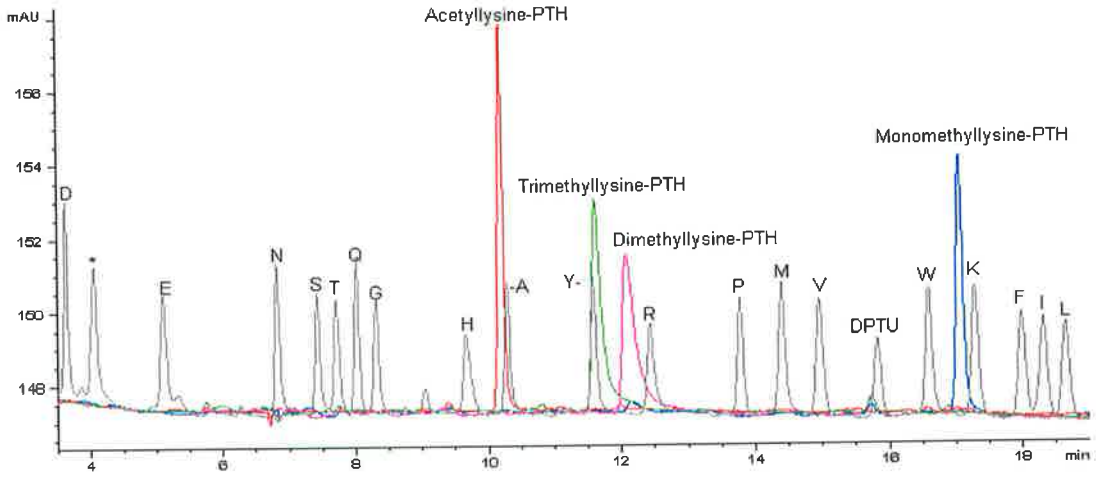



**Figure 5.4**: Retention times of PTH derivatives of acetyl-, trimethyl-, dimethyl-, and monomethyllysine with respect to standard amino acids.

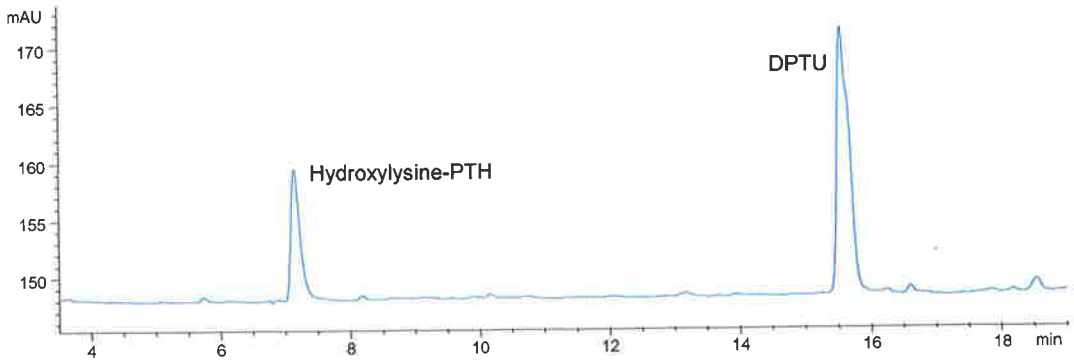



**Figure 5.5**: Retention time of the PTH derivative of hydroxylysine with respect to DPTU.

The presence of post-translational modifications to lysine residues and heterogeneous C-terminal proteolysis results in complex peptide maps (Fig 5.2). The ß-amylase enzymes purified from germinated barley are therefore unsuitable for

comparative peptide mapping. In order to determine the amino acid differences between the Sd1 and Sd2L enzymes, complete peptide maps with baseline separation of peptides are required to ensure all amino acid substitutions are detected. ß-amylase in mature grain has not been exposed to the high sugar concentrations generated during germination and has not been subjected to proteolysis, and therefore may be better suited to high resolution peptide mapping.

### 5.3.2 Purification and characterisation of ß-amylase enzymes from barley grain

Three forms of ß-amylase representing the range in thermostability profiles were purified from mature barley grain. Dr D.E. Evans (Evans et al. 1997) provided purified Sd1 and Sd2L enzymes. The low stability enzyme (Sd2L) was isolated from cv. Schooner and the intermediate stability (Sd1) enzyme from cv. Franklin. The thermostable Sd3 ß-amylase was purified from accession CPI 77146/33 of *H.v.* ssp. *spontaneum* as outlined below.

#### 5.3.2.1 Purification of the Sd3 ß-amylase From *H. spontaneum*

The purification procedure for isolating the Sd3 ß-amylase is shown in Figure 5.6. Mature barley grain was milled, extracted and subsequently fractionated by ammonium sulphate precipitation. The bulk of the ß-amylase activity was in the 20-60% cut, which was applied to a DEAE-cellulose DE-52 column at pH 7.0. Only trace levels of enzyme activity were detected in the unbound fraction, and the bound ß-amylase was eluted isocratically with 500mM NaCl. Concentrated ß-amylase preparations were applied to a DEAE-cellulose DE-52 column at pH 8.5 and eluted with a 150mM NaCl gradient. Fractions containing ß-amylase activity were diluted 1:5 with 1% glycine and 5µL analysed by IEF as shown in Figure 5.7.

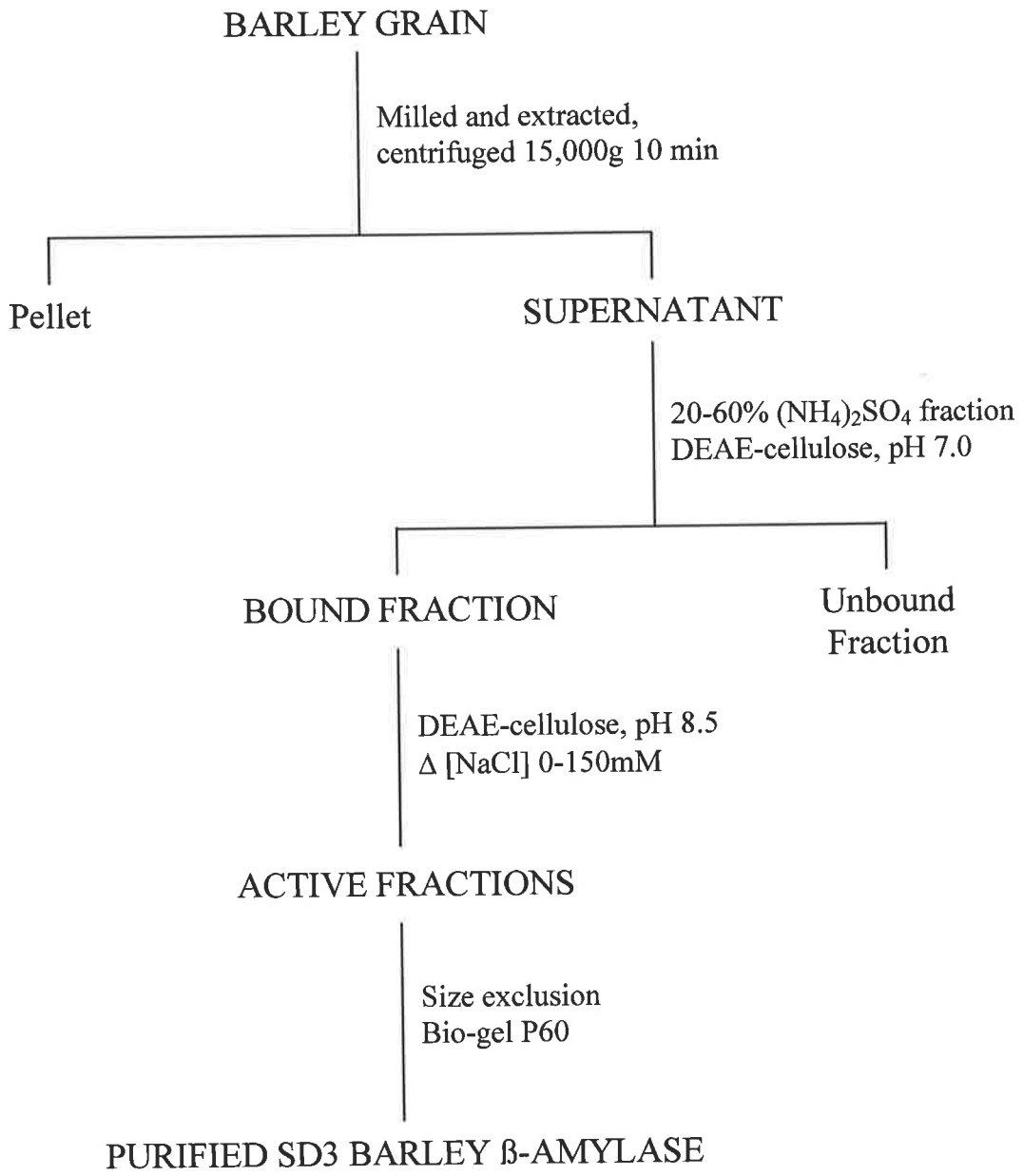



**Figure 5.6**: Purification schedule of the Sd3 ß-amylase from *Hordeum spontaneum* ssp. *vulgare* CPI 77146-33.

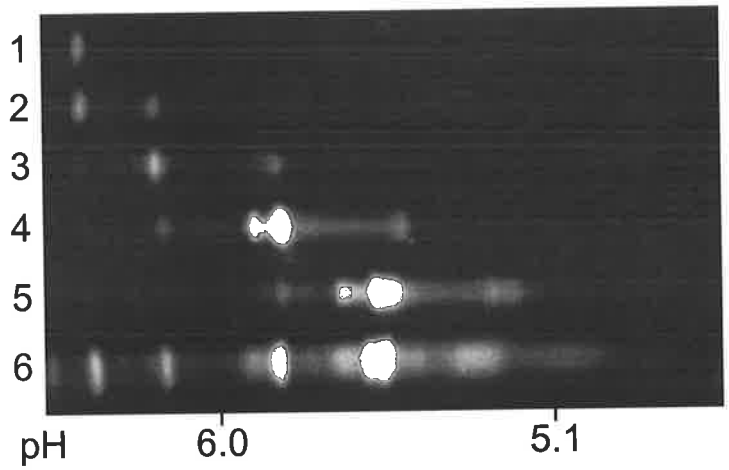



**Figure 5.7**: Isoelectric focusing of Sd3 ß-amylase fractions from anion-exchange chromatography. Lane 1: fraction 30, 2: fraction 37, 3: fraction 47, 4: fraction 51, 5: fraction 60, 6: crude extract. ß-amylase bands were detected by activity staining with starch/iodine.

The fractions exhibit differences in composition of ß-amylase isoforms. All fractions containing enzyme activity were pooled to yield a ß-amylase preparation consistent with the isoelectric heterogeneity observed in crude extracts (Figure 5.7, lane 6). No attempt was made to isolate the individual charged species. The pooled fractions were further purified by size exclusion chromatography on a Bio-gel P60 column (Figure 5.8). ß-amylase elutes as a single major peak, with baseline separation from a minor contaminant species eluting at fraction 60. The purified Sd3 ß-amylase exhibits an apparent molecular mass of 60 kDa and was free of contaminant species as determined by SDS-PAGE (Figure 5.9). The Sd3 ß-amylase was blocked to N-terminal protein sequencing, and no secondary sequences were detected. The molecular mass of the Sd3 enzyme was determined to be 59,568 Da by MALDI mass spectrometry.

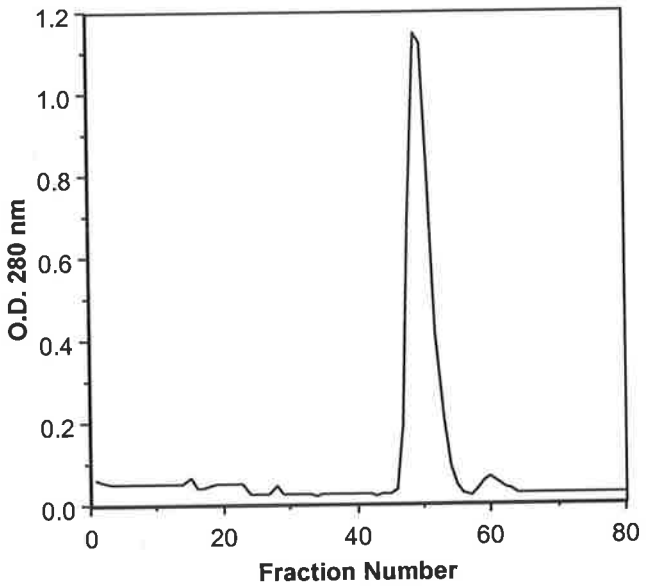



**Figure 5.8**: Size exclusion chromatography of ß-amylase fractions isolated from ion-exchange chromatography.

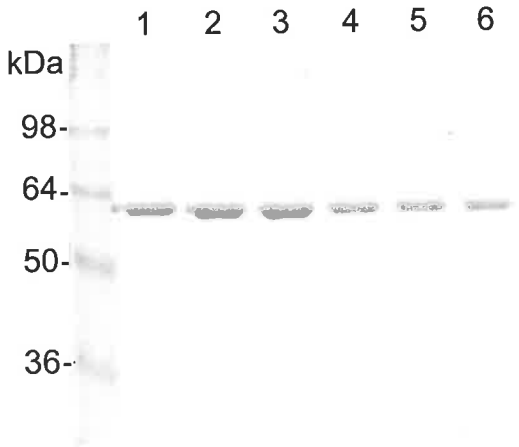



**Figure 5.9**: SDS-PAGE of selected fractions isolated from size exclusion chromatography stained with Coomassie Blue R250. Lanes 1-6 contain fractions 48-53 from Figure 5.8.

SDS-PAGE of the Sd1, Sd2L and Sd3 preparations after storage at -20°C revealed a minor polypeptide with a slightly lower molecular weight (Figure 5.10). The second species was not present immediately after purification but appeared after storage. The band exhibits immunological identity to anti-ß-amylase antibodies and is therefore considered to be a post-purification degradation product. Each purified enzyme remained blocked to N-terminal

protein sequencing and no secondary sequences were detected, suggesting the degradation is localised to the C-terminus of the proteins.

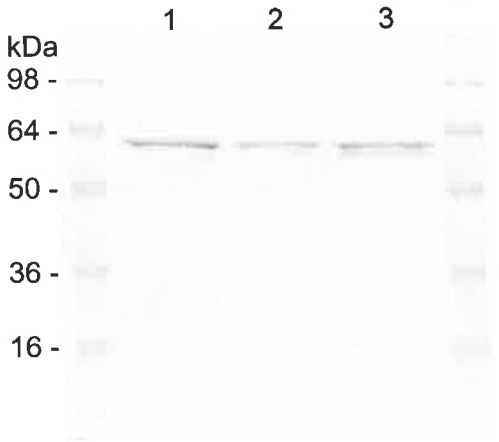



**Figure 5.10**: SDS-PAGE of the three purified forms of ß-amylase showing the production of a minor degradation product on storage at -20°C. Lane 1: Sd1, 2: Sd2L, 3: Sd3.

### 5.3.2.2 Thermostability of Purified ß-amylase Enzymes

The rate of thermal inactivation for the pure enzymes is shown in Figure 5.11. The Sd2L ß-amylase purified from cv. Schooner exhibited rapid thermal inactivation, consistent with results from crude extracts. The purified Sd1 ß-amylase shows an intermediate response to thermal inactivation, and the Sd3 ß-amylase remains the most thermostable form of the enzyme. The purified enzymes were incubated at a range of temperatures and the $T_{50}$ temperatures determined to be Sd2L = 56.8°C, Sd1 = 58.5°C, and Sd3 = 60.8°C. The thermostability profiles are consistent with results obtained from barley extracts, and as expected, the purified enzymes are less stable under these experimental conditions. For

example, after 10 min at 60°C only 10% of the purified Sd3 activity remains, compared to 35% in barley extracts (Chapter 4).

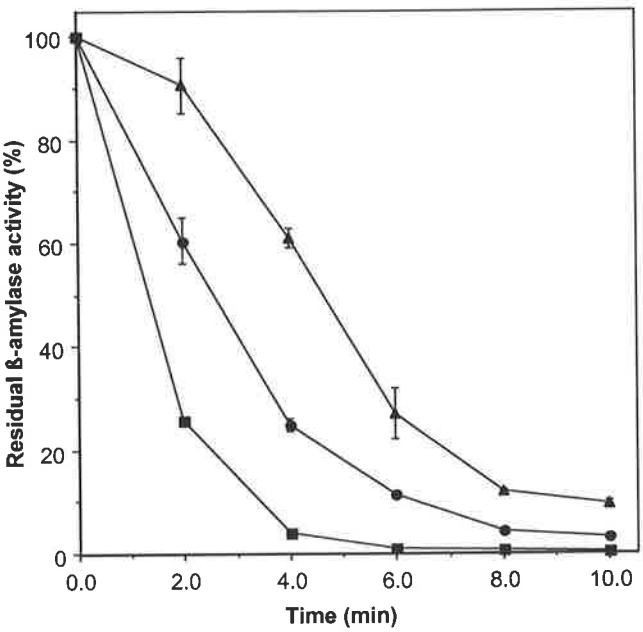



**Figure 5.11**: Irreversible thermal inactivation of purified barley ß-amylase enzymes incubated at 60°C. —●— Sd1, —■— Sd2-L, —▲— Sd3. Activity is expressed as a percentage of initial activity and the standard deviation of three independent determinations is shown.

### 5.3.2.3 Primary Structure Analysis

Protein sequencing revealed all three forms of ß-amylase to be blocked to Edman degradation, and no secondary sequences were detected. The amino acid sequence differences between the three forms of ß-amylase were determined by comparative peptide mapping. The ß-amylase enzymes were proteolytically digested and the resulting peptides were separated by RP-HPLC. Isolated peptides were analysed by mass spectrometry (MS) and peptides exhibiting differences in mass and/or retention time were sequenced. In all three peptide maps an additional small peptide not shown in Figures 5.12, 5.13 and 5.16 was identified, exhibiting a retention time of 19 minutes and a mass of 388.3 amu, consistent with the

expected Lys C fragment of residues 80 – 83. All expected Lys C peptides were identified with the exception of fragment 288 – 293, and the C-terminal peptide 511 – 535.

### 5.3.2.4 Comparative Peptide Mapping of the Sd1 and Sd2L ß-amylases

Peptide maps of the Sd1 and Sd2L enzymes are shown in Figure 5.12 and the ESI-MS analyses of the resulting peptides are detailed in Table 5.2. Peptide 3 exhibited a mass of 761.7, 42 amu higher than expected and was blocked to Edman degradation, consistent with the presence of N-acetyl methionine at the N-terminus of both Sd1 and Sd2L ß-amylase enzymes. Peak 14 in the Sd1 map contained a peptide 28 amu less than expected, and sequencing confirmed the mutation of valine at position 430 to alanine. The Sd1 peptide 15b exhibited significant shifts in retention time in addition to mass changes with respect to the homologous peptide from the Sd2L enzyme. Direct sequencing failed to identify any amino acid substitutions due to the length of the peptide. The peptide was digested with trypsin and

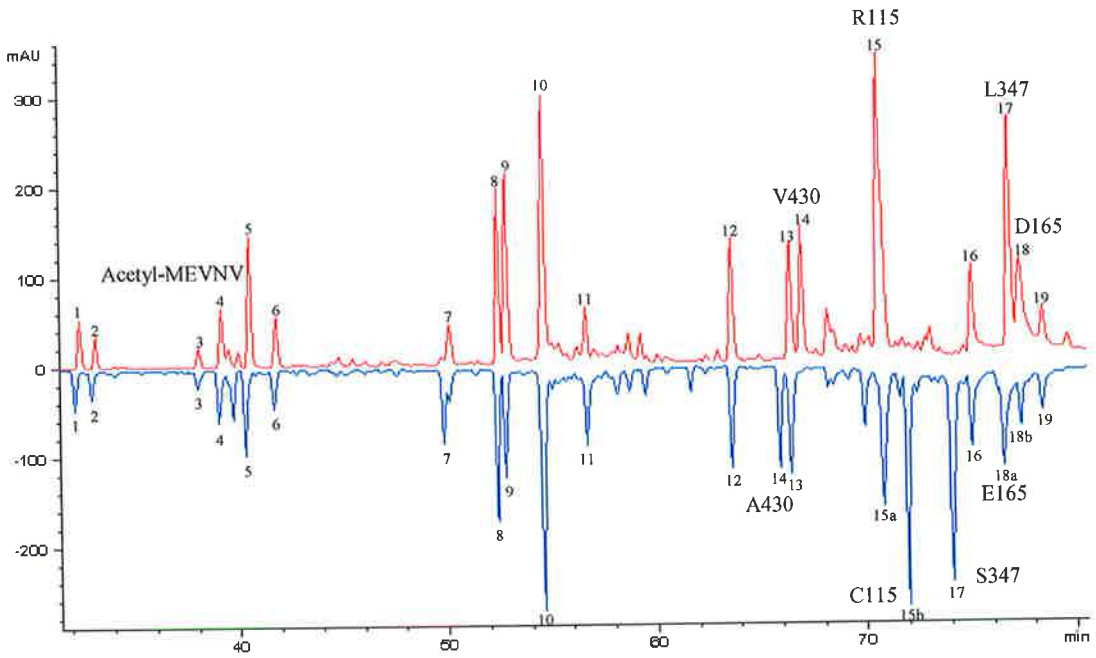



**Figure 5.12**: Comparison of endo Lys-C peptide maps of Sd1 and Sd2L ß-amylases separated by RP-HPLC detected at 214nm. The Sd1 map is shown as negative peaks (blue) and the Sd2L as positive peaks (red). Peptides containing amino acid substitutions are labelled. Peak 4 corresponds to the N-terminal peptide.

compared to a digest of the homologous Sd2L peptide (data not shown). Sequence analysis of the resulting peptides confirmed the Sd1 enzyme contained the mutation of arginine to cysteine at position 115, consistent with the 10 amu difference observed between the original peptides and with the decreased isoelectric point of the Sd1 ß-amylase.

**Table 5.2**: Analysis of Sd1 ß-amylase peptides by ESI-MS. Expected masses are derived from the cDNA clone from *Bmy*1-Sd2L variety Hiproly (Kreis *et al.* 1987), adjusted for carboxymethyl cysteine.

| Peak | MH+ (mass) | | Fragment | Mutation |
|---|---|---|---|---|
| | Observed | Expected | | |
| 1 | 1267.6 | 1267.64 | 271 - 281 | |
| 2 | 1186.0 | 1185.67 | 30 - 39 | |
| 3 | 761.7 | 719.39 | 1 - 6 | N-acetyl Met |
| 4 | 884.6 | 884.45 | 210 - 216 | |
| 5 | 1003.6 | 1003.45 | 64 - 71 | |
| 6 | 726.8 | 726.42 | 282 - 287 | |
| 7 | 1004.7 | 1004.58 | 72 - 79 | |
| 8 | 3319.0 | 3318.68 | 303 - 331 | |
| 9 | 1189.9 | 1189.62 | 294 - 302 | |
| 10 | 4530.0 | 4528.09 | 217 - 256 | |
| 11 | 3686.6 | 3683.69 | 475 - 510 | |
| 12 | 1728.4 | 1728.97 | 257 - 270 | |
| 13 | 2656.0 | 2655.36 | 7 - 29 | |
| 14 | 2723.0 | 2750.43 | 411 - 433 | Val430Ala |
| 15a | 4603.6 | 4601.35 | 434 - 474 | |
| 15b | 9241.9 | 9231.39 | 84 - 164 | Arg115Cys |
| 16 | 2541.2 | 2540.32 | 40 - 63 | |
| 17 | 8916.0 | 8942.32 | 332 - 410 | Leu347Ser |
| 18a | 5006.8 | 4990.34 | 165 - 209 | Asp165Glu |

Peptide 17 comprises residues 332 – 410 and sequencing identified the mutation of leucine to serine at position 347 and confirmed the 26 amu decrease in the Sd1 peptide. Direct sequencing of peptide 18a identified the mutation of aspartic acid to glutamic acid at position 165 of the Sd1 ß-amylase, consistent with the 16 amu difference observed between the homologous Sd1 and Sd2L peptides. The peak labeled 18b contained a large ß-amylase peptide beginning at position 165, and is presumed to result from incomplete proteolysis.

Peak 19 contains a polypeptide derived from the Lys C proteinase. A total of four amino acid differences were therefore detected in the Sd1 ß-amylase with respect to the Sd2L sequence.

### 5.3.2.5 Comparative Peptide Mapping of the Sd1 and Sd3 ß-amylases

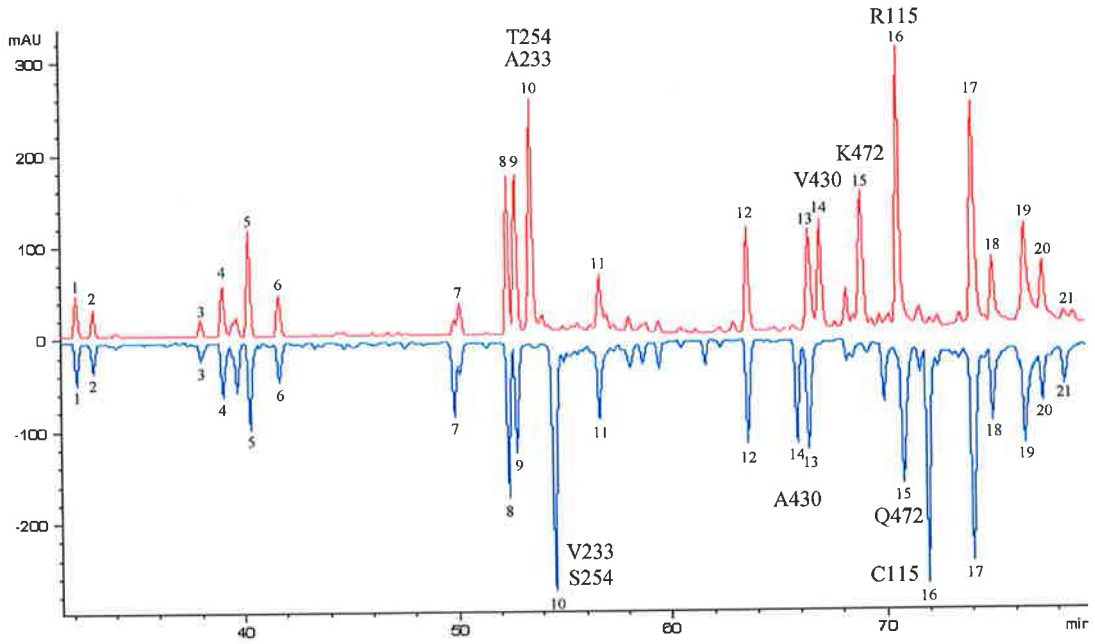



**Figure 5.13**: Comparison of endo Lys-C peptide maps of Sd1 and Sd3 ß-amylases separated by RP-HPLC detected at 214nm. The Sd1 map is shown as negative peaks (blue) and the Sd3 as positive peaks (red) and peptides containing amino acid substitutions are labelled.

The comparative peptide maps of the Sd1 and Sd3 ß-amylase enzymes are shown in Figure 5.13, and the characterisation of the individual peptides is detailed in Table 5.3. Peak 3 exhibited a mass 42 amu higher than expected and was blocked to Edman degradation, consistent with the presence of N-acetyl methionine at the N-terminus of both Sd1 and Sd3 enzymes. Direct sequencing failed to identify the amino acid substitutions in peptides 10 and 16, which were subjected to further peptide mapping (Figures 5.14 and 5.15). Analysis of peptide 14 identified the substitution of valine for alanine at position 430 in the Sd3 enzyme,

consistent with the 28 amu increase observed in the Sd3 derived peptide. Peptide 15 did not exhibit a significant mass difference compared to the homologous Sd1 peptide, however sequencing identified the mass conserved substitution of lysine for glutamine at position 472 of the Sd3 enzyme. This amino acid substitution was also consistent with the increased isoelectric point of the Sd3 ß-amylase. Peak 20 contained a large peptide that did not exhibit a mass consistent with any expected peptides. Sequence analysis confirmed the peptide originated from ß-amylase beginning at position 165 and resulted from incomplete proteolysis at Lys209. Peak 21 contained polypeptides derived from the endo Lys C proteinase.

**Table 5.3**: Comparison of Sd3 ß-amylase peptides with Sd1 peptides by ESI-MS.

| Peak | MH+ (mass) | | Fragment | Mutation |
|---|---|---|---|---|
| | Sd3 | Sd1 | | |
| 1 | 1267.9 | 1267.64 | 271 - 281 | |
| 2 | 1185.0 | 1185.67 | 30 - 39 | |
| 3 | 761.7 | 761.39 | 1 - 6 | N-acetyl Met |
| 4 | 884.5 | 884.45 | 210 - 216 | |
| 5 | 1003.8 | 1003.45 | 64 - 71 | |
| 6 | 726.7 | 726.42 | 282 - 287 | |
| 7 | 1004.2 | 1004.58 | 72 - 79 | |
| 8 | 3319.7 | 3318.68 | 303 - 331 | |
| 9 | 1189.9 | 1189.62 | 294 - 302 | |
| 10 | 4516.0 | 4528.09 | 217 - 256 | V233A,S254T |
| 11 | 3683.9 | 3683.69 | 475 - 510 | |
| 12 | 1728.9 | 1728.97 | 257 - 270 | |
| 13 | 2656.0 | 2655.36 | 7 - 29 | |
| 14 | 2751.0 | 2722.40 | 411 - 433 | Ala430Val |
| 15 | 4604.0 | 4601.35 | 434 - 474 | Gln472Lys |
| 16 | * | 9231.39 | 84 - 164 | Cys115Arg |
| 17 | 8916.0 | 8916.26 | 332 - 410 | |
| 18 | 2541.0 | 2540.32 | 40 - 63 | |
| 19 | 5006.8 | 5004.34 | 165 - 209 | |

* Confident mass assignment could not be made for peptide 16, additional peptide mapping (Figure 5.13) and sequencing confirmed C115R.

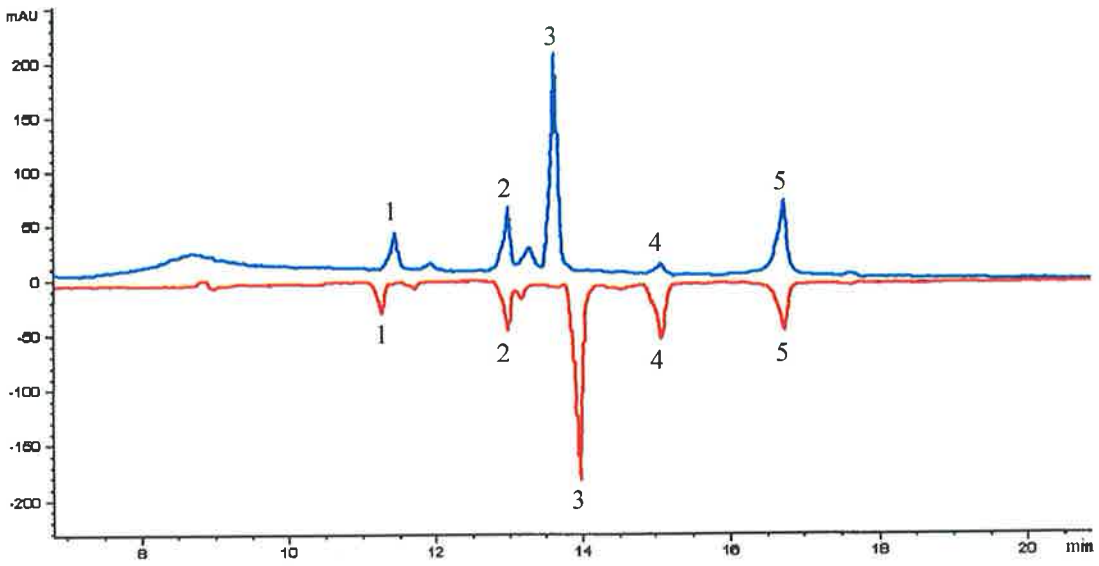



**Figure 5.14**: Comparison of tryptic digests of the peptide number 10 containing amino acids 217 - 256 from the Sd1 and Sd3 enzymes. Digests were separated by RP-HPLC and the eluent monitored at 214nm. The Sd1 map is shown as negative peaks (red) and the Sd3 peptide map as positive peaks (blue).

Peptide 10 from Figure 5.13 comprises residues 217 – 256, mass spectrometry identified a reduction of 12 amu in the Sd3 peptide, however direct sequencing was unable to identify sequence differences between the Sd1 and Sd3 enzymes due to the length of the peptide. The homologous Sd1 and Sd3 peptides were proteolytically cleaved with trypsin and the separation of the resulting peptides by RP-HPLC is shown in Figure 5.14. The isolated peptides were analysed by direct sequencing. Peptide 1 consists of residues 248 – 256, and includes the mutation of serine to threonine at position 254 in the Sd3 ß-amylase. Peak 2 contains the peptide from 243 – 247 that exhibits no differences between the Sd1 and Sd3 enzymes. Peak 3 contains the peptide from 217 – 242 and includes the substitution of valine for alanine at position 430. The two amino acid substitutions are consistent with the observed differences in the masses of the original peptides. Peak 4 contains residual uncleaved peptide 217 - 256, and peak 5 contains trypsin.

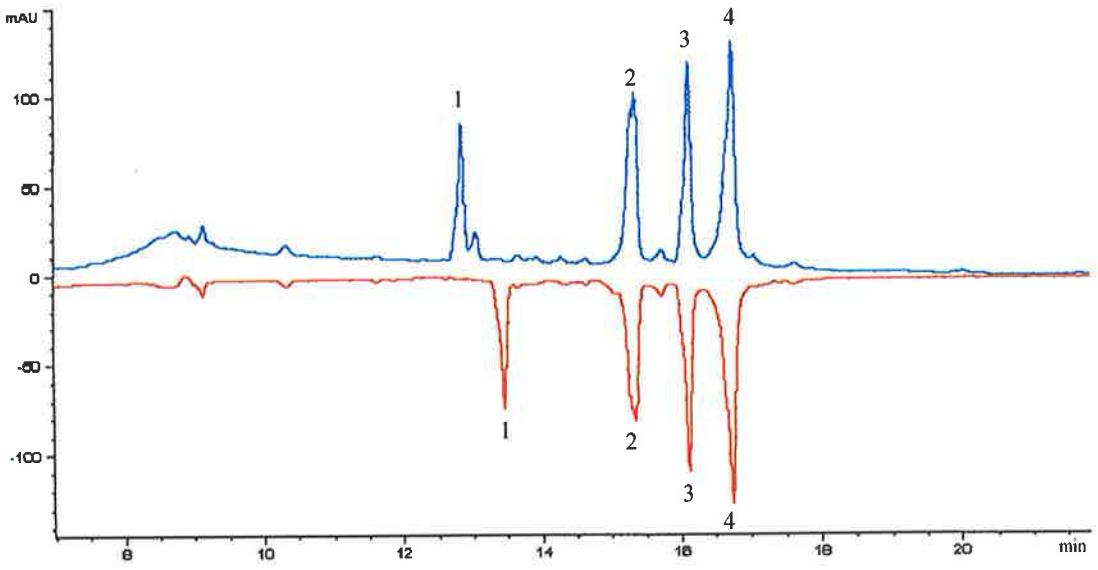



**Figure 5.15**: Comparison of tryptic digests of peptide 16 containing amino acids 84 - 164 from the Sd1 and Sd3 enzymes. Digests were separated by RP-HPLC and the eluent monitored at 214nm. The Sd1 map is shown as negative peaks (red) and the Sd3 peptide map as positive peaks (blue).

Peptide 16 from Figure 5.13 comprises residues 84 – 164. Analyses by mass spectrometry and direct sequencing were unable to identify sequence differences between Sd1 and Sd3 enzymes due to the length of the peptides. The peptides were proteolytically cleaved with trypsin and the separation of the resulting peptides by RP-HPLC is shown in Figure 5.15. There is a significant shift in retention time of the first peptide, and sequencing peptide 1 identified the substitution of arginine for cysteine in the Sd3 enzyme at position 115. The peptide consisted of residues 111-128 and was not cleaved by trypsin at arginine 115, presumably due to the inhibitory effect of aspartic acid at position 116. The amino acid substitution R115C is also consistent with the increased isoelectric point of the Sd3 ß-amylase. The remaining three peaks are identical in the Sd1 and Sd3 chromatograms, with peptide 2 comprised of residues 147 – 160, peptide 3 of residues 129 – 146, and peak 4 contained a peptide of residues 84 – 110 co-eluting with trypsin.

### 5.3.2.6 Comparative Peptide Mapping of the Sd2L and Sd3 ß-amylases

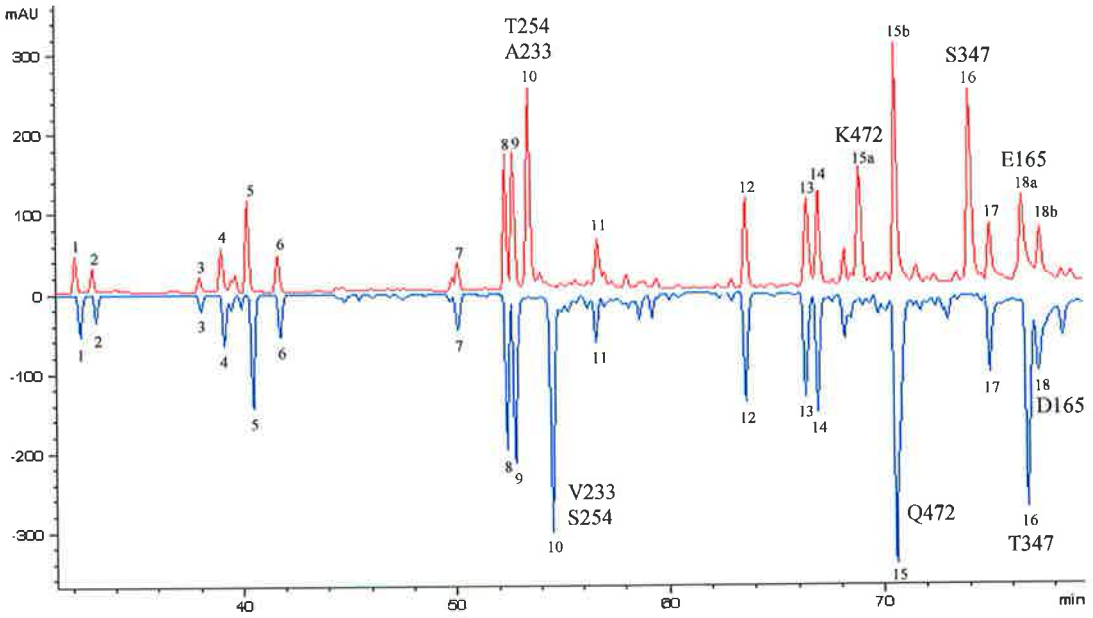



Figure 5.16: Comparative endo Lys-C peptide maps of Sd2L and Sd3 ß-amylases separated by RP-HPLC monitored at 214nm. The Sd2L map is shown as negative peaks (blue) and the Sd3 as positive peaks (red) and peptides exhibiting amino acid substitutions are labelled.

The sequence differences between the thermostable Sd3 ß-amylase and the thermolabile Sd2L ß-amylase were determined by comparative peptide mapping as shown in Figure 6.16, and the analysis of the individual peptides is detailed in Table 5.4. The Sd3 and Sd2L enzymes both contain N-acetyl methionine in the N-terminal peptide (peak 3). Peptide 10 is comprised of residues 217 – 256 and further proteolysis with trypsin revealed the Sd3 homolog to contain mutations of valine 233 to alanine and threonine 254 to serine, consistent with the observed mass difference. The Sd3 peptide 15a was found to contain residues 434 – 474 and included the substitution of lysine for glutamine at position 472, which does not alter the mass of the peptide (Table 5.4), and is consistent with the increased isoelectric point of the Sd3 ß-amylase. The corresponding Sd2L fragment appears in peak 15, coeluting with

peptide 84 – 164. Peptide 16 is comprised of amino acids 332 – 410 and exhibited a reduction of 26 amu in the Sd3 digest, and sequence analysis confirmed the substitution of serine for leucine in the Sd3 ß-amylase. The Sd3 peak labelled 18a contains the peptide spanning amino acids 165 – 209 and exhibits a significant decrease in retention time compared to the Sd1 peptide labelled 18. Sequence analysis identified the substitution of glutamic acid for aspartic acid in the Sd3 ß-amylase at position 165, consistent with the 16 amu difference observed between the two peptides. The Sd3 ß-amylase therefore contains a total of five amino acid substitutions compared to the thermolabile Sd2L ß-amylase.

Table 5.4: Comparison of Sd3 ß-amylase peptides with masses derived from the Sd2L sequence (Kreis *et al.* 1987) adjusted for carboxymethyl cysteine.

| Peak | MH+ (mass) | | Fragment | Mutation |
|---|---|---|---|---|
| | Observed | Expected | | |
| 1 | 1267.9 | 1267.64 | 271 - 281 | |
| 2 | 1185.0 | 1185.67 | 30 - 39 | |
| 3 | 761.7 | 719.39 | 1 - 6 | N-acetyl Met |
| 4 | 884.5 | 884.45 | 210 - 216 | |
| 5 | 1003.8 | 1003.45 | 64 - 71 | |
| 6 | 726.7 | 726.42 | 282 - 287 | |
| 7 | 1004.2 | 1004.58 | 72 - 79 | |
| 8 | 3319.7 | 3318.68 | 303 - 331 | |
| 9 | 1189.9 | 1189.62 | 294 - 302 | |
| 10 | 4516.0 | 4528.09 | 217 - 256 | V233A,S254T |
| 11 | 3683.9 | 3683.69 | 475 - 510 | |
| 12 | 1728.9 | 1728.97 | 257 - 270 | |
| 13 | 2656.0 | 2655.36 | 7 - 29 | |
| 14 | 2751.0 | 2750.43 | 411 - 433 | |
| 15a | 4604.0 | 4601.35 | 434 - 474 | Gln472Lys |
| 15b | * | 9231.39 | 84 - 164 | |
| 16 | 8916.0 | 8942.32 | 332 - 410 | Leu347Ser |
| 17 | 2541.0 | 2540.32 | 40 - 63 | |
| 18a | 5006.8 | 4990.34 | 165 - 209 | Asp165Glu |

### 5.3.2.7 ß-amylase Sequence Analysis

The amino acid differences between the four allelic forms of barley ß-amylase are shown in Table 5.5, and compared to the homologous amino acids in the soybean ß-amylase. The Sd1 enzyme contains four mutations with respect to the thermolabile Sd2L enzyme. The substitution of R115C is consistent with the reduced isoelectric point of the Sd1 enzyme, and is likely to be responsible for the increased level of bound ß-amylase in *Bmy*1-Sd1 barley varieties. The Sd2H ß-amylase exhibits only two amino acids different to the Sd2L enzyme, neither of which alter the net charge of the proteins. The thermostable Sd3 ß-amylase contains five amino acid substitutions with respect to the Sd2L enzyme, including the substitution of Gln472Lys that is consistent with the increased isoelectric point of the Sd3 ß-amylase.

**Table 5.5**: Comparison of the amino acid substitutions between the four barley enzymes. The amino acids present at the homologous position in soybean ß-amylase are also shown.

| Position | Sd2L | Sd1 | Sd2H[1] | Sd3 | Soybean[2] |
|---|---|---|---|---|---|
| 115 | Arg | Cys | Arg | Arg | Leu |
| 165 | Asp | Glu | Asp | Glu | Asp |
| 233 | Val | Val | Ala | Ala | Ala |
| 254 | Ser | Ser | Ser | Thr | Thr |
| 347 | Leu | Ser | Ser | Ser | Ser |
| 430 | Val | Ala | Val | Val | Asn |
| 472 | Gln | Gln | Gln | Lys | Lys |
| 527 | Ile | Ile | Met | Ile | * |

1:Yoshigi *et al.* (1994)
2: Totsuka and Fukazawa (1993)
* The soybean ß-amylase terminates at position 489 of the barley sequence.

Soybean ß-amylase shares 67% identity and 80% similarity with the Sd2L enzyme from barley, but exhibits significantly higher thermostability (Yoshigi *et al.* 1995). A comparison of the amino acid substitutions between the barley enzymes (Table 5.5) indicates that as the level of thermostability increases, so does the homology to the soybean sequence. The only mutation in the Sd1 ß-amylase that is present in the soybean enzyme is L347S. The more thermostable Sd2H ß-amylase also contains L347S and V233A in common with the soybean enzyme. The highly thermostable Sd3 ß-amylase exhibits four mutations L347S, V233A, S254T, and Q472K in common with the soybean enzyme.

Examination of the amino acid sequences of ß-amylase from a range of species (Figure 5.17) further indicates that the thermolabile Sd2L form of the enzyme has diverged more recently. The amino acids at positions 115, 165 and 430 in the barley sequence are not conserved in the other species examined. However at position 347, the Sd2L barley ß-amylase is the only enzyme exhibiting leucine, with the remaining barley sequences and those from rice, rye, potato and soybean exhibiting serine at the homologous position. The mutation of A233V is restricted to the Sd2L and Sd1 enzymes, with the remaining ß-amylase sequences retaining alanine at this position. At position 254 the barley alleles exhibit serine, with the exception of the thermostable Sd3 enzyme, which contains threonine consistent with the ß-amylase sequence from other plant species. At position 472 the Sd3 enzyme exhibits lysine, which is also present in the enzyme from soybean and potato while the rice and rye sequences exhibit conservative mutations to the other basic residues (His and Arg respectively). The Sd1, Sd2L and Sd2H sequences all contain glutamine at this position. Based on these comparisons the thermostable Sd3 enzyme from wild barley is more closely related to an ancestral protein than the enzymes from cultivated barley, which appear to have diverged more recently.

```
          1                                                                80
Sd2L      V..N....VKGNYVQVYVMLPLDAVSVNNRFEKGDELRAQLRKLVEAGVDGVMVDVWWGLVEGKGPKAYDWSAYKQLFEL
Sd1       V..N....VKGNYVQVYVMLPLDAVSVNNRFEKGDELRAQLRKLVEAGVDGVMVDVWWGLVEGKGPKAYDWSAYKQLFEL
Sd2H      V..N....VKGNYVQVYVMLPLDAVSVNNRFEKGDELRAQLRKLVEAGVDGVMVDVWWGLVEGKGPKAYDWSAYKQLFEL
Sd3       V..N....VKGNYVQVYVMLPLDAVSVNNRFEKGDELRAQLRKLVEAGVDGVMVDVWWGLVEGKGPKAYDWSAYKQLFEL
Rice      MAGN....LLANYVQVNVMLPLDVVTVHNKFEKVDETRAQLKKLTEAGVDGIMVDVWWGLVEGKGPGSYDWEAYKQLFRL
Rye       MAGN....MLANYVQVYVMLPLDVVSVDNKFEKGDEIRAQLKKLTEAGVDGVMIDVWWGLVEGKGPKAYDWSAYKQVFDL
Potato    MAPIPGVMPIGNYVSLYVMLPLGVVNADNVFPDKEKVEDELKQVKAGGCDGVMVDVWWGIIEAKGPKQYDWSAYRELFQL
Soybean   MATSDSNMLL.NYVPVYVMLPLGVVNVDNVFEDPDGLKEQLLQLRAAGVDGVMVDVWWGIIELKGPKQYDWRAYRSLFQL

          81                                                               160
Sd2L      VQKAGLKLQAIMSFHQCGGNVGDAVNIPIPQWVRDVGTRDPDIFYTDGHGTRNIEYLTLGVDNQPLFHGRSAVQMYADYM
Sd1       VQKAGLKLQAIMSFHQCGGNVGDAVNIPIPQWVRDVGTCDPDIFYTDGHGTRNIEYLTLGVDNQPLFHGRSAVQMYADYM
Sd2H      VQKAGLKLQAIMSFHQCGGNVGDAVNIPIPQWVRDVGTRDPDIFYTDGHGTRNIEYLTLGVDNQPLFHGRSAVQMYADYM
Sd3       VQKAGLKLQAIMSFHQCGGNVGDAVNIPIPQWVRDVGTRDPDIFYTDGHGTRNIEYLTLGVDNQPLFHGRSAVQMYADYM
Rice      VQEAGLKLQAIMSFHQCGGNVGDIVNIPIPQWVRVVGVNDPDIFYTNRGGARNIEYLTLGVDDQPLFHGRTAIQMYVDYM
Rye       VHEAGLKLQAIMSFHQCGGNVGDVVNIPIPQWVRDVGATGPTFFYTNRSGTRNIEYLTLGVDDQPLFHGRTAVQMYADYM
Potato    VKKCGLKIQAIMSFHQCGGNVGDAVFIPIPQWILQIGDKNPDIFYTNRAGNRNQEYLSLGVDNQRLFQGRTALEMYRDFM
Soybean   VQECGLTLQAIMSFHQCGGNVGDIVNIPIPQWVLDIGESNHDIFYTNRSGTRNKEYLTVGVDNEPIFHGRTAIEIYSDYM

          161                                                              240
Sd2L      TSFRENMKDFLDAGVIVDIEVGLGPAGEMRYPSYPQSHGWSFPGIGEFICYDKYLQADFKAAAAAVGHPEWEFPN.DVGQ
Sd1       TSFRENMKEFLDAGVIVDIEVGLGPAGEMRYPSYPQSHGWSFPGIGEFICYDKYLQADFKAAAAAVGHPEWEFPN.DVGQ
Sd2H      TSFRENMKDFLDAGVIVDIEVGLGPAGEMRYPSYPQSHGWSFPGIGEFICYDKYLQADFKAAAAAVGHPEWEFPN.DAGQ
Sd3       TSFRENMKEFLDAGVIVDIEVGLGPAGEMRYPSYPQSHGWSFPGIGEFICYDKYLQADFKAAAAAVGHPEWEFPN.DAGQ
Rice      KSFRENMAEFLDAGVIVDIEVGLGPAGEMRYPSYPQSQGWVFPGIGEFICYDKYLEADFKAEAAKAGHPEWELPD.DAGE
Rye       ASFRENMKKFLDAGTIVDIEVGLGPAGEMRYPSYPQSQGWVFPGIGEFICYDKYLEADFKGAAAKAGHPEWELPD.DAGE
Potato    ESFRDNMADFLKAGDIVDIEVGCGAAGELRYPSYPETQGWVFPGIGEFQCYDKYMVADWKEAVKQAGNADWEMPGKGAGT
Soybean   KSFRENMSDFLESGLIIDIEVGLGPAGELRYPSYPQSQGWEFPRIGEFQCYDKYLKADFKAAVARAGHPEWELPD.DAGK

          241                                                              320
Sd2L      YNDTPERTQFFRDNGTYLSEKGRFFLAWYSNNLIKHGDRILDEANKVFLGYKVQLAIKISGIHWWYKVPSHAAELTAGYY
Sd1       YNDTPERTQFFRDNGTYLSEKGRFFLAWYSNNLIKHGDRILDEANKVFLGYKVQLAIKISGIHWWYKVPSHAAELTAGYY
Sd2H      YNDTPERTQFFRDNGTYLSEKGRFFLAWYSNNLIKHGDRILDEANKVFLGYKVQLAIKISGIHWWYKVPSHAAELTAGYY
Sd3       YNDTPERTQFFRDNGTYLTEKGRFFLAWYSNNLIKHGDRILDEANKVFLGYKVQLAIKISGIHWWYKVPSHAAELTAGYY
Rice      YNDTPEKTRFFTDNGTYVTEKGKFFLTWYSNKLIKHGDKILDEANKVFLGCRVQLAIKISGIHWWYRVPNHAAELTAGYY
Rye       YNDTPEKTQFFKENGTYLTEKGKFFLSWYSNKLIKHGDKILDEANKVFLGCRVQLAIKVSGIHWWYRVPNHAAELTAGYY
Potato    YNDTPDKTEFFRPNGTYKTDMGKFFLTWYSNKLIIHGDQVLEEANKVFVGLRVNIAAKVSGIHWWYKVENHAAELTAGFY
Soybean   YNDVPESTGFFKSNGTYVTEKGKFFLTWYSNKLLNHGDQILDEANKAFLGCKVKLAIKVSGIHWWYKVENHAAELTAGYY

          321                                                              400
Sd2L      NLHDRDGYRTIARMLKRHRASINFTCAEMRDLEQSSQAMSAPEELVQQVLSAGWREGLNVACENALPRYDPTAYNTILRN
Sd1       NLHDRDGYRTIARMLKRHRASINFTCAEMRDSEQSSQAMSAPEELVQQVLSAGWREGLNVACENALPRYDPTAYNTILRN
Sd2H      NLHDRDGYRTIARMLKRHRASINFTCAEMRDSEQSSQAMSAPEELVQQVLSAGWREGLNVACENALPRYDPTAYNTILRN
Sd3       NLHDRDGYRTIARMLKRHRASINFTCAEMRDSEQSSQAMSAPEELVQQVLSAGWREGLNVACENALPRYDPTAYNTILRN
Rice      NLDNRDGYRTIARMLTRHRACVNFTCAEMRDSEQSSEAKSAPEELVQQVLSAGWREGLHVACENALGRYDATAYNTILRN
Rye       NLDDRDGYRTIARMLTRHHASMNFTCAEMRHSEQSEEAKNAPEELVQQVLSAGWREGLHVACENALGRYDATAYNTILRN
Potato    NVAGRDGYRPIARMLARHHATLNFTCLEMRDSEQPAEAKSAPQELVQQVLSSGWKEYIDVAGENALPRYDATAYNQMLLN
Soybean   NLNDRDGYRPIARMLSRHHAILNFTCLEMRDSEQPSDAKSGPQELVQQVLSGGWREDIRVAGENALPRYDATAYNQIILN

          401                                                              480
Sd2L      ARPHGINQSGPPEHKLFGFTYLRLSNQLVEGQNYVNFKTFVDRMHANLP....RDPYVDPMAPLPRSGPEISIEMILQAA
Sd1       ARPHGINQSGPPEHKLFGFTYLRLSNQLVEGQNYANFKTFVDRMHANLP....RDPYVDPMAPLPRSGPEISIEMILQAA
Sd2H      ARPHGINQSGPPEHKLFGFTYLRLSNQLVEGQNYVNFKTFVDRMHANLP....RDPYVDPMAPLPRSGPEISIEMILQAA
Sd3       ARPHGINQSGPPEHKLFGFTYLRLSNQLVEGQNYVNFKTFVDRMHANLP....RDPYVDPMAPLPRSGPEISIEMILQAA
Rice      SRPHGINKNGPPEYKLFGFTYLRLSDELLEGQNYSTFKTFVKRMHANLV....SATNVDPLEPLQRSMPEMPIGKILQAA
Rye       ARPKGINENGPPQHKLYGFTYLRLSNELQEGQNYATFQTFVEKMHANLG....HDPTVDPVAPLERSKPEMPIEMILKAA
Potato    VRPNGVNLNGPPKLKMSGLTYLRLSDDLLQTDNFELFKKFVKKMHADLD..PSPNAISPAV..LERSNSAITIDELMEAT
Soybean   AKPQGVNNNGPPKLSMFGVTYLRLSDDLLQKSNFNIFKKFVLKMHADQDYCANPQKYNHAITPLKPSAPKIPIEVLLEAT

          481                          512
Sd2L      QPKLQPFPFQEHTDLPV......G
Sd1       QPKLQPFPFQEHTDLPV......G
Sd2H      QPKLQPFPFQEHTDLPV......G
Sd3       KPKLQPFPFQEHTDLPV......G
Rice      HPKLAPFPFDENTDLPV
Rye       RPKLEPFPFDKNTDLPVKDHTDVGDEVFLAPV
Potato    KGSR.PFPWYDVTDMPVD.....GSNPF...D
Soybean   KPTL.PFPWLPETDMKVD.....G
```

**Figure 5.17**: Comparison of the ß-amylase amino acid sequences determined for the four forms of the enzyme from barley with the sequences from rice, rye, potato and soybean. The polymorphic residues in the barley enzymes are in bold and underlined.

### 5.3.3 Structural Analysis of Barley ß-amylase

Three dimensional structures of the four forms of barley ß-amylase were developed based on the crystal structure of an engineered form of barley ß-amylase (BBA-7) resolved at 2.5Å resolution (Mikami *et al.* 1999). BBA-7 was based on a clone of the Sd2H enzyme with five N-terminal amino acids deleted, and methionine added to the N-terminus, presumably due to the *E.coli* translation initiation codon (ATG). The amino acid numbering in the model structures is based on the full-length barley sequence. The recombinant ß-amylase was also mutated at seven sites to include M185L, S295A, I297V, S350P, S351P, Q352D and A376S (Yoshigi *et al.* 1995), resulting in homology to the four naturally occurring forms of barley ß-amylase ranging from 97.3 to 98.1% positional identity.

The crystal structure of the engineered BBA-7 ß-amylase does not extend beyond position 504 of the amino acid sequence. This was due to the disordered structure of the extended C-terminal loop (Mikami *et al.* 1999), presumably resulting from the glycine-rich repeats which would be expected to impart high levels of main chain flexibility. For this reason, the barley ß-amylase models were developed based on the proteolytically cleaved form of the enzyme present in germinated barley, comprising residues 2 – 489, as determined in section 5.3.1.1.

#### 5.3.3.1 Evaluation of the Barley ß-amylase Structures

Molecular modeling using a template structure that is almost identical in primary sequence is expected to yield models that do not deviate significantly from the original structure. However, analysis of stereochemistry and database related checks were performed to ensure the structures were suitable for detailed molecular analysis. Homology based models were also constructed using the soybean ß-amylase structure as a template, and using

both soybean and BBA-7 ß-amylase simultaneously. The resulting structures were discarded in favor of the models based on only the BBA-7 enzyme.

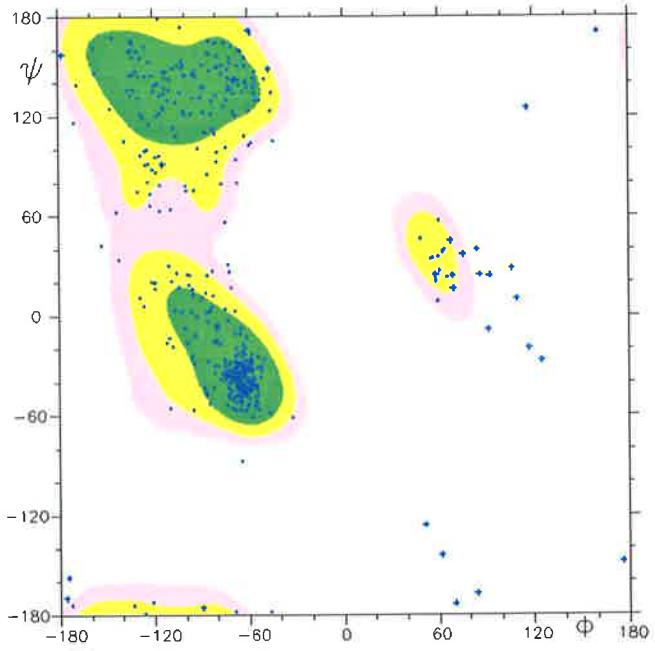



**Figure 5.18:** Ramachandran plot of the Sd3 ß-amylase. Glycine residues are indicated by plus symbols, all other residues are plotted as dots. The $\phi$ and $\psi$ angles of all residues are within allowed regions of low energy.

The RMS Z-scores for all improper dihedrals in the four barley ß-amylase structures were within normal ranges. All bond lengths were in agreement with standard bond lengths using a tolerance of 4σ, and bond length deviation was distributed normally from standard bond lengths (values taken from Engh and Huber 1991). Bond angles were also shown to deviate normally from mean standard bond angles (values taken from Engh and Huber, 1991). The RMS Z-scores of bond angles for the four structures ranged from 0.989 to 0.994, very close to the value of 1.0 expected for normally restrained data sets. Backbone conformation analysis also generated values consistent with well-refined protein structures. Ramachandran plots (Figure 5.18) of the main chain conformation angles showed that approximately 90% lie within the core region, and 100% within allowed regions (Ramachandran *et al.* 1963). As

expected from the very high level of homology, each of the four modeled structures are almost identical to the experimentally determined structure of the BBA-7 ß-amylase. The RMS distance between each of the four models and the original structure was 0.11Å for Cα atoms. Energy minimisation of the four barley ß-amylase structures resulted in final energies ranging from –27227 KJ $mol^{-1}$ for the Sd2H structure, to –26919 KJ $mol^{-1}$ for the Sd1 structure.

The average B-factor values for all buried protein atoms was outside the normal range in each of the four modeled structures. However the average atomic B-factors for buried atoms were also outside the expected range in the mutant BBA-7 ß-amylase, and high in the soybean ß-amylase structure resolved at 2.2 Å, suggesting this may be an intrinsic property of ß-amylase enzymes.

### 5.3.3.2 Molecular Localisation of Amino Acid Substitutions

The active site of ß-amylase is comprised of three long loops forming a deep pocket close to the centre of the carboxyl end of the ß-barrel. One wall of the pocket is formed by a flexible loop (L3) that extends into the solvent during substrate capture and product release, but closes upon substrate binding, shielding the catalytic groups and the reaction centre from the solvent (Mikami *et al.* 1994). The active site is shown in the open and closed conformations with loop L3 coloured red in Figures 5.19a and 5.19b, respectively.

The global structure of barley ß-amylase is represented in Figure 5.19c. The protein forms a single domain comprising an $(\alpha/ß)_8$ barrel core exhibiting extended loops on the C-terminal side of the ß-barrel, with each loop connecting the C-terminus of a ß-strand to the N-terminus of the next α-helix. The seven amino acid mutations identified between the four allelic forms of the enzyme are shown as ball and stick structures in Figure 5.19c. Each of the mutations occurs close to the surface of the protein and are distal to the active site.

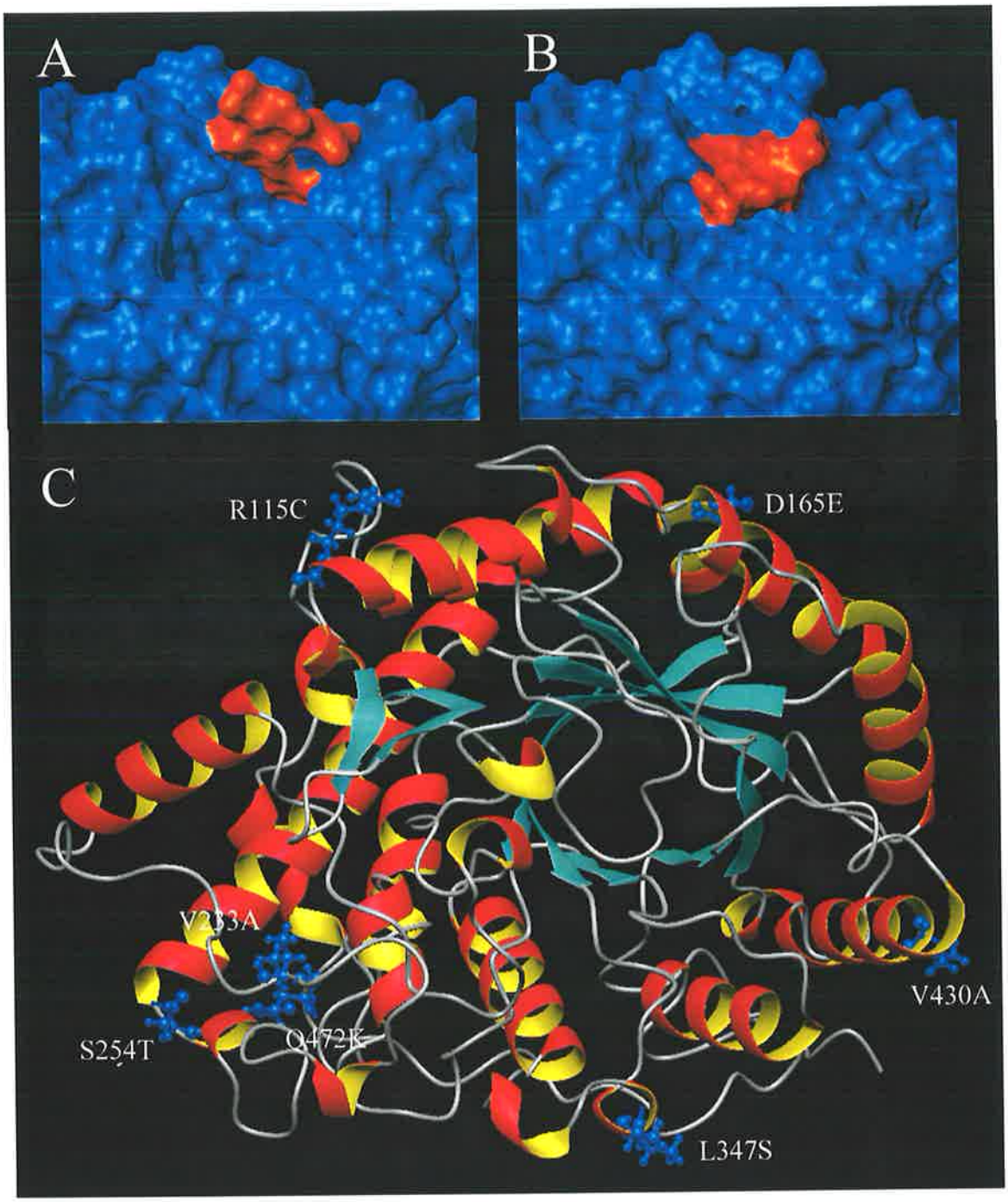



**Figure 5.19:** (A) View into the active site pocket of ß-amylase showing the van der Waals protein surface. The hinged loop L3 (designated red) is shown in the unliganded open conformation. (B) The hinged loop L3 in the closed conformation showing the van der Waals surface extending over the reaction centre, allowing the methyl groups of Val95 to interact with those of Leu381. (C) Ribbon representation of the Sd2L barley ß-amylase, looking towards the carboxyl end of the $(\alpha/ß)_8$ core with loop L3 centred above the barrel. The seven variant residues in the allelic forms of ß-amylase are shown as ball and stick structures.

Three of the mutations occur within α-helices or turns. The substitution Arg115Cys is in an α-helix within loop L3, and Asp165Glu and Val430Ala occur in α3 and α8 respectively. These three mutations do not result in changes to helix dipoles (Sali *et al.* 1988), helix capping (Richardson and Richardson 1988), or helix forming propensity (Chou and Fasman 1978). Therefore the mutations of Arg115Cys and Val430Ala in the Sd1 ß-amylase, and Asp165Glu in the Sd1 and Sd3 enzymes are not expected to contribute to the observed changes in thermostability. The substitution of Arg115Cys in the Sd1 enzyme occurs on the surface of the protein and the respective side chains project into the solvent, consistent with the decreased isoelectric point of the Sd1 enzyme. The side chain of Cys115 is not close enough to any other cysteine residue to allow the formation of an intramolecular disulphide bond, however the location of the side chain does allow the formation of an intermolecular disulphide bond.

The amino acid substitution of Val233Ala occurs in loop L4 which exhibits higher than average main chain flexibility. In the thermolabile Sd1 and Sd2L enzymes, the side chain of Val233 extends into a cavity delimited by the cyclic side chains of Phe204, Pro230, Phe245 and Phe246 (Figure 5.20a). The interatomic distances between CG1 of V233 and the β-carbon of Pro230, CG of Phe245 and CE2 of Phe204 are 3.2Å, 3.35Å and 3.34Å respectively. The substitution of the smaller side chain of alanine at position 233 in the thermostable Sd2H and Sd3 enzymes yields improved side chain packing into the cavity (Figure 5.20b), with the interatomic distances to the β-carbon of Ala233 increased to 4.01Å, 4.31Å and 3.83Å respectively. The mutation of Val233Ala is therefore expected to increase the thermostability by decreasing the entropy of refolding in the Sd2H and Sd3 enzymes.

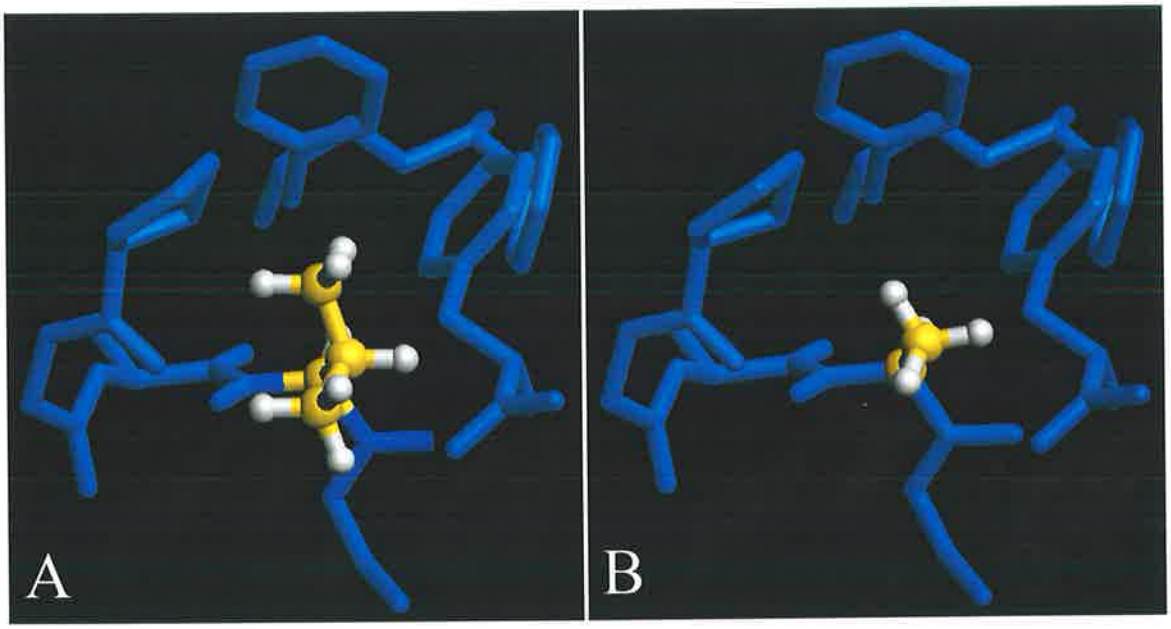



**Figure 5.20:** The spatial arrangement of amino acids (clockwise from the left) Pro230, Phe245, Phe246 and Phe204 with respect to (A) valine and (B) alanine at position 233.

The thermolabile Sd2L ß-amylase is the only enzyme to contain leucine at position 347 instead of serine. This position is within loop L6 which also exhibits high main chain flexibility. The side chains of the respective amino acids project directly into the solvent. Although the side chains are not involved in intramolecular hydrogen bonding, serine at this position is expected to stabilise the flexible loop through decreased surface hydrophobicity.

The amino acid substitution of Ser254Thr is present only in the thermostable Sd3 ß-amylase. This mutation also occurs within a flexible loop structure (L4), and is surface exposed. However, this substitution does not significantly alter the main chain conformation or influence hydrogen bonding, and represents a small increase in surface hydrophobicity. Therefore this mutation is not predicted to be responsible for the observed thermostability difference between the Sd2H and Sd3 enzymes.

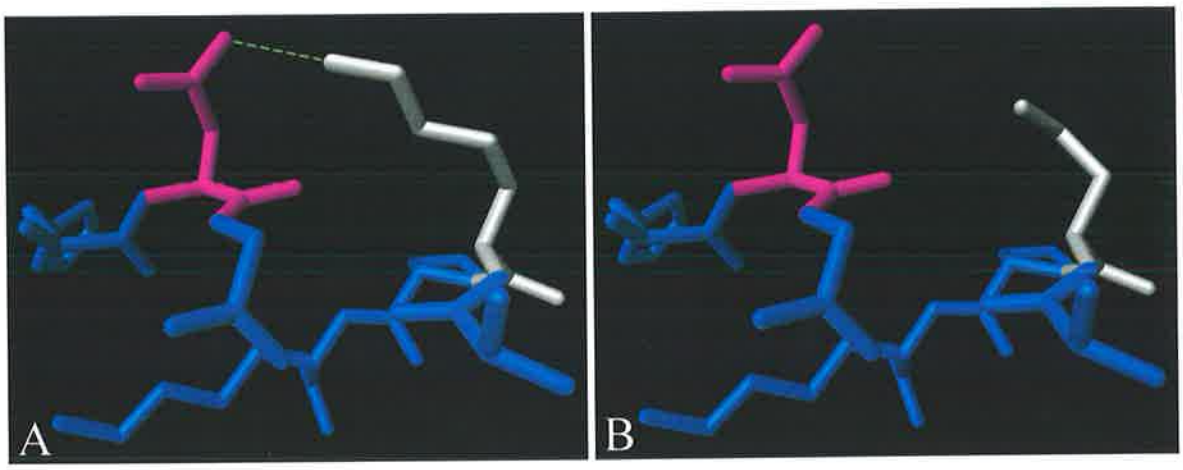



**Figure 5.21:** Interaction of aspartic acid 319 with lysine (A) and glutamine (B) at position 472 of barley ß-amylase. Aspartic acid is shown in magenta and residue 472 is in grey. The interchain hydrogen bond formed between NZ of Lys 472 and OD2 of Asp319 is shown as a green dashed line.

The amino acid substitution of Gln472Lys occurs in the highly flexible C-terminal loop of the thermostable Sd3 ß-amylase. The side chains of the two residues are surface exposed, therefore the presence of Lys472 is consistent with the observed increase in isoelectric point of the Sd3 ß-amylase. Figure 5.21 shows this substitution also results in the formation of an interchain hydrogen bond between NZ of Lys 472 and OD2 of Asp319 in loop L5. This additional hydrogen bond is concluded to be responsible for the increased thermostability of the Sd3 ß-amylase by decreasing the flexibility of the C-terminal and L5 loops, and is also the basis of the increased isoelectric point of the Sd3 enzyme.

## 5.4 Discussion

The proteolytic processing of barley ß-amylase was analysed by N- and C-terminal protein sequencing of Sd2L and Sd1 enzymes purified from 5 day germinated barley. The C-terminal sequence data exhibited high levels of background signal and two minor cleavage sites, indicating the presence of ragged ends from incomplete or non-specific hydrolysis. This is consistent with the stepwise nature of ß-amylase proteolytic processing. The most abundant C-terminus was at glycine 489, which precisely delimits the start of the glycine rich repeat region. This cleavage site has subsequently been shown to maximise the substrate affinity and thermostability of each form of barley ß-amylase (Ma *et al.* 2000). This differs from the sites G495, G506 and G517 cleaved by the cysteine endoproteinases EP-A and EP-B (Davy *et al.* 2000) and is also different to the cleavage by proteinases from mature grain (Lundgaard and Svensson 1987). These differences strongly support the view that there is a role in the post-translational modification of ß-amylase for proteinases other than or in addition to the cysteine endoproteinases (Lundgaard and Svensson 1987).

This is the first report of aminopeptidase processing of ß-amylase during germination, with the removal of $NH_2$-acetyl methionine and glutamic acid. The cleavage was very specific, with detailed analysis failing to detect any further degradation in two independent preparations of each of the Sd2L and Sd1 enzymes from 5 day germinated barley. Aminopeptidase enzymes have been purified from mature barley grain, however little is known about their *in vivo* substrates or substrate specificities (Doi and Kawakami 1996 and 1997).

Peptide maps generated from the Sd2L and Sd1 enzymes purified from germinated barley exhibited incomplete hydrolysis despite optimisation of the proteolysis conditions. Analysis of selected peptides revealed the presence of similarly modified lysine residues at positions 270 and 433. The epsilon amino group of lysine is highly reactive and a number of

post-translational modifications to lysine have been characterised (Lapolla *et al.* 1994, Deyl and Miksik 1997, Tsiboli *et al.* 1997). The naturally occurring modified forms monomethyl-, dimethyl-, trimethyl-, acetyl- and hydroxylysine were manually derivatised and shown to exhibit chromatographic behaviour different to the modified lysine at positions 270 and 433. Mass spectrometry of peptides containing the modified residues yielded molecular masses greater than 200 amu higher than expected, consistent with the presence of advanced glycation end products probably formed through the reaction of maltose with the epsilon amino group of lysine through Maillard reactions (Maillard 1912). Examination of the three-dimensional structure of barley ß-amylase shows the side chains of Lys270 and Lys433 are on the surface of the protein, facilitating post-translational modification. The high concentrations of reducing sugars in germinating barley result in significant production of Maillard products in malt and these are important determinants of beer flavour (Wittmann and Eichner 1989). Modification of the epsilon amino group of lysine residues may contribute to the charge heterogeneity observed in ß-amylase from germinated barley, and also the kinetic differences observed between purified and recombinant forms of the enzyme (Ma *et al.* 2000).

The Sd3 barley ß-amylase was purified to homogeneity from *H.v.* ssp *spontaneum* CPI 77146-33 and shown to have a molecular mass of 59,568 Da by MALDI-MS. The thermostability of the purified Sd2L, Sd1 and Sd3 enzymes was consistent with the results obtained from crude extracts. The Sd2L enzyme exhibited rapid denaturation, the Sd1 enzyme exhibited an intermediate level of stability, and the Sd3 ß-amylase was significantly more thermostable. The $T_{50}$ temperatures for the three purified enzymes were 56.8°C for the Sd2L enzyme, 58.5°C for the Sd1 enzyme, and 60.8°C for the Sd3 ß-amylase. The relative differences between the enzymes have subsequently been confirmed in an associated study (Ma *et al.* 2000).

Comparative peptide mapping of the three purified forms of barley ß-amylase revealed mutations to seven residues within the ß-amylase enzyme. All three forms of the enzyme were shown to contain acetyl-methionine at the N-terminus, consistent with the blocked N-terminal sequencing of each preparation. The sequence of the Sd2L enzyme was identical to the sequence deduced from cDNA clones isolated from cv. Hiproly (Kreis *et al.* 1987) and cv. Adorra (Errkilä *et al.* 1998). The Sd1 enzyme contained four substitutions of Arg115Cys, Asp165Glu, Leu347Ser, and Val430Ala. Subsequent analysis of genomic and cDNA sequences for the *Bmy*1-Sd1 allele have confirmed these mutations (Kaneko *et al.* 2000, Ma *et al.* 2001). The Sd3 enzyme contained five substitutions with respect to the Sd2L sequence, comprising Asp165Glu, Val233Ala, Ser254Thr, Leu347Ser, and Gln472Lys. Comparison of these sequences with the published Sd2H ß-amylase sequence (Yoshigi *et al.* 1994) and the sequences from soybean, rice, rye and potato suggests a correlation between thermostability and divergence from an ancestral enzyme. The low thermostability Sd2L enzyme differs from the soybean sequence at six of the seven variable residues, the Sd1 at six, the Sd2H at five, and the highly thermostable Sd3 at only three of the polymorphic residues. Therefore, in the absence of selection pressure for increased thermostability there has been an accumulation of random mutations in the *Bmy*1 gene that have decreased the structural stability of the enzyme. The presence of a 126 base pair insertion within intron III of only the Sd2L allele (Chapter 4) also supports the view that the low thermostability form has diverged most recently.

Homology based modelling procedures were successfully used to develop accurate three-dimensional structures of the allelic forms of barley ß-amylase. These structures were used to localise the amino acid substitutions and provide a complete structural basis for the functional differences between the enzymes. The Sd1 ß-amylase exhibits a decreased isoelectric point due to the substitution of cysteine for arginine at position 115. The side chain of Cys115 is surface exposed, allowing the formation of intermolecular disulphide bonds.

This structural feature fully explains the increased level of enzyme bound to the endosperm protein matrix via disulphides in barley varieties containing the Sd1 ß-amylase (Chapter 3). The slightly higher thermostability of the Sd1 enzyme is attributed to the substitution of leucine to serine at position 347, which results in decreased surface hydrophobicity. The presence of C115 has also been shown to be responsible for the increased affinity for starch in the Sd1 ß-amylase enzyme, however the authors conclude this mutation exerts its effect through the formation of an additional hydrogen bond (Ma *et al.* 2001). Analysis of the effect of the mutation R115C on electrostatic potential demonstrates a large change in surface charge (not shown), adjacent to the substrate binding sites determined by automated docking (Laederach *et al.* 1999). This major change in the surface electostatic potential is far more likely to have an effect on substrate specificity than the additional hydrogen bond suggested by Ma *et al.* (2001), which is a significant distance from the binding sites and does not alter the main chain conformation.

The Sd2H ß-amylase exhibits the same isoelectric point as the Sd2L enzyme, reflecting the lack of mutations to charged residues. It contains the favourable serine at position 347, but exhibits thermostability greater than the Sd1 enzyme due to the improved side chain packing of alanine at position 233 in place of valine. The role of the amino acid substitutions in stabilising the Sd1 and Sd2H ß-amylase enzymes has since been confirmed in site directed mutagenesis studies (Ma *et al.* 2001).

The Sd3 ß-amylase contains both favourable residues at positions 233 and 347, but exhibits a further increase in thermostability due to increased interchain hydrogen bonding in the C-terminal loop due to the mutation of glutamine to lysine at position 470. This amino acid substitution is also responsible for the increased isoelectric point of the Sd3 ß-amylase. Each of these amino acid changes are structurally conservative and do not significantly alter

the main chain conformation, therefore the stabilising mutations can be predicted to have simple additive effects.

Despite advances in protein engineering and structural biology, a set of general principles for predicting the outcome of specific mutations is yet to emerge. For many published examples of stabilising mutations there are also reports of similar substitutions resulting in destabilising effects. A growing body of evidence indicates a dominant factor in determining the effect of mutations is context, both in terms of the immediate structural elements and the protein as a whole. In the model protein T4 lysozyme it has been shown that structural stability is dominated by rigid components and that the flexible, solvent-exposed regions contribute little (Mathews 1993). This tolerance to change occurs because conformational flexibility often allows the protein to minimize the effects of a potentially deleterious substitution by locally readjusting to give an alternative structure that is energetically comparable to the wild-type (Mathews 1993). ß-amylase represents a significantly different structural class of proteins, and the results of mutagenesis based studies (Okada *et al.* 1995, Yoshigi *et al.* 1995), analysis of the flexible C-terminal loop of ß-amylase (Ma *et al.* 2000), taken in conjunction with the present study, suggests mutations within flexible regions of $(\alpha/ß)_8$ barrel proteins can significantly contribute to increases in stability.

# Chapter 6: The Impact of ß-amylase Alleles on Malt Quality and Applications in Barley Breeding

## 6.1 Introduction

A detailed review of the published literature addressing ß-amylase biochemistry and its influence on malt quality has suggested that its thermolabile nature may be a limiting factor in the hydrolysis of starch during brewing (Chapter 1). The subsequent Chapters have identified and characterised genetic variation for barley ß-amylase, and demonstrated significant differences in the thermostability of the alternative forms of the enzyme. The current Chapter includes an assessment of the impact of ß-amylase thermostability variation on starch hydrolysis in terms of malt quality.

Wort fermentability is a key functional specification for malt quality. Fermentability, measured as apparent attenuation limit (AAL), is a direct measure of the ability of the wort to be fermented by yeast. AAL is largely a measure of the level of fermentable sugars, however it may be influenced by a number of factors including nitrogen and micronutrient levels, but yeast typically converts all fermentable sugars into ethanol. In this Chapter, the effect of ß-amylase thermostability on fermentability was examined by QTL mapping of AAL in a mapping population segregating for the thermolabile (Sd2L) and thermostable (Sd2H) forms of ß-amylase. The levels of fermentable and nonfermentable sugars were also measured. If ß-amylase exerts a significant affect on these parameters, then a QTL is expected coincident with the *Bmy*1 structural gene. This hypothesis was further tested by an evaluation of the relationship between ß-amylase thermostability and AAL in commercial malts.

AAL is a relatively expensive and time consuming malt quality assay, and is therefore only applied to advanced lines in barley breeding programs. This form of evaluation severely limits the selection pressure that can be applied to breeding populations for this key trait. The

demonstrated role of ß-amylase in determining fermentability indicates that selection for specific *Bmy*1 alleles will provide effective selection for AAL. The development of methods to screen for ß-amylase could then be applied to larger population sizes, such as early generation lines and segregating populations. A suite of different *Bmy*1 screening methods were developed and evaluated, providing practical selection tools with a range of technical requirements and advantages to breeding programs.

The novel Sd3 ß-amylase identified in *H. spontaneum* is significantly more thermostable than the other forms of the enzyme (Chapter 4). This is expected to result in a greater capacity for starch hydrolysis under mashing conditions. This is particularly relevant in brewing practices using high levels of starch adjuncts, such as Japanese dry beers and Happoshu, which may use up to 75% starch adjunct (Inoue 1996). The Sd3 ß-amylase allele was introgressed into elite malting quality germplasm to begin the development of novel barley varieties specifically targeting these end uses.

## 6.2 Materials and Methods

### 6.2.1 Barley samples

A rapid ß-amylase thermostability test was used to analyse 179 elite lines from the 1998 Stage 3 trials of the South Australian Barley Improvement Program. The material was sourced from a range of sites including winter and summer nurseries to ensure a wide range of grain protein contents, which is typical of early generation trials.

Grain protein was determined by the SA Barley Improvement Program using an NIRSystems 6500 scanning spectrophotometer (NIRSystems Inc., Perstop Analytical, Silver Spring, MD, USA). Samples were scanned as whole grain and absorbance data was measured in reflectance mode from 400 – 2500 nm. All samples were scanned in duplicate. The spectra

were averaged and partial least squares regression applied to find the model best fitting the calibration against Kjeldahl protein determinations.

$BC_3$ lines derived from *Hordeum vulgare* ssp. *spontaneum* CPI 77146-33 (Sd3 ß-amylase, Chapter 4) and cv Clipper as the recurrent parent were provided by Dr Tony Brown, CSIRO Division of Plant Industry, Canberra. The SA Barley Improvement Program contributed to the barley hybridisations performed for the introgression of the Sd3 ß-amylase allele from *Hordeum vulgare* ssp. *spontaneum* CPI 77146-33.

### 6.2.2 Micromalting and malt quality analysis

Micromalting and standard malt quality analyses was performed by the Waite Malting Quality Evaluation Laboratory. Barley samples of 100g were micromalted in duplicate in an automated micromalting unit (Phoenix Systems, Adelaide, Australia) employing a standard malting program (Evans *et al.* 1997a). Wort fermentability was determined as apparent attenuation limit (AAL). Fresh yeast (S.A. Breweries, Adelaide, Australia) was shaken with wort prepared by the small scale EBC hot water extract (HWE) method (Analytica-EBC 1998) for 24 hours at 25°C. The specific gravity was measured before and after fermentation, and AAL calculated from the difference in the values.

### 6.2.3 Commercial Malt

Samples of 42 commercial malts with associated data for fermentability (AAL by EBC methods, Analytica-EBC, 1998) and diastatic power (by IOB methods, Recommended Methods of Analysis, 1997) were provided by Barrett Burston Malting Company Pty. Ltd., Melbourne, Australia. Haruna Nijo malt was supplied by Mr Ken Fukuda, Sapporo Breweries Ltd, Japan.

### 6.2.4 HPAEC analysis of wort sugar profiles

Sugar profiles were analysed using a DX-500 HPLC system (Dionex, Sunnyvale, CA, USA) fitted with a SIL-10AD autoinjector (Shimadzu, Kyoto, Japan) and an ED-40 pulsed amperometric detector (Dionex). Fermentable and nonfermentable sugars were separated on 250 x 4 mm Carbopac PA1 and PA10 columns respectively, using linear sodium acetate gradients at a flow rate of 0.6 mL $min^{-1}$. Data was analyzed with Peaknet Chromatography Workstation version 4.3 (Dionex).

Glucose, fructose, sucrose, maltose, maltotriose, maltotetraose and maltohexaose were used as standards. Wort samples were centrifuged and diluted in deionised water before injection. Wort was diluted 1:200 for analysis of fermentable sugars and diluted 1:5 for analysis of non-fermentable sugars. Samples were transferred to 96 well ELISA plates for automated injection of 10μL per sample.

### 6.2.5 Molecular Mapping

Apparent attenuation limit and levels of fermentable and nonfermentable sugars were mapped in the Galleon x Haruna nijo population using the methods described in Section 3.2.5.

### 6.2.6 ß-amylase Thermostability Assay

A method was developed to assess ß-amylase thermostability with cost and throughput appropriate for application within breeding programs. Extracts were prepared by incubating 100mg samples of barley flour with 1.0mL extraction buffer containing 100mM maleic acid, 1mM disodium EDTA, 0.02% sodium azide, 1mg/mL BSA (Sigma, St Louis, U.S.A.), 0.02% $NaN_3$ and 143mM 2-mercaptoethanol for 1hr at room temperature with constant mixing. Samples were then incubated at 60°C for 10 min, chilled immediately on ice and centrifuged at 10,000g for 5 min at 4°C. A 1:150 dilution (using extraction buffer

minus reducing agent) was performed in 1mL x 96 well Beckman blocks (Beckman, San Diego, CA, USA). 50µl from each cell was then transferred to a second Beckman block for enzyme assay. Enzyme activity was determined by the addition of 50µl of the substrate *p*-nitrophenyl maltopentaoside (PNPG5, Megazyme). One unit of ß-amylase activity is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute in the presence of excess α-glucosidase under the defined assay conditions. After 10 min, the assay was stopped by the addition of 750µl 1%Trisma base. 300µl aliquots of each sample were transferred to an ELISA plate, and absorbance values read at 405nm in a model 3355 microplate reader (Bio-Rad).

### 6.2.7 PCR Analysis of *Bmy*1 Intron III

PCR analysis of Bmy1 intron III was performed by the method of Errkila *et al.* (1998), as described in Section 4.2.8.

### 6.2.8 CAPS Assays

The cleaved amplified polymorphic site (CAPS) assays were performed by Mr Maxime Paris, Murdoch University, WA. The *Msp* I restriction site, C↓CGG, is present in the genomic sequences of both Sd2H and Sd3 alleles and absent in both Sd2L and Sd1 alleles. Primers *Bmy*1 2142F 5'-GCA CGA TAA TAT ATA CCA TTG CC-3' and β-amy 2413R 5'-TTG TTG GAG TAC CAT GCA AGG AA-3' and β-amy 2413R were used to amplify the region containing the restriction site to yield a 271 bp fragment. Digests were performed by adding 25 µL of a digestion mixture (2.5 units *Msp* I, 400 mM NaCl, 40 mM $MgCl_2$, 160 mM DTT) to 15 µL of amplified product followed by incubation at 37°C for two hours. Restriction products were resolved by electrophoresis on 2% agarose gels.

### 6.2.9 SNP Analysis

Single nucleotide polymorphism (SNP) assays were developed in conjunction with this project by Mr Maxime Paris, Murdoch University, WA (Paris and Eglinton 2002). Briefly, the four alleles were identified by genotyping two SNPs using a duplex Single Nucleotide Primer Extension (SNuPE) assay (Figure 6.1). Two genotyping primers with their 3' ends directly flanking the selected SNPs were annealed to the amplified target sequences and extended by single dideoxynucleotides complementary to the polymorphic nucleotides. Extended primers were analysed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) and incorporated nucleotides were identified by the increase in mass of the extended primers.

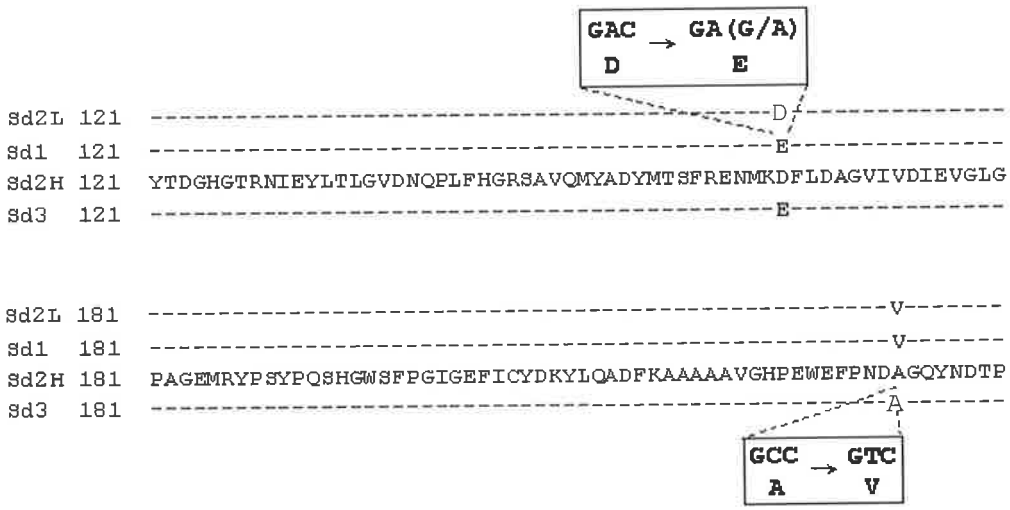



**Figure 6.1:** Section of the β-amylase amino acid sequence used in the duplex SNuPE assay enabling identification of β-amylase alleles.

## 6.3 Results

### 6.3.1 Impact of ß-amylase Alleles on Wort Fermentability

Wort fermentability is an important functional parameter for malt quality that is gaining priority in Australia's export markets. Fermentability reflects the level of fermentable sugars available to the yeast for alcohol production. Fermentability of malt extracts is typically assessed by determining the change in specific gravity after a small scale fermentation, and is referred to as the apparent attenuation limit (AAL).

#### 6.3.1.1 Molecular Mapping of AAL

The Galleon x Haruna nijo mapping population was micromalted and analysed for AAL. Wort samples were also analysed by HPAEC to quantitate fermentable sugars (glucose, fructose, sucrose, maltose, maltotriose – shown in Figure 6.2) and non-fermentable sugars (maltotetraose and larger linear and branched maltodextrins). Correlation analysis showed that there was no simple relationship between AAL and the assessed sugars. Adjusting the fermentable sugar levels to correct for differences in malt protein gave no improvement in the regression results.

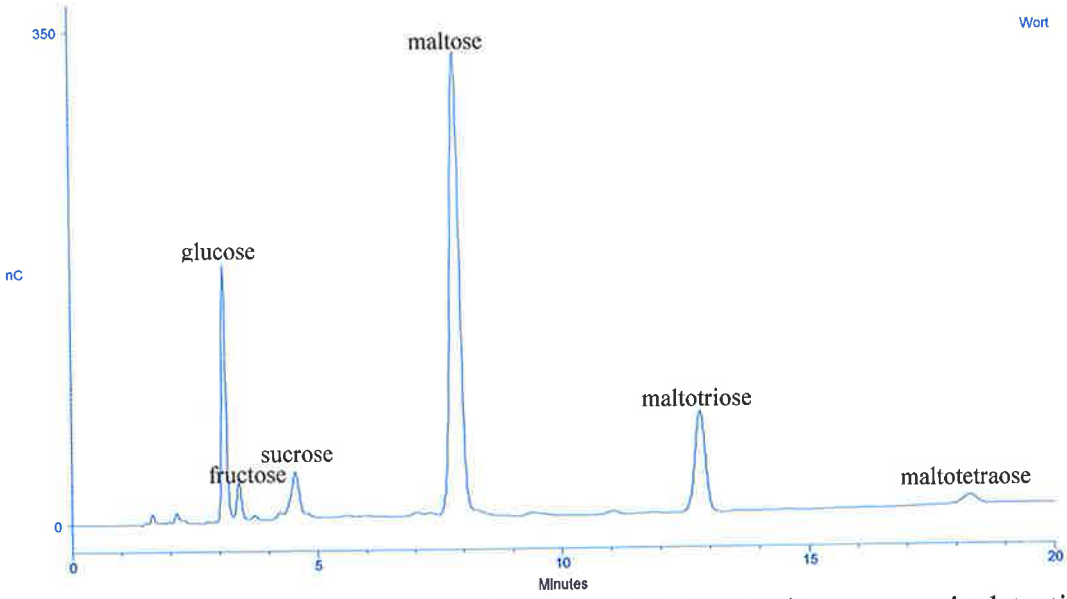



**Figure 6.2:** Separation of wort sugars by HPAEC with pulsed-amperometic detection.

QTL analysis of AAL in the Galleon x Haruna nijo population identified two genomic regions significantly associated with fermentability. One QTL is positioned on chromosome 3H, and the second is present on the long arm of chromosome 4H, coincident with the *Bmy*1 locus (Figure 6.3). The low thermostability Sd2L allele was associated with low fermentability, and the thermostable Sd2H allele from Haruna nijo was associated with high fermentability. Genetic analysis of the sugar profiles showed that level of maltotetraose (the smallest of the non-fermentable dextrins) is significantly associated with the *Bmy*1 locus (Figure 6.3). This suggests that the thermolabile Sd2L ß-amylase from Galleon results in incomplete conversion of starch to fermentable sugars and therefore yields increased levels of maltotetraose, resulting in lower values for fermentability. The more thermostable Sd2H ß-amylase from Haruna nijo results in more effective hydrolysis, yielding significantly lower levels of residual non-fermentable sugars.

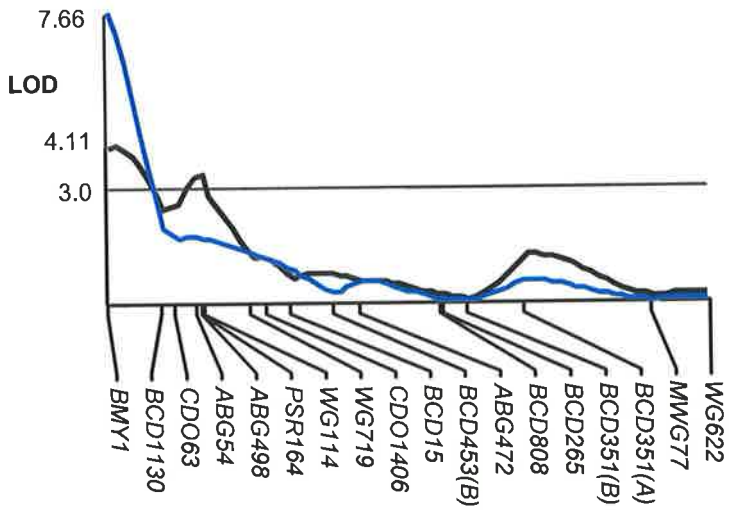



**Figure 6.3**: Interval analysis on chromosome 4H of wort fermentability measured as AAL (black trace) and wort maltotetraose concentration (blue trace) in the Galleon x Haruna nijo mapping population. Both traits show significant QTL coincident with the *Bmy*1 locus encoding ß-amylase.

The second QTL for fermentability was identified on chromosome 3H, and does not coincide with the map position of enzymes involved in either starch synthesis or hydrolysis, but does correspond to the location of the *sdw* dwarfing gene. Analysis of the non-fermentable sugar profiles of the Galleon x Haruna nijo population shows the level of small branched (α-1,6 linked) dextrins is also significantly associated with the *sdw* locus on chromosome 3H (data not shown). It is likely that this locus does not have a direct biochemical role in determining malt quality, but exerts pleiotropic effects through control of plant development and adaptation.

#### 6.3.1.2 Analysis of Commercial Malt Samples

The relationship between ß-amylase thermostability and wort fermentability was also investigated in 43 commercial malts (Figure 6.4). The malt samples are divided into three discrete populations, consistent with their ß-amylase alleles. The same three groups are observed whether residual ß-amylase is expressed as a percentage of activity prior to heat treatment, or in absolute terms of units/g after heat treatment (not shown). The malt samples containing Sd1 and Sd2H ß-amylases yield AAL between 80-84% under standard EBC mash conditions. The malt samples containing Sd2L ß-amylase show the lowest levels of fermentability and do not exhibit AAL above 81.5%.

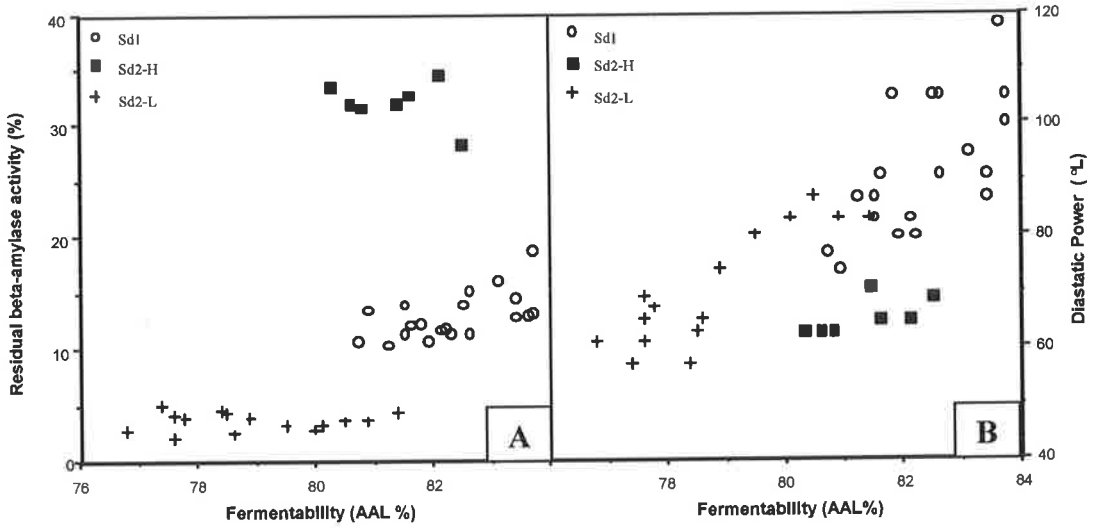



**Figure 6.4:** (A) The relationship between ß-amylase thermostability and AAL in 42 commercial malts. (B) The relationship between diastatic power and AAL.

The production of fermentable sugars from starch is accomplished by the combined activity of the diastatic enzymes, therefore the level of DP has a significant impact upon fermentability. The relationship between DP and AAL is plotted in Figure 6.4, and shows a positive correlation ($r=0.76$, $p<0.01$). A major contribution to the variation in this association is from the high thermostability Sd2H malts, which generate relatively high AAL from low diastatic power. The removal of the Sd2H samples from Figure 6.4 increases the correlation coefficient for DP and AAL ($r=0.87$, $p<0.01$). This suggests that the increase in thermostability results in more efficient starch degradation compared to the Sd1 and Sd2L malts with similar levels of DP. This is supported by anecdotal brewery evidence where Sd2H malt has been observed to produce higher fermentability than its DP specification would indicate.

ß-amylase represents a large component of DP, and as expected, the relationship between ß-amylase activity and AAL is very similar to the DP relationship, with $r=0.72$ ($p<0.01$) for all samples, and $r=0.79$ ($p<0.01$) within the Sd2L and Sd1 malts (not shown).

The detailed characterisation of ß-amylase in the current study has provided a better understanding of the biochemistry underlying the key functional specifications for malt quality. The Australian malt and malting barley industry services a diverse range of end-users with different quality requirements. The range of quality profiles can be divided into 6 major groups based on DP and fermentability specifications (Table 6.1). The different properties of the allelic forms of ß-amylase underpin each of these ideotypes (Table 6.1). Knowledge of the role of the alternative alleles in determining malt quality allows for targeted breeding of varieties to suite specific end-user requirements.

**Table 6.1:** Target quality for malt variety ideotypes for the production of the different beer types in Australia's major malt and malting barley markets.

| Target Market | Adjunct | DP (°WK) | AAL (%) | ß-amylase | Current desirable variety |
|---|---|---|---|---|---|
| 1. Low alcohol | sugar | 200 | <80 | Sd2L | Dhow |
| 2. Mid alcohol | sugar | 225 | 80 | Sd2L | Schooner |
| 3. Aust. Beer | sugar | 250 | 80 | Sd2L or Sd1 | Schooner, Sloop, Stirling, Gairdner |
| 4. Chinese beer | rice | 275-325 | 82 | Sd1 or Sd2H | Sloop, Harrington, Arapiles, Gairdner |
| 5. Japanese dry beer | rice | 300-350 | 83 | Sd2H, Sd1 or Sd3 | Franklin, Arapiles, Harrington, Alexis, Haruna Nijo |
| 6. Happoshu | rice | 400 | 84 | Sd3, Sd2H | AC Oxbow |

### 6.3.2 Development of Selection Methods for ß-amylase Alleles

#### 6.3.2.1 IEF Screening

IEF was the method initially used to identify the allelic forms of ß-amylase (Chapters 2 and 3). This approach tests barley grain, and the method can be used on half grains allowing the remaining half to be germinated for crossing. The IEF method offers clear identification of heterozygotes, but cannot differentiate the Sd2L and Sd2H forms of ß-amylase. Sample throughput is limited to 100 per day, which adds to the significant cost of the IEF gels, which range from $A0.40 per sample for the individual components, up to $1.40 per sample for pre-cast gels. While the IEF system has been employed extensively in the current study, the limitations of this method preclude its use for routine implementation in breeding programs.

#### 6.3.2.2 Thermostability Assay

A rapid small scale ß-amylase thermostability assay was developed as described in Section 6.2.6. Thermostability assays were based on the decay curves presented in Chapter 3, initially employing a two point assay with a measurement of ß-amylase activity in grain extracts before and after heating at 60°C for 10 minutes. The Betamyl assay (Megazyme) was scaled down and adapted to 96 well format for sample dilutions and the enzyme assay using deep well (1 mL) polypropylene plates. Absorbance measurements were then made in microtitre plates using an automated microplate reader. The method allows for excellent reproducibility with an average coefficient of variation for the microplate method of 2.6%.

Analysis of 179 elite breeders lines showed a clear differentiation of the samples based on their *Bmy*1 allele (Table 6.2). The Sd2L lines exhibit residual ß-amylase activity less than 2% of the initial activity, the Sd1 lines retain 2.4 - 8% activity, and the Sd2H lines retain 10 – 20% of the initial ß-amylase activity after incubation for 10 minutes at 60°C.

The level of ß-amylase activity in mature grain is strongly correlated with the level of grain protein (Giese and Hopp 1984). It was therefore considered possible to halve the number of assays required by correcting for variation in initial ß-amylase activity by expressing residual activity after heat treatment on the basis of grain protein. Grain protein levels were determined by NIR, and the relationship between grain protein and ß-amylase activity for the 179 test lines showed a strong positive correlation, $r^2 = 0.92$ (Figure 6.5).

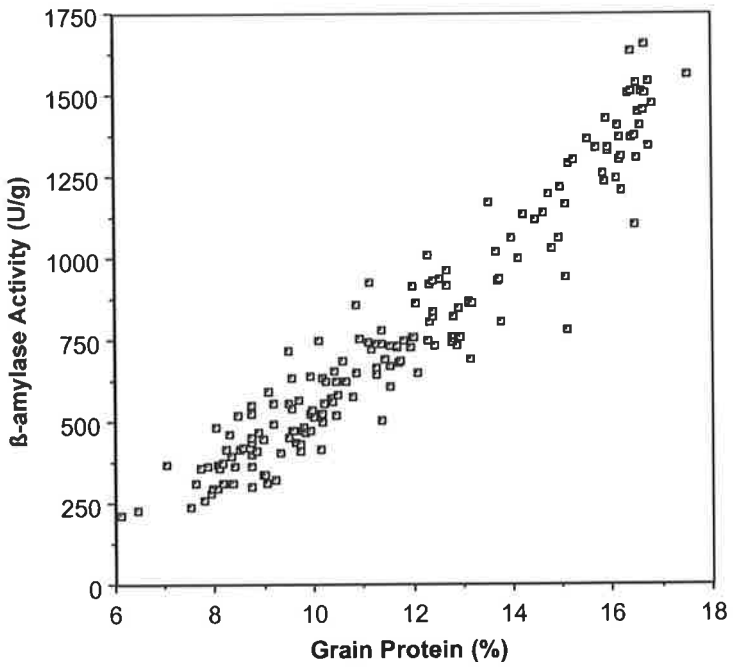



**Figure 6.5:** Association between grain protein (%) and ß-amylase activity (U/g), $r^2 = 0.92$.

Based on this linear relationship, residual activity was expressed per milligram of grain protein to correct for variation in initial ß-amylase activity between samples. This allowed all genotypes tested to be successfully resolved into one of the three allelic forms of ß-amylase, consistent with the results from the 2 point assay (Table 6.2). Of the 179 entries, 128 were classified as Sd1, 50 were classified as Sd2L and 11 as Sd2H. The high

representation of the Sd1 allele is consistent with the large proportion of progeny from the Sd1 varieties Sloop and Franklin within this germplasm.

The rapid thermostability assay provides the level of throughput required for early generation screening, based on 200 samples per day analysed in duplicate. The relatively low cost of consumables for the method, at approximately $0.33 per sample, is also amenable to early generation screening. The method is also an informative adjunct to AAL testing of elite lines, and may form part of evaluation of new introductions or parental material. For these types of applications, this method has been evaluated and implemented by the SA Barley Improvement Program (Logue *et al.* 1999).

**Table 6.2:** Distribution of the three ß-amylase alleles in 179 test lines based on the rapid small-scale ß-amylase thermostability assay.

| Sd1 | | | | Sd2L | | Sd2H |
|---|---|---|---|---|---|---|
| 88S28-1-1 | WI3158 | WI3195 | WI3140 | Barque | WI3044 | Arapiles |
| B%1302 | WI3159 | WI3196 | WI3141 | Chebec | WI3050 | Sapporo 93013 |
| Fitzgerald | WI3160 | WI3197 | WI3142 | Dash | WI2976 | Sapporo 93110/8 |
| Franklin | WI3161 | WI3198 | WI3143 | CMOKoru123 | WI2978 | Sapporo 93113/15 |
| Gairdner | WI3162 | WI3199 | WI3144 | Fr*Che/1016 | WI3102 | Sapporo 95203 |
| Jubilant | WI3163 | WI3200 | WI3145 | Galleon | WI3184 | Sapporo 95274 |
| Molloy | WI3164 | WI3203 | WI3146 | Mundah | WI3184 | Unicorn |
| Monarch | WI3165 | WI3204 | WI3147 | Schooner | WI3185 | VB9536 |
| Profit | WI3166 | WI3205 | WI3181 | Skiff | WI3185 | VB9720 |
| Sapporo | WI3167 | WI3206 | WI3182 | Tantangara | WI3187 | WI3188 |
| 95223 | WI3168 | WI3207 | WI3183 | VB9613 | WI3187 | WI3245 |
| Sloop | WI3169 | WI3210 | WI3186 | VB9614 | WI3189 | |
| Tilga | WI3170 | WI3211 | WI3190 | VB9726 | WI3189 | |
| VB9527 | WI3171 | WI3212 | WI3191 | VB9728 | WI3193 | |
| VB9610 | WI3172 | WI3213 | WI3236 | VB9729 | WI3201 | |
| VB9615 | WI3173 | WI3214 | WI3237 | WA0563 | WI3202 | |
| VB9623 | WI3174 | WI3215 | WI3238 | WB146-14 | WI3208 | |
| VB9703 | WI3175 | WI3216 | WI3239 | WB213 | WI3217 | |
| VB9710 | WI3176 | WI3218 | WI3240 | WB217 | WI3222 | |
| VB9725 | WI3177 | WI3219 | WI3241 | WB219 | WI3229 | |
| VB9727 | WI3178 | WI3220 | WI3242 | WB223 | WI3230 | |
| Venture | WI3179 | WI3221 | WI3244 | WB227 | WI3235 | |
| Vic 9524 | WI3180 | WI3225 | WI3246 | WB228 | WI3243 | |
| WB220 | WI3181 | WI3226 | WI3247 | WI2986 | WI3254 | |
| WB226 | WI3182 | WI3227 | WI3248 | WI3084 | | |
| WB229 | WI3183 | WI3228 | WI3249 | Wyalong | | |
| WB230 | WI3186 | WI3157 | WI3250 | | | |
| WI3071 | WI3190 | WI3194 | WI3251 | | | |
| WI3072 | WI3191 | WI3231 | WI3252 | | | |
| WI3073 | WI3192 | WI3232 | WI3253 | | | |
| WI3076 | WI3223 | WI3233 | WI3255 | | | |
| WI3099 | WI3224 | WI3234 | WI3139 | | | |

### 6.3.2.3 PCR Screening of *Bmy*1 Intron III

The identification of a 126 base pair insertion within intron III of the Sd2L allele by Errkila *et al.* (1998), provides an opportunity to identify this allele with a simple PCR marker. This assay has been implemented within the SA Barley Improvement Program for marker assisted selection particularly in allele enrichment of complex cross $F_1$'s and backcrossing strategies.

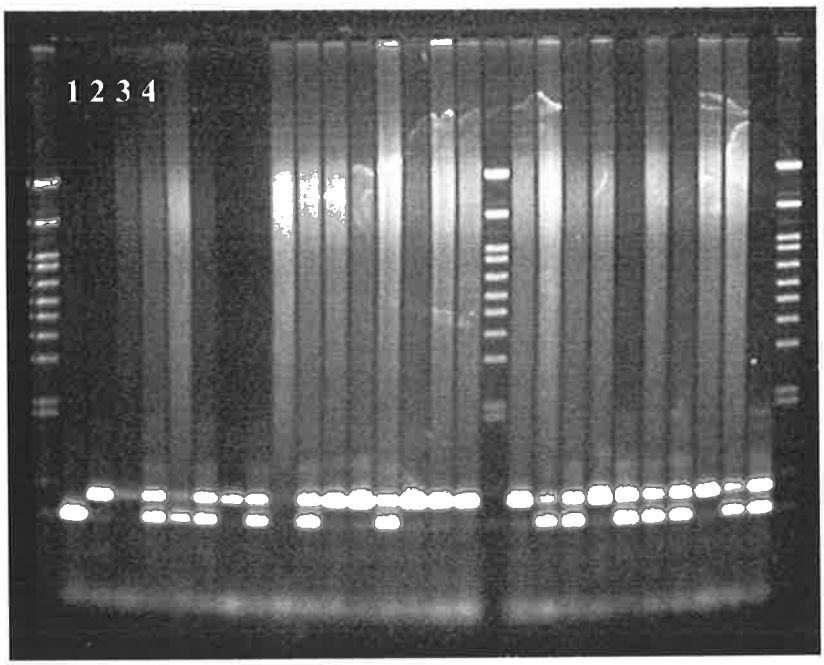



**Figure 6.6:** Bmy1 intron III analysis of a $BC_1F_1$ population with the Sd2L line as the recurrent parent. Analysis was performed by Ms Patricia Warner. Lane 1: Sd1, lane 2: Sd2L, lane 3: Sd2L, lane 4: heterozygote.

The intron III PCR analysis is a robust assay with a tolerance to variable template DNA quality, and it provides clear assignment of heterozygotes as shown in Figure 6.6. However the inability of the assay to discriminate between the Sd1, Sd2H and Sd3 alleles significantly limits its application.

### 6.3.2.4 Cleaved Amplified Polymorphic Sequence (CAPS) Assay

The amino acid sequence data presented in Chapter 5 was used to deduce the single nucleotide polymorphisms (SNP) in the *Bmy*1 gene. The amino acid substitution A233V in the Sd2H and Sd3 enzymes corresponds to a $C^{698}\rightarrow T$ mutation, which also represents an enzymatic restriction polymorphism. A cleaved amplified polymorphic sequence (CAPS) assay, enabling discrimination between the lower thermostability alleles (Sd2L and Sd1) and high thermostability alleles (Sd2H and Sd3) was developed based on this restriction site polymorphism in the coding region of the *Bmy*1 gene. PCR products containing the *Msp* I restriction site (C↓CGG) were cleaved into 171 bp and 100 bp fragments, indicating the presence of either Sd2H or Sd3 alleles. The presence of intact fragments indicated the presence of either Sd2L or Sd1 alleles (Figure 6.7). The CAPS assay was validated using IEF to analyse the doubled haploid progeny shown in Figure 6.7, and as expected, the two methods were in agreement.

The CAPS assay is slightly more expensive than a simple PCR based assay due to the additional endonuclease restriction step, however the assay is still cost effective, and allows the level of throughput required for routine implementation for marker assisted selection. While the assay does not provide complete discrimination between the four *Bmy*1 alleles of interest, it is significantly more informative than the intron III analysis approach. The CAPS assay is therefore considered to be the most appropriate screening technique for routine implementation in barley breeding programs.

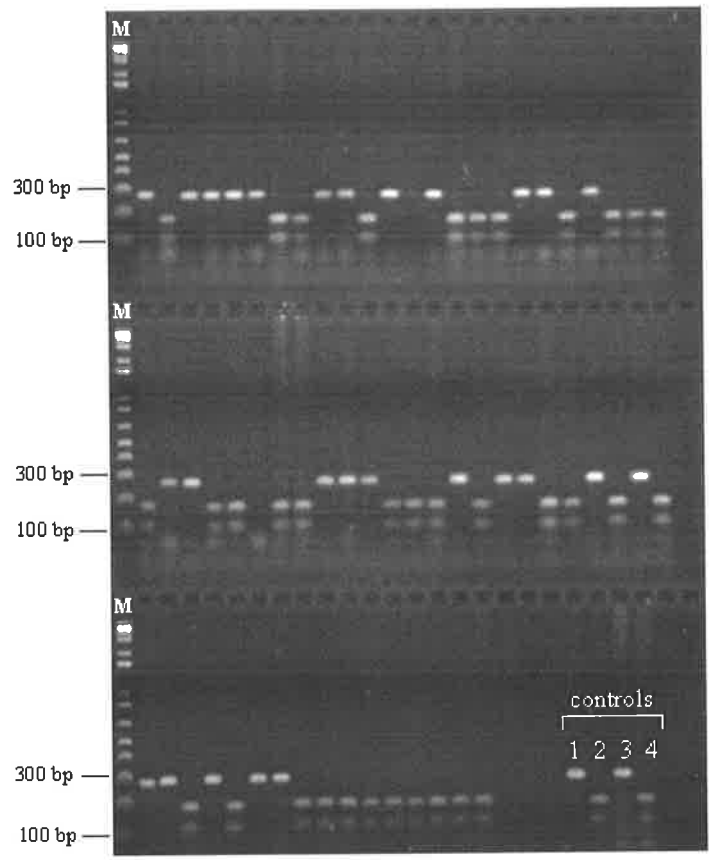



**Figure 6.7:** Doubled haploid progeny segregating for Sd1 and Sd3 ß-amylase assayed using the CAPS marker. Sd1 genotypes yield 271 bp products and Sd3 genotypes yield 171 and 100 bp products. Controls 1: Fitzgerald (Sd1); 2: AB75 (Sd3); 3: Stirling (Sd2L); 4: Haruna-Nijo (Sd2H). M: 1kb ladder (Gibco Life Technologies). Analysis was performed by Mr Maxime Paris, Murdoch University, WA.

### 6.3.2.5 SNP analysis

The amino acid mutations D165E and V233A identified in Chapter 5 were used as the basis for developing SNP assays to identify each of the Sd2L, Sd1, Sd2H and Sd3 ß-amylase alleles. The assay development and validation was performed by Mr Maxime Paris, Murdoch University, WA, as described in Paris and Eglinton (2002). The four *Bmy*1 alleles were identified by genotyping two SNPs using a duplex Single Nucleotide Primer Extension (SNuPE) assay. Two genotyping primers with their 3' ends directly flanking the selected

SNPs were annealed to the amplified target sequences and extended by single dideoxynucleotides complementary to the polymorphic nucleotides. Extended primers were analysed using MALDI-ToF MS and, making use of the inherent molecular weight difference between DNA bases, incorporated nucleotides were identified by the increase in mass of the extended primers as shown in Figure 6.8.

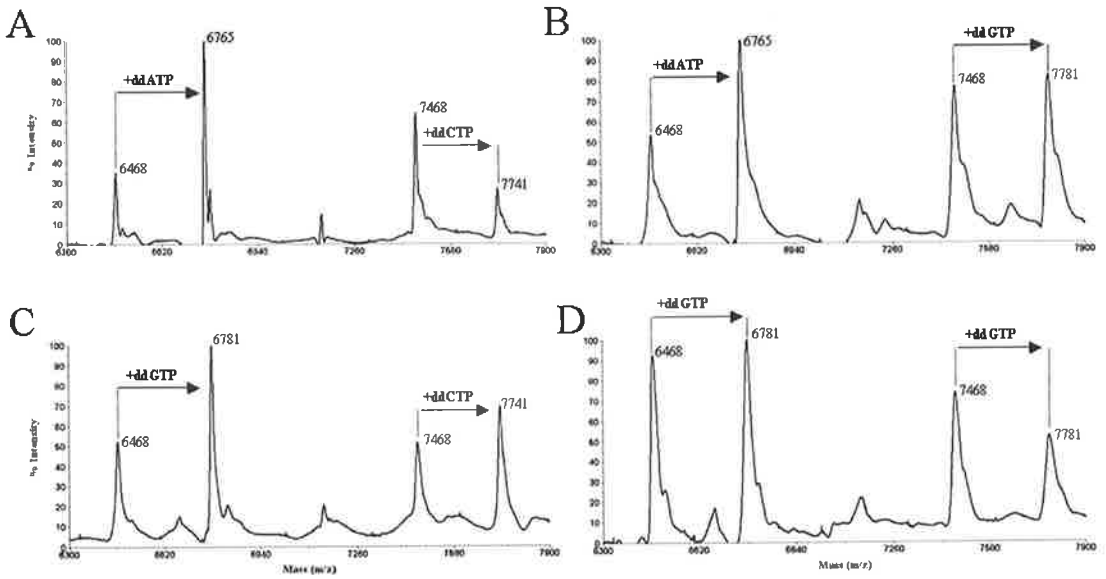



**Figure 6.8:** Duplex SNuPE assays used to identify β-amylase alleles from barley. A. Sd2L allele. The first genotyping primer (6468 Da) is extended by a single ddATP to generate a 6765 Da product and the second genotyping primer (7468 Da) is extended by a single ddCTP to generate a 7741 Da product. B. Sd1 allele. The first genotyping primer (6468 Da) was extended by a single ddATP to generate a 6765 Da product and the second genotyping primer (7468 Da) was extended by a single ddGTP to generate a 7781 Da product. C. Sd2H allele. The first genotyping primer (6468 Da) is extended by a single ddGTP to generate a 6781 Da product and the second genotyping primer (7468 Da) is extended by a single ddCTP to generate a 7741 Da product. D. Sd3 allele. Both genotyping primers (6468 Da and 7468 Da) were extended by single ddGTPs to generate products of 6781 Da and 7781 Da.

The SNuPE assay was validated by testing 40 lines that had been analysed with the rapid small-scale ß-amylase thermostability assay (Table 6.2). The assay correctly assigned the ß-amylase allele for each of the validation lines (data not shown).

The SNuPE assay is the most powerful of the selection tools developed for screening barley ß-amylase. The test offers discrimination of the Sd2L, Sd1, Sd2H and Sd3 alleles, and also allows assignment of heterozygotes. The technique is significantly more expensive than the other PCR based assays, and also requires MALDI-ToF MS, however the assay system could be adapted to any other SNP detection platform.

### 6.3.3 Introgression of the Sd3 ß-amylase Allele

A series of lines derived from *H. spontaneum* accessions backcrossed to cv Clipper were screened with IEF to identify lines carrying the novel ß-amylase alleles identified in Chapter 3. The $BC_3$ line AB75 derived from *H. spontaneum* accession CPI 77146-33 was found to carry the Sd3 ß-amylase allele (data not shown). AB75 was used as the donor for the Sd3 introgression. The introgression strategy comprised backcrossing into the current Australian malting cultivar Gairdner, and backcross derivatives of Sloop, as well as intercrossing hybrids with a range of elite malting quality lines (Table 6.3). Half grains from a total of 371 $BC_1F_1$ and $TC_1F_1$ individuals from 11 of the 16 unique crosses were screened with IEF for the presence of the Sd3 ß-amylase allele as shown in Figure 6.9.

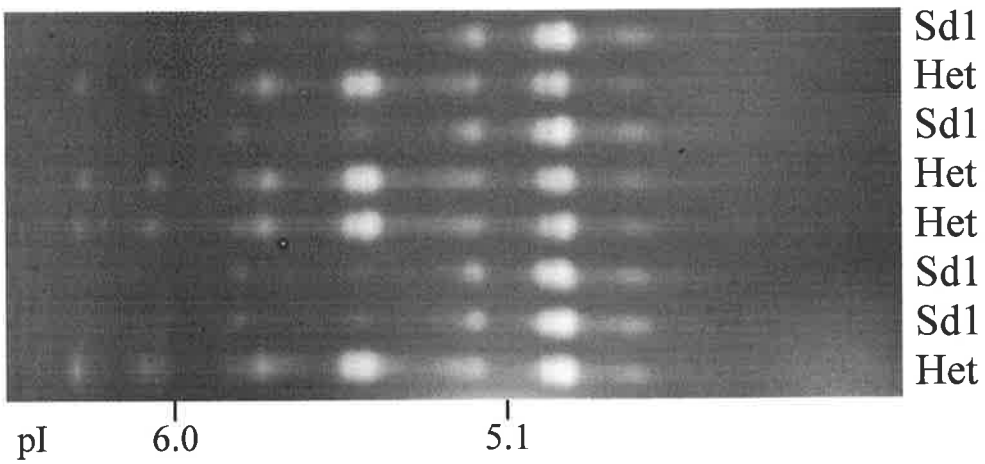



**Figure 6.9:** IEF screening of $BC_1F_1$ individuals segregating for Sd3 ß-amylase from AB75 and Sd1 ß-amylase from the recurrent parent Gairdner.

Individuals heterozygous for the Sd3 allele were used for subsequent crossing, and the $F_2$ generations grown in the field as bulks. Single plant selections were taken from the subsequent $F_3$ bulks and grown as 2 row plots (0.52m), four metres in length, within single replicate trials at Strathalbyn, South Australia, in 2000 and 2001. Lines within the 2 row experiments were selected on the basis of agronomic observations, grain size, and NIR predicted malt extract and diastatic power. As summarised in Table 6.3, a total of 1172 lines derived from AB75 were evaluated, and 646 lines were selected for promotion to preliminary yield trials.

The Sd3 ß-amylase introgression strategy also aimed to pyramid the thermostable ß-amylase with the 'anthocyanin free' mutation from the variety Caminant (Jende-Strid 1997), to develop a unique malting quality profile. In addition to the BX99S;054 and 055 populations listed in Table 6.3, a doubled haploid population was produced. 48 $F_1$ seeds from the cross Caminant/Sloop $BC_2F_3$//AB75/WI3143/3/Gairdner/Keel were screened with IEF and 23 individuals heterozygous for the Sd3 allele were used as donor plants for doubled haploid production (performed by Dr Phil Davies, SARDI, SA).

**Table 6.3:** Summary of the populations developed for the introgression of the Sd3 ß-amylase allele into elite malting quality germplasm. The total number of lines tested and the number of lines selected for promotion to preliminary yield trials are shown. The Sd3 donor line AB75 is shown in bold within the pedigrees.

| Cross Code | Pedigree | Total Lines | Lines Selected |
|---|---|---|---|
| BX98S;156 | Guardian/Sloop$BC_1$/3/Osiris/Sloop$BC_1$//Halcyon/Sloop $BC_1$/4/**AB75**/WI3143 | 63 | 25 |
| BX98S;286 | **AB75**/Gairdner//Gairdner/Keel | 81 | 44 |
| BX99S;041 | **AB75**/WI3182/5/WI2875-1/Harrington-9//WI3182/4/DH115/Sloop $BC_3$/3/Gairdner | 140 | 49 |
| BX99S;042 | **AB75**/WI3182/5/WI2875-1/Harrington-9//WI3182/4/DH115/Sloop$BC_3$/3/Halcyon/SloopBC1//Keel/Sloop | 98 | 38 |
| BX99S;043 | **AB75**/WI3182/4/WI2875-1/Harrington-9//WI3182/3/Gairdner/Keel | 90 | 37 |
| BX99S;044 | **AB75**/WI3182/4/WI2875-1/Harrington-9/3/WI3182//Pitcher/Keel | 110 | 29 |
| BX99S;045 | **AB75**/WI3182/4/WI2875-1/Harrington-9//WI3182/3/Rifle/TG*Harrington-58 | 67 | 33 |
| BX99S;046 | **AB75**/WI3182/4/WI2875-1/Harrington-9//WI3182/3/VB9729/TG*Harrington-59 | 70 | 14 |
| BX99S;047 | **AB75**/WI3181/4/WI2875-1/Harrington-11//WI3148/3/DH115/Sloop $BC_3$//Gairdner | 99 | 23 |
| BX99S;048 | **AB75**/WI3181/5/WI2875-1/Harrington-11//WI3148/4/DH115/Sloop $BC_3$/3/Halc/SloopBC1//Keel/Sloop | 245 | 105 |
| BX99S;049 | **AB75**/WI3181/4/WI2875-1/Harrington-11//WI3148/3/Gairdner/Keel | 107 | 29 |
| BX99S;050 | **AB75**/WI3181/4/WI2875-1/Harrington-11//WI3148/3/Pitcher/Keel | 97 | 31 |
| BX99S;051 | **AB75**/WI3181/4/WI2875-1/Harrington-11//WI3148/3/Rifle/TG*Harrington-58 | 97 | 40 |
| BX99S;052 | **AB75**/WI3181/4/WI2875-1/Harrington-11//WI3148/3/VB9729/TG*Harrington-59 | 107 | 47 |
| BX99S;054 | Caminant/Sloop $BC_2F_3$//**AB75**/WI3182/3/Rifle/WI3102 | 191 | 47 |
| BX99S;055 | Caminant/Sloop $BC_2F_3$//**AB75**/WI3182/3/WB235 | 110 | 55 |
| | TOTAL | 1772 | 646 |

Of the lines tested in double row experiments in 2000, 51 lines were evaluated in stage 1 trials of the SA Barley Improvement Program in 2001. Field trials were conducted at Pinery and Maitland, South Australia, and consisted of plots six rows wide (1.23m), 4m in length, arranged as unreplicated designs with control cultivars sown every seventh plot. The *Bmy*1 genotype of each of the 51 lines was determined by analysing 4 individuals from each line by either IEF or SNP assays. Micromalting and quality analysis of the grain samples from these trials will be the first measure of the influence of the Sd3 ß-amylase from wild barley on malt quality in elite germplasm.

## 6.4 Discussion

Wort fermentability is a key functional specification for malt quality. The genetic basis of fermentability was investigated with the aim of validating the role of ß-amylase in influencing this trait. QTL mapping demonstrated the association between the higher thermostability Sd2H ß-amylase, increased AAL, and decreased levels of the non-fermentable dextrin maltotetraose. This association was further supported by analysis of commercial malt samples. The comparison showed the low thermostability Sd2L ß-amylase was consistently associated with lower levels of fermentability, whereas malt samples containing the high thermostability Sd2H ß-amylase exhibited significantly higher fermentability than would be predicted based on the levels of diastatic power in the malt samples. Investigations demonstrating the role of ß-amylase in determining wort fermentability have subsequently been repeated (Kaneko *et al.* 2000). These results support the view that selection for specific *Bmy*1 alleles is an effective strategy to apply selection pressure for wort fermentability. This is of practical significance to barley breeding programs because the existing methods for measuring fermentability are constrained by throughput, large sample requirements and assay costs, and are therefore only be applied to the most advanced breeding lines.

Genetic analysis of fermentability and dextrin profiles also demonstrated the importance of adaptation as a determinant of malt quality. QTL for AAL and the level of small branched (α–1,6) dextrins were identified coincident with the *sdw* dwarfing gene on chromosome 3H. It is concluded that this locus does not have a direct biochemical role in determining malt quality, but exerts pleitropic effects through control of plant development and adaptation. Both the *sdw* locus and the ari-eGP dwarfing gene on chromosome 5H have since been reported to influence fermentability in other genetic backgrounds (Meyer *et al.* 2000).

Maltose is the major product of starch hydrolysis in germinating barley. Despite significant variation in wort maltose levels within the Galleon x Haruna nijo mapping population, no significant QTL were detected. This is not unexpected, given that wort maltose levels are influenced by a number of biochemical pathways and environmental effects, including variations in starch quantity and quality, and the hydrolysis of both linear and branched dextrins. Analysis of carefully matched near isogenic lines, only differing in their *Bmy*1 alleles, could be expected to yield a significant association between maltose levels and the *Bmy*1 gene due to the absence of confounding effects.

Fermentability, determined as apparent attenuation limit (AAL), is a measure of the ability of wort to ferment. AAL is considered to be essentially a measure of the level of fermentable sugars, since it is assumed that the yeast typically converts most available sugars to ethanol. However in the present study, no correlation was observed between the levels of fermentable sugars and AAL. Similar studies have also reported poor correlations (Collins *et al.* 1995, Meyer *et al.* 2000), and there are a number of possible explanations. Although this study and those of Collins *et al.* 1995 and Meyer *et al.* 2000 have all considered the influence of nitrogen nutrition, this has only been assessed quantitative terms, and specific amino acid compositions have not been determined. The levels of residual fermentable sugars in the fermented wort samples was not determined in the mapping population, therefore limiting factors other than the fermentable sugars cannot be excluded. Another potential confounding factor is the use of specific gravity measurements in assessing fermentability. A direct measure of ethanol production could be used to exclude the possibility of certain conditions inducing yeast cells to consume sugars for growth and metabolism at the expense of alcohol production. These conclusions suggest investigations into the limiting factors in achieving high fermentability in the small scale AAL assay may identify novel characteristics to

improve malt quality, and more importantly, determine how accurately the AAL assay reflects the functional requirements of the brewing industry.

AAL is a relatively expensive and time-consuming malt quality assay, and is therefore only applied to advanced lines. The development of techniques for selection of specific *Bmy*1 alleles in early generations of breeding programs would allow increased selection pressure for wort fermentability, and targeted development of new varieties to suite specific end user requirements. Five alternative methods for screening ß-amylase were developed and evaluated. The IEF and thermostability screening methods are suitable for incorporation into malt quality evaluation laboratories, and complement the existing AAL method by providing increased sample throughput and independence from environmental effects. The *Bmy*1 intron III assay and the CAPS assay are suitable for DNA based marker screening programs employing gel-based detection systems. The intron III assay allows selection for the Sd2L allele, appropriate for the low fermentability requirements of low alcohol and sugar adjunct brewing styles, and the CAPS assay allows selection for the high thermostability Sd2H and Sd3 alleles required for the high fermentability end uses. Both assays also provide assignment of heterozygotes, and are therefore suitable for marker assisted backcrossing or $F_1$ allele enrichment strategies. The SNuPE based assays for the detection of SNPs are the most powerful of the selection tools available to screen for ß-amylase. SNP detection provides discrimination of all four *Bmy*1 alleles of practical interest, as well as the unambiguous identification of heterozygotes. This method also offers high throughput, and is therefore amenable to all current marker assisted selection strategies. The range of techniques underlying the five different selection tools provides flexibility in the way ß-amylase screening can be performed, and ensures methods are appropriate for resource levels available to different barley breeding programs.

The highly thermostable Sd3 ß-amylase identified in *H. spontaneum* has been introgressed into elite malting quality germplasm using marker assisted selection. Germplasm containing the Sd3 allele has been provided to a number of barley breeding programs, including the Western Australian Department of Agriculture, the Victorian Institute of Dryland Agriculture, Ackermann Seeds (Germany), Syngenta (France), and Advanta Seeds (UK), and these groups are now also introgressing the thermostable ß-amylase into elite germplasm. Within the SA Barley Improvement Program, a range of different genetic backgrounds and different agronomic profiles have been used in the introgression strategy, and this germplasm will form the basis for the development of commercial varieties containing the Sd3 ß-amylase. A detailed evaluation of the malt quality of the first elite lines from this program is planned. It is envisaged that AAL and simulated mashing studies will be modified to include the exogenous addition of starch to fully assess the impact of the novel Sd3 ß-amylase allele.

# Chapter 7: General Discussion

The identification and characterisation of genetic variation in barley ß-amylase has demonstrated that targeted allele discovery is a feasible strategy to identify novel alleles of economic significance. The integration of genetics and functional analysis provides a framework for fundamental studies to deliver practical outcomes for crop improvement. The success of this approach suggests genetic variation in other key targets should be characterised in terms of functional diversity. α-Amylase is an ideal candidate for allele mining, as it is a key determinant of quality in brewing systems using high levels of starch based adjuncts, and has already been shown to exhibit allelic variation (Brown *et al.* 1982). Another potential target is the limit dextrinase inhibitor, which is likely to offer an opportunity to alter fermentable sugar production if novel alleles conferring reduced levels of enzyme inhibition can be identified. While there are clear opportunities to further improve and control malting quality in barley through alternative alleles, this principle can be applied to other species and phenotypic characteristics where the roles of particular genes or proteins are well understood.

The examples in the current study are based on structural genes, however complex traits such as abiotic stress tolerance may be more strongly influenced by regulatory genes. While determining the key control points of signal transduction pathways presents significant technical challenges, the characterisation of transcription factors such as the DREB family (Liu *et al.* 1998), provides opportunities to apply allele discovery to this class of genes.

The strategy of functional diversity analysis also offers an alternative way to investigate the role of genes or proteins that are not well characterised. Serpins have been associated with malt quality parameters, but even their endogenous function in higher plants has not been defined. Exploiting genetic variation in serpins may provide a useful approach

to further examine the physiological function of serpins in barley, and the impact of these proteins in malt and beer quality. The development of near isogenic lines containing alternative alleles offers a significant tool in the analysis of even very complex gene actions. Reducing the influence of genetic background is particularly useful in the analysis of traits such as quality and abiotic stress tolerance, as highlighted by the success of advanced backcross QTL analysis approaches (Bernacchi *et al.* 1998).

The analysis of ß-amylase polymorphism and ecogeographic correlations has implications for sampling wild populations for functional diversity analysis or allele discovery. In the present study, the polymorphism in ß-amylase was consistent with the general structure of *H. spontaneum* populations, exhibiting high variability, genetic differentiation across environments, and a relatively high proportion of unique alleles. Diversity estimates are often used to develop sampling structures within germplasm collections or wild populations, and these strategies can increase the genetic diversity within a given sample size. While a relatively small sample size may be developed that reflects the genetic variability of wild populations, the absence of rare or unique alleles significantly reduces the effectiveness of these sampling techniques for allele discovery. High throughput gene sequencing or protein characterisation methods are therefore important components of functional diversity analysis to maximise the sample size and therefore the probability of identifying novel alleles.

The potential importance of rare alleles is highlighted by the evolutionary relationships of the ß-amylase alleles. The sequence comparisons indicate that the novel and highly thermostable Sd3 ß-amylase is the most closely related to an ancestral enzyme, and therefore an accumulation of random mutations in the *Bmy*1 gene has progressively decreased the structural stability of the enzyme. ß-amylase could be considered to be selectively neutral in the context of *H. spontaneum* adaptation, the domestication of barley and the early

breeding of the crop species, and has only recently been subjected to selection in barley breeding. This has resulted in the absence of key genetic variation in current breeding germplasm, limiting the capacity of traditional breeding methods to respond to changes in quality requirements. The principle of losing genetic diversity through domestication and selection has been demonstrated for barley ß-amylase, but it is also expected to apply to most traits relating to processing quality. The loss of genetic variation may be even more important in selecting for novel end-uses that have only recently become breeding targets, for example pearling quality, high beta-glucan content, and alternative starch types. In order to maximise the genetic progress for these traits it will be necessary to exploit the genetic variation within *H. spontaneum* to identify favourable alleles that have not progressed through the bottlenecks of domestication and traditional selection. This subsequently presents plant breeders with the challenges of introgression, rather than the traditional methods of crossing and selection within existing adapted germplasm.

Marker assisted selection is a rapidly developing field, and the application of microsatellites and other anonymous marker systems have significantly improved the capability for genetic analysis and selection. Backcrossing has traditionally been viewed as a conservative breeding strategy, however in combination with MAS it can now be used as a rapid and aggressive plant breeding strategy. Despite these advances, diagnostic markers are widely regarded as the ultimate selection tool, overcoming the limitations posed by lack of polymorphism and recombination between the marker and target gene. This study has developed diagnostic markers for ß-amylase alleles and implemented these in introgression strategies. The subsequent breeding lines have exhibited relatively poor adaptation, considering *H. spontaneum* only contributes between 1.6 – 3.125 % of the pedigree. It is now apparent that for introgression from poorly adapted exotic germplasm, a single diagnostic marker is not the most effective selection tool. For maximum efficiency, a panel of markers is

required to select for individuals carrying small introgression segments to minimise linkage drag from the poorly adapted donor parent.

There are surprisingly few examples of successful barley improvement from wild relatives. The Australian feed barley Tantangara carries resistance to leaf scald from *H. spontaneum*, and its release in 1995 is possibly still the only commercial example of a variety developed from the introgression of a specific trait from wild barley. However, targeted allele discovery coupled with innovative introgression techniques such as advanced backcross QTL analysis, introgression segment length analysis, and recurrent parent genome selection, are likely to deliver significant genetic improvements from *Hordeum spontaneum* and other crop relatives.

# REFERENCES


Abbott, D.C., Brown, A.H.D. and Burdon, J.J. (1992). Genes for scald resistance from wild barley (*Hordeum vulgare* ssp *spontaneum*) and their linkage to isozyme markers. Euphytica **61**: 225-231.

Ahokas, H. and Erkkila, M.J. (1992). Barley ß-amylase and ß-glucanase activities at germination in vulgare-type lines from backcrosses of wild, spontaneum-type strains with cv. Adora. Agricultural Science in Finland **1**: 339-350.

Ahokas, H. and Naskali, L. (1990a). Geographic variation α-amylase, ß-amylase, ß-glucanase, pullulanase and chitinase activity in germinating *Hordeum spontaneum* barley from Israel and Jordan. Genetika **82**: 73-78.

Ahokas, H. and Naskali, L. (1990b). Variation of α-amylase, ß-amylase, ß-glucanase, pullulanase, proteinase, and chitinase activity in germinated samples of the wild progenitor of barley. J.Inst.Brew. **96**: 27-31.

Allison, M.J. (1973). Genetic studies on the ß-amylase isozymes of barley. Genetica **44**: 1-15.

Allison, M.J. (1978). Amylase activity and endosperm hardness of high lysine barleys. J.Inst.Brew. **84**: 231-232.

Allison, M.J. and Ellis, R.P. (1973). The inheritance of ß-amylase in developing barley grain. Biochem.Genet. **10**: 165-173.

Allison, M.J. and Swanston, J.S. (1974). Relationships between ß-amylase polymorphisms in developing, mature and germinating grains of barley. J.Inst.Brew. **80**: 285-291.

Arends, A.M., Fox, G.P., Henry, R.J., Marschke, R.J. and Symons, M.H. (1995). Genetic and environmental variation in the diastatic power of Australian barley. J.Cer.Sci. **21:** 63-70.

Asano, K., and Hashimoto, N. (1980). Isolation and characterization of foaming proteins of beer. J.Am.Soc.Brew.Chem. **38:** 129-137.

ASBC (1992). Methods of Analysis. American Society of Brewing Chemists, St Paul, Minnesota, USA.

Aspegren, K., Mannonen, L., Ritali, A., Puupponen-Pimia, R., Kurton, U., Salmenkallio-Martila, M., Kauppinen, V. and Teeri, T.H. (1995). Secretion of a heat-stable beta-glucanase from transgenic, suspension-cultured barley cells. Mol.Breed. **1**: 91-99.

Ballance, G.M., Meredith, W.O.S. and LaBerge. D.E. (1976). Distribution and development of endo-ß-glucanase activities in barley tissues during germination. Can.J.Plant Sci. **56**: 459-466.

Bamforth, C.W. (1982). Barley ß-glucans. Their role in malting and brewing. Brewers Digest **57**: 22-27.

Bamforth, C.W. (1985). The foaming properties of beer. J.Inst.Brew. **91**: 370-383.

Bamforth, C.W. (1986). Enzymes from the grist. The Brewer (427-434).

Bamforth, C.W. and Barclay, A.H.P. (1993). Malting Technology and the Uses of Malt. In: Barley: Chemistry and Technology. St Paul, Minnesota, USA, American Association of Cereal Chemists Inc. Chap 7.

Bamforth, C.W. and Martin, H.L. (1983). The degradation of ß-glucan during malting and mashing: The role of ß-glucanase. J.Inst.Brew. **89**: 303-307.

Barber, M.G., Jackson, E.A. and Smith, D.B. (1994). Total and individual barley (1→3),(1→4)-ß-D-glucanase activities in some green and kilned malts. The Journal of Cereal Science **100**: 91-97.

Bendelow, V.M. (1964). Inheritance of free ß-amylase in barley. Canadian Journal of Plant Science **44**: 550-554.

Bernacchi, D., Beck-Bunn, T., Eshed, Y., Lopez, J., Petiard, V., Uhlig, J., Zamir, D., and Tanksley, S. (1998). Advanced backcross QTL analysis in tomato. I. Identification of QTLs for traits of agronomic importance from Lycopersicon hirsutum. Theor.Appl.Genet. 97(3): 381-397.

Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984). Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography **336**: 93-104.

Bilderback, D.E. (1974). Amylases from aleurone layers and starchy endosperm of barley seeds. Plant Physiol. **53**: 480-484.

Bjork, I., Jackson, C.M., Jornvall, H., Lavine, K.K., Nordling, K. and Salsgiver, W.J. (1982). The active site of antithrombin: Release of the same proteolytically cleaved form of the inhibitor from complexes with factor IXa, factor Xa, and thrombin. J.Biol.Chem. **257**: 2406-2411.

Brandt, A., Svendsen, I. and Hejgaard, J. (1990). A plant serpin gene. Structure, organisation and expression of the gene encoding barley protein Z4. Eur.J.Biochem. **194**: 499-505.

Briggs, D.E. (1989). Barley Germination: Biochemical Changes and Hormonal Control. In: Cereal Science and Technology. Aberdeen, Aberdeen University Press. 369-395.

Brown, A.H.D. (1992). Genetic variation and resources in cultivated barley and wild *Hordeum*.. In Barley Genetics VI, L. Munck ed, p 669-682. Munksgaard International Publishers Ltd., Copenhagen, Denmark.

Brown, A.H.D., Nevo, E., Zohary, D. and Dagan, O. (1978). Genetic variation in natural populations of wild barley (*Hordeum spontaneum*). Genetica. **49**: 97-108.

Brown, A.H.D. and Jacobson, J.V. (1982). Genetic basis and natural variation of α-amylase isoenzymes in barley. Gen.Res.Camb. **40**: 315-324.

Bush, D.S., Biswas, A.K. and Jones R.L. (1989). Gibberellic-acid-stimulated $Ca^{2++}$ accumulation in endoplasmic reticulum of barley aleurone: $Ca^{2++}$ transport and steady state levels. Planta. **178**: 411-420.

Callis, J. and Ho, T.H.D. (1983). Multiple molecular forms of the gibberellin induced α–amylase from the aleurone layers of barley seeds. Arch.Biochem.Biophys. **224**: 224-234.

Cannell, M., Karp, A., Isaac, P.G. and Shewry, P. (1992). Chromosomal assignment of genes in barley using telosomic wheat-barley addition lines. Genome 35: 17-23.

Chalmers, K.J., Waugh, R., Watters, J., Forster, B.P., Nevo, E., Abbott, R.J. and Powell, W. (1992). Grain isozyme and ribosomal DNA variability in *Hordeum spontaneum* populations from Israel. Theor.Appl.Genet. **84**: 313-322.

Cheong, C.G., Eom, S.H., Chang, C., Shin, D.H., Song, H.K., Min, K., Moon, J.H., Kim, K.K., Hwang, K.Y. and Suh, W. (1995). Crystalization, molecular replacement solution, and refinement of tetrameric ß-amylase from sweet potato. Proteins: Struct.Func.Genet. **21:** 105-117.

Chou P.Y. and Fasman G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Advances in Enzymology **47:** 45-147.

Clegg, M.T., Brown, A.H.D. and Whitfield (1984). Chloroplast DNA diversity in wild and cultivated barley: Implications for genetic conservation. Genet.Res. **43**:339-343.

Collins, H.M., Logue, S.J., Lance, R.C.M., Barritt, S., Pascoe, P., Fukuda, K. and Barr, A.R. (1995). Comparison of two different methods for determining fermentability in a range of barley varieties. Proceedings of the 45th Australian Cereal Chemistry Conference, Adelaide, 10-14 September, Pages 453-457.

Cooperman, B.S., Stauridi, E., Nickbarg, E., Rescorla, E., Schechter, N.M. and Rubin, H. (1993). Antichymotrypsin interaction with chymotryspin. J.Biol.Chem. **268**: 23616-23625.

Corke, H. (1992). Inheritance of protein content and seed weight in crosses between wild barley (*Hordeum spontaneum*) and cultivated barley (*Hordeum vulgare*). Cer.Res.Comm. **20**(3-4): 241-247.

Corke, H. and Atsmon, D. (1990). Wild barley (*Hordeum spontaneum* Koch) and its potential utilization in barley protein improvement. Israel Journal of Botany **39**(3): 271-286.

Coventry, S., Barr, A.R, Jefferies, S.P., Chalmers, K., Logue, S. and Langridge, P. (1999). Validating quantitative trait loci for diastatic power in populations of malting barley (Hordeum vulgare L.). Proc. 9th Australian Barley Technical Symposium, Melbourne.

Curioni, A., Pressi, G., Furegon, L. and Peruffo, A.D.B. (1995). Major proteins of beer and their precursors in barley: Electrophoretic and immunological studies. J.Agric. Food Chem. **43**: 2620-2626.

Dahl, S.W., Rasmussen, S.K. and Hejgaard, J. (1996). Heterologous expression of three plant serpins with distinct inhibitory functions. J.Biol.Chem. **271**(41): 25083-25088.

Daussant, J., Sadowski, J. and Ziegler, P. (1994). Cereal ß-amylases: Diversity of the ß-amylase isozyme status within cereals. J.Plant Physiol. **143**: 585-590.

Davy, A., Sorenson, M.B., Svendsen, I., Cameron-Mills, V. and Simpson, D.J. (2000). Prediction of protein cleavage sites by the barley cysteine endoproteinases EP-A and EP-B based on the kinetics of synthetic peptide hydrolysis, Plant Physiol. **122:** 137-145.

DeCordt, S., Hendrickx, M., Maesmans, G. and Tobback, P. (1994). The influence of polyalcohols and carbohydrates on the thermostability of α-amylase. Biotech.Bioeng. **43:** 107-114.

Degan, F.D., Rocher, A., Cameron-Mills, V. and Wettstein, D. (1994). The expression of serine carboxypeptidases during maturation and germination of the barley grain. Proc.Natl.Acad.Sci.USA. **91**: 8209-8213.

Delcour, J.A. and Verschaeve, S.G. (1987). Malt diastatic activity. Part II. A modified EBC diastatic power assay for the selective estimation of *beta*-amylase activity. Time and temperature dependence of the release of reducing sugars. J.Inst.Brew. **93**: 296-301.

Deyl, Z. and Miksik, I. (1997). Post-translational non-enzymatic modifications of proteins. 1. Chromatography of marker adducts with special emphasis to glycation reactions. J.Chromatog. B. **699**(1-2): 287-309.

Doan, D.N.P. and Fincher, G.B. (1992). Differences in the thermostabilities of barley (1→3,1→4)-ß-glucanases are only partly determined by N-glycosylation. FEBS Lett. **309**(3): 265-271.

Doi, H. and Kawakami, M. (1996). Purification of aminopeptidase from Japanese classified barley flour. Journal of Food Biochemistry.

Doi, H. and Kawakami, M. (1997). Purification of aminopeptidase from Australian classified barley. International Journal of Biochemistry and Cell Biology **29**(2): 345-352.

EBC, Analytica-EBC, 8th ed (1998). Verlag Hans Carl, Nürnberg.

Edmunds, M.D., Allan, G.R., Fincher, G.B. and Stuart, I.M. (1994). The quantitation of (1→3,1→4)-ß-glucanase isoenzymes during malting using an enzyme-linked immunosorbent assay. Convention of the Institute of Brewing (ANZ section), Sydney.

Enevoldsen, B.S. (1978). Degradation of starch by amylases in beer brewing. J.Jap.Soc.Starch Sci. **25**: 89-99.

Enevoldsen, B.S. and Bathgate, G.N. (1969). Structural analysis of wort dextrins by means of ß-amylase and the debranching enzyme, pullulanase. J.Inst.Brew. **75**: 433-443.

Enevoldsen, B.S. and Schmidt, F. (1974). Dextrins in brewing. Studies on the singly-branched and multiply-branched dextrins in brewing. J.Inst.Brew. **80**: 520-533.

Engh, R. and Huber, R. (1991). Accurate bond and angle parameters for X-ray protein structure refinement. Acta Crystallogr. **A47**: 392-400.

Erkkila, M.J., Leah, R., Ahokas, H. and Cameron-Mills, V. (1998). Allele dependent barley grain ß-amylase activity. Plant Physiol. **117:** 679-685

Evans, D.E. and Hejgaard, J. (1999). The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. J. Inst. Brew. **105**(3): 159-169

Evans, D.E., Lance, R.C.M. and MacLeod, L.C. (1994). The importance of protein Z in barley, malt and finally beer quality. Proc. 44th Australian Cereal Chemistry Conference, Ballarat, 11th-16th September. p187-192.

Evans, D.E., MacLeod, L.C., Eglinton, J.K., Gibson, C.E, Zhang, X., Wallace, W., Skerritt, J.H. and Lance R.C.M. (1997). Measurement of *Beta*-amylase in malting barley (*Hordeum vulgare* L.). Part 1: Development of a quantitative ELISA for *Beta*-amylase. J.Cer.Sci. **26**(2): 229-239.

Evans, D.E., MacLeod, L.C. and Lance, R.C.M. (1995). The importance of protein Z to the quality of barley and malt for brewing. Proceedings of the 25th EBC, Brussels.

Evans, D.E. and Sheehan, M.C. (2002). Don't be fobbed off: The substance of beer foam - A review. JASBC 60(2): 47-57.

Faris, J.D., Laddomada, B. and Gill, B.S. (1998). Molecular mapping of segregation distortion in *Aegilops tauschii*. Genetics 149(1): 319-327

Feuerstein, U., Brown, A.H.D. and Burdon, J.J. (1990). Linkage of rust resistance genes from wild barley (*Hordeum spontaneum*) with isozyme markers. Plant Breeding **104**: 318-324.

Fincher, G.B. (1975). Morphology and chemical composition of barley endosperm cell walls. J.Inst.Brew. **81**: 116-122.

Fincher, G.B., Lock, P.A., Morgan, M.M., Lingelbach, K., Wettenhall, R.E.H., Brandt, A. and Thomsen, K.K. (1986). Primary structure of a (1→3,1→4)-ß-glucan 4-glucanohydrolase from barley aleurone. Proc.Natl.Acad.Sci.USA **83**: 2081-2085.

Fincher, G.B. and Stone, B.A. (1993). Physiology and Biochemistry of Germination in Barley. In: Barley: Chemistry and Technology. St Paul, Minnesota, USA, American Association of Cereal Chemists Inc. Chapter 6.

Forster, B.P., Thomson, D.M., Watters, J. and Powell, W. (1991). Water soluble proteins of mature barley endosperm: genetic control, polymorphism, and linkage drag with ß-amylase and spring/winter habit. Theor.Appl.Genet. **81**: 787-792.

Gibson, C.E., Evans, D.E. and Proudlove, M.O. (1995). Protein Z4 and beer foam. Ferment: 81-84.

Gibson, T.S. and Solah, V. (1995). Diastatic power in malted barley: contributions of malt parameters to its development and the potential of barley grain ß-amylase to predict malt diastatic power. J.Inst.Brew. **101**: 277-280.

Giese, H. and Hejgaard, J. (1984). Synthesis of salt-soluble proteins in barley. Pulse labelling study of grain filling in liquid cultured detached spikes. Planta **161**:172-177.

Giese, H. and Hopp, H.E. (1984). Influence of nitrogen nutrition on the amount of hordein, protein Z and ß-amylase messenger RNA in developing endosperms of barley. Carlsberg Res.Commun. **49**: 365-383.

Grime, K.H. and Briggs, D.E. (1996). The release of bound ß-amylase by macromolecules. J.Inst.Brew. **102**: 261-270.

Guerin, J.R., Lance, R.C.M. and Wallace, W. (1992a). Release and activation of barley ß-amylase by malt endopeptidases. J.Cer.Sci. **15**: 5-14.

Guerin, J.R., Lance, R.C.M. and Wallace, W. (1992b). Chromosomal location of a malt endopeptidase gene. Genet.Res.Camb. **59**: 179-181.

Guex, N. and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modelling. Electrophoresis **18**: 2714-2723.

Hamilton, D.M. and Lewis, M.J. (1974). Factors affecting wort extract and attenuation. M.B.A.A. Technical Quarterly **11**(1): 31-35.

Hara-Nishimura, I., Nishimura, M. and Daussant, J. (1986). Conversion of free ß-amylase to bound ß-amylase on starch granules in the barley endosperm during desiccation phase of seed development. Protoplasma **134**: 149-153.

Hardie, D.G. (1975). Control of carbohydrase formation by gibberellic acid in barley endosperm. Phytochemistry. **14**: 1719-1722.

Harlan, J.R. and Zohary, D. (1966). Distribution of wild wheats and barley. Science. **153**: 1074-1080.

Harlow, E. and Lane, D. Antibodies. A laboratory manual. Cold Spring Harbour Press, Cold Spring Harbour, New York (1988).

Harthill, J.E. and Thomsen, K.K. (1995). Analysis of glycan structures of barley (1→3,1→4)-ß-D-glucan4-glucanohydrolase isoenzyme EII. Plant Physiology and Biochemistry **33**(1): 9-18.

Hayes, P.M., Liu, B.H., Knapp, S.J., Chen, F., Jones, B., Blake, T., Franckowiak, J., Rasmussen, D., Sorrells, M., Ullrich, S.E., Wesenburg, D. and Kleinhofs, A. (1993). Quantitative trait locus affects and environmental interaction in a sample of North American germplasm. Theor.Appl.Genet. **87:** 392-401

Hejgaard, J. (1976). Free and protein bound ß-amylases of barley grain. Characterisation by two-dimensional immunoelectrophoresis. Physiol.Plant. **38**: 293-299.

Hejgaard, J. (1977). Origin of a dominant beer protein. Immunochemical identity with a ß-amylase associated protein from barley. J.Inst.Brew. **83**: 94-96.

Hejgaard, J. (1978). 'Free' and 'bound' ß-amylases during malting of barley. Characterisation by two-dimensional immunoelectrophoresis. J.Inst.Brew. **84**: 43-46.

Hejgaard, J. (1982). Purification and properties of protein Z - a major albumen of barley endosperm. Physiol.Plant. **54**: 174-182.

Hejgaard, J. (1984). Gene products of barley chromosomes 4 and 7 are precursors of the major antigenic beer protein. J.Inst.Brew. **90**: 85-87.

Hejgaard, J. and Boisen, S. (1980). High lysine proteins in Hiproly barley breeding: Identification, nutritional significance and new screening methods. Hereditas. **93**: 311-320.

Hejgaard, J. and Carlsen, S. (1977). Immunoelectrophoretic identification of a heterodimer ß-amylase in extracts of barley grain. J.Sci.Food Agric. **28**: 900-904.

Hejgaard, J. and Kaersgaard, P. (1983). Purification and properties of the major antigenic beer protein of barley origin. J.Inst.Brew. **89**: 402-410.

Hejgaard, J., Rasmussen, S.K., Brandt, A. and Svendson, I. (1985). Sequence homology between barley endosperm protein Z and protease inhibitors of the α1-antitrypsin family. FEBS Lett. **180**: 89-94.

Henry, R.J. (1990). Quantitative analysis of barley (1→3),(1→4)-ß-glucanase isoenzymes by high-performance liquid chromatography. J.Cer.Sci. **12**: 187-192.

Henson, C.A. and Stone, J.M. (1988). Variation in α-amylase and α-amylase inhibitor activities in barley malts. J.Cer.Sci. **8**: 39-46.

Hoj, P., Hoogenraad, N.J., Hartman, D.J., Yannakena, H. and Fincher, G.B. (1990). Identification of individual (1→3,1→4)-ß-D-glucanase isoenzymes in extracts of germinated barley using specific monoclonal antibodies. J.Cer.Sci. **11**(3): 261-268.

Holwerda, B.C., Jana, S., Crosby, W.L. and Rogers, J.C. (1993). Large differences in genetic variation of multigene families in barley. Journal of Heredity **84**(1): 29-33.

Hooft, R.W.W., Vriend, G., Sander, C., Abola, E.E. (1996). Errors in protein structures. Nature **381**:272-272.

Ingverson, J., Englyst, A. and Jorgensen, K.G. (1989). Evaluation of malting quality in a barley breeding programme. Use of α-amylase and β-glucan levels in malt as preselection tools. J.Inst.Brew. **95**(2): 99-103.

Inoue, T. (1996). Brewing Industry Requirements for Malting Barley in Japan. Proceedings of the V IOC and VII IBGS, Saskatoon, Saskatchewan, Canada, University Extension Press. pp 30-35.

IOB (1997). Recommended Methods of Analysis. Institute of Brewing, London.

Jahoor, A. and Fishbeck, G. (1987). Genetical studies of resistance to powdery mildew in barley lines derived from *Hordeum spontaneum* collected from Israel. Plant Breeding **99**: 265-273.

Jana, S. and Pietrzak, L.N. (1988). Comparative assessment of genetic diversity in wild and primitive cultivated barley in a centre of diversity. Genetics **119**: 981-990.

Jende-Strid, B. (1997). Proanthocyanidin-free malting barley – a solution of the beer haze problem. Proceedings of the 26th EBC Congress, Maastricht.

Jonassen, I. (1980). Characterisation of Hiproly barley. 1. Isolation and characterisation of two water-soluble high lysine proteins. Carlsberg Res.Commun. **45**: 47-58.

Jensen, L.G., Olsen, O., Kops, O., Wolf, N., Thomsen, K.K. and von Wettstein, D. (1996). Transgenic barley expressing a protein engineered, thermostable (1,3-1,4)-ß-glucanase during germination. Proc.Natl.Acad.Sci.USA. **93**: 3487-3491.

Jones, B.L. and Marinac, L.A. (1995). A comparison of barley and malt polypeptides that inhibit green malt endoproteinases. J.A.S.B.C. **53**(4): 160-166.

Jones, B.L. and Marinac, L.A. (1997). Purification, identification and partial characterisation of a barley protein that inhibits green malt endopeptidases. J.A.S.B.C. **55**(2): 58-64.

Jorgensen, O.B. (1965). Barley malt α-glucosidase. VI. Localization and development during barley germination. Acta Chem.Scand. **19**: 1014-1015.

Kaneko, T., Kihara, M. and Ito, K. (2000). Genetic analysis of ß-amylase thermostability to develop a DNA marker for malt fermentability improvement in barley, *Hordeum vulgare* L. Plant Breeding **119:** 197-201.

Kauffman, J.A., Mills, E.N.C., Brett, G.M., Fido, R., Tatham, A.S., Shewry, P.R., Onishi, A., Proudlove, A. and Morgan, M.R.A. (1994). Immunological characterisation of barley polypeptides in lager foam. J.Sci.Food Agric. **66**: 345-355.

Khursheed, B. and Rogers, J.C. (1988). Barley α-amylase genes: Quantitative comparison of steady state mRNA levels from individual members of the two different families expressed in aleurone cells. J.Biol.Chem. **263**: 18953-18960.

Kihara, M., Kaneko, T. and Ito, K. (1998). Genetic variation of ß-amylase thermostability among varieties of barley, *Hordeum vulgare* L., and relation to malting quality. Plant Breeding **117:** 425-428.

Kobyljanskij, V.D. (1967). Biological features of wild species of barley with a view to their utilization in breeding. Armen.Biol.J. **20**: 41-51.

Koradi, R., Billeter, M. and Wüthrick, K. (1996). MOLMOL: A program for display and analysis of macromolecular structures. J.Mol.Graph. **14**: 51-55.

Kosambi, D.D. (1944). The estimation of map distances from recombination values. Ann.Eugen. 12: 172-175.

Kreis, M., Williamson, M., Buxton, B., Pywell, J., Hejgaard, J. and Svendson, I. (1987). Primary structure and differential expression of ß-amylase in normal and mutant barleys. Eur.J.Biochem. **169**: 517-525.

Kreis, M., Williamson, M.S., Shrewry, P.R., Sharp, P. and Gale, M. (1988). Identification of a second locus encoding ß-amylase on chromosome 2 of barley. Genet.Res.Camb. **51**: 13-16.

LaBerge, D.E. and Marchylo, B.A. (1983). Heterogeneity of the ß-amylase enzymes of barley. ASBC J. **41**: 120-124.

Laederach, A., Dowd, M.K., Coutinho, P.M. and Reilly, P.J. (1999). Automated docking of maltose, 2-deoxymaltose, and maltotetraose into the soybean ß-amylase active site. Proteins: Struct.Funct.Genet. **37:** 166-175.

Lander, E.S. and Botstein, D. (1989). Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics. **121:** 185-199.

Langridge, P., Karakousis, A., Collins, N., Kretschmer, J. and Manning, S. (1995). A consensus linakge map of barley. Mol.Breed. 1: 389-395.

Lapolla, A., Fedele, D. and Traldi, P. (1994). Studies on advanced glycation end products by recent mass spectrometric techniques. Amino Acids **6**: 65-96.

Lee, W.J. and Pylor, R.E. (1984). Barley malt limit dextrinase: Varietal, environmental and malting effects. ASBC J. **42**: 11-17.

Lenoir, P., MacGregor, W., Moll, M. and Daussant, J. (1984). Identification de la R-enzyme de'orge et du malt par focalisation isoelectrique. C.R.Acad.Sc.Paris. **298**: 243-248.

Letters, R. (1977). ß-glucans in brewing. Eur.Brew.Conf., Amsterdam.

Lin, Y. and Yu, Z. (1990). Correlation analysis of ß-amylase activity in mature barley seeds and malt diastatic power. Hereditas (Beijing) **12**(4): 12-14.

Litts, J.C., Simmons, C.R., Karrer, E.E., Huang, N., and Rodriguez, R.L. (1990). The isolation and characterisation of a barley 1,3-1,4-ß-glucanase gene. Eur.J.Biochem. **194**: 831-838.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10**(8): 1391-1406.

Logue, S.J., Eglinton, J.K., Margach, C., Roumeliotis, S., Tansing, P., Evans, D.E. and Barr, A.R. (1999). Application of ß-amylase thermostability analysis in a barley breeding program. Proceedings of the 9th Australian Barley Technical Symposium, Melbourne, Australia. P3.4.1-3.4.5.

Loi, L., Ahluwalia, B. and Fincher, G.B. (1988). Chromosomal location of genes encoding barley (1→3,1→4)-β–glucan 4-glucanohydrolases. Plant Physiol. **87**(2): 300-302.

Loi, L., Barton, P.A. and Fincher, G.B. (1987). Survival of barley (1→3,1→4)-ß-glucanase isoenzymes during mashing and kilning. J.Cer.Sci. **5**: 45-50.

Longstaff, M.A. and Bryce, J.H. (1993). Development of limit dextrinase in germinated barley (*Hordeum vulgare*). Evidence of proteolytic activation. Plant Physiol. **101**: 881-889.

Lundgard, R. and Svensson, B. (1986). Limited proteolysis in the carboxy-terminal region of barley ß-amylase. Carlsberg Res.Commun. **51**: 487-491.

Lundgard, R. and Svensson, B. (1987). The four major forms of barley ß-amylase. Purification, characterisation and structural relationship. Carlsberg Res.Commun. **52**: 313-326.

Lundgard, R. and Svensson, B. (1989). A 39kD barley seed protein of the serpin superfamily inhibits α-chymotrypsin. Carlsberg Res.Commun. **54**: 173-180.

Ma, Y., Eglinton, J.K., Evans, D.E., Logue, S.J. and Langridge, P. (2000). Removal of the four C-terminal glycine-rich repeats enhances the thermostability and substrate binding affinity of barley ß-amylase. Biochemistry **39** (44): 13350-13355.

Ma, Y.F., Evans, D.E., Logue, S.J., and Langridge, P. (2001). Mutations of barley ß-amylase that improve substrate binding affinity and thermostability. Mol.Genet. Genomics. **266**: 345-352

MacGregor, A.W. Constraints on starch breakdown during mashing. Proc. 7th Australian Barley Technical Symposium, Perth, 1995. pp 151-155.

MacGregor, A.W. (1996). Malting and brewing science: Challenges and opportunities. J.Inst.Brew. **102**: 97-102.

MacGregor, A.W. and Ballance, D.L. (1980). Hydrolysis of large and small starch granules from normal and waxy barleys by α-amylases from barley malt. Cereal Chem. **57**: 397-402.

MacGregor, A.W. and Lenoir, C. (1987). Studies on α-glucosidase in barley and malt. J.Inst.Brew. **93**: 334-337.

MacGregor, A.W., Bazin, S.L., Macri, L.J and Babb, J.C. (1999). Modelling the contribution of alpha-amylase, beta-amylase and limit dextrinase to starch degradation during mashing. J.Cer.Sci. **29**(2): 161-169.

MacGregor, A.W., Macri, L.J., Schroeder, S.W. and Bazin, S.L. (1994). Purification and characterisation of limit dextrinase inhibitors from barley. J.Cer.Sci. **20**: 33-41.

MacGregor, A.W., Marchylo, B.A. and Kruger, J.E. (1988). Multiple α–amylase components in germinated cereal grains determined by isoelectric focusing and chromatofocusing. Cereal Chem. **65**: 326-333.

MacLeod, L.C., Lance, R.C.M. and Brown, A.H.D. (1991). Chromosomal mapping of the *Glb1* locus encoding (1→3),(1→4)-ß-D-glucan 4-glucanohydrolase EI in barley. J.Cer.Sci. **13**: 291-298.

Macri, L.J., MacGregor, A.W., Schroeder, S.W. and Bazin, S.L. (1993). Detection of a limit dextrinase in barley. J.Cer.Sci. **18**: 103-106.

Maillard, L.C. (1912). Action des acid amines sur les sucres; formation des melanoidines par voie methodique. C.R.Acad.Sci. **154**: 66-68.

Manley, K.F. and Elliot, R.W. (1993). RI manager, a microcomputer program for analysis of data from recombinant inbred strains. Mamm.Gen. 1: 123-126.

Manners, D.J. and Yellowlees, D. (1973). Studies on debranching enzymes. Part 1.The limit dextrinase activity of extracts of certain higher plants and commercial malts. J.Inst.Brew. **79**: 377-385.

Mather, D.E., Tinker, N.A., LaBerge, D.E., Edney, M., Jones, B., Rossnagel, B.G., Legge, W.G., Briggs, K.G., Irvine, R.B., Falk, D.E. and Kasha, K.J. (1997). Regions of the genome that affect grain and malt quality in a North American two-row barley cross. Crop Sci. **37:** 544-554.

Mathews, B.W. (1993). Structural and genetic analysis of protein stability. Annu. Rev. Biochem. **62**: 139-160

McLeary, B.V. and Codd, R. (1989). Measurement of ß-amylase in cereal flours and commercial enzyme preparations. J.Cer.Sci. **9:** 17-33.

Meyer, R.C., Swanston, J.S., Young, G.R., Lawrence, P.E., Bertie, A., Ritchie, J., Wilson, A., Brosnan, J., Pearson, S., Bringhurst, T., Steele, G., Powell, W. and Thomas, W.T.B. (2000). Genetic approaches to improving distilling quality in barley. Proceedings of the VIII International Barley Genetics Symposium, 22-27 October, Adelaide, Australia. Pages 107-114.

Michelmore, R.W., Paran, I. and Kesseli, R.V. (1991). Identification of markers linked to disease resistance by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc.Nat.Acad.Sci. USA. **88:** 9828-9832.

Mikami, B., Hehre, E.J., Sato, M., Katsube, Y., Hirose, M., Morita, Y. and Sacchettini, J.C. (1993). The 2.0-Å resolution structure of soybean ß-amylase complexed with α-cyclodextrin. Biochemistry **32:** 6836-6845.

Mikami, B., Degano, M., Hehre, E.J. and Sacchettini, J.C. (1994). Crystal structure of soybean ß-amylase reacted with ß-maltose and maltal: active site components and their apparent roles in catalysis. Biochemistry **33:** 7779-7787.

Mikami, B., Yoon, H., and Yoshigi, N. (1999). The crystal structure of the sevenfold mutant of barley ß-amylase with increased thermostability at 2.5 Å resolution. J.Mol.Biol. **285:** 1235-1243.

Mikola, J. (1983). Proteinases and peptidases in germinating cereal grains. In: Seed Proteins. J. Daussant, J. Moss and J. Vaughan (eds) pp 35-52, Academic Press, London.

Mulcahy, D.L. and Mulcahy, G.B. (1983). Gametophytic self-incompatibility reexamined. Science 220: 1247-1251.

Munck, L., Mundy, J. and Vaag, P. (1985). Characterisation of enzyme inhibitors in barley and their tentative role in malting and brewing. ASBC Journal **43**(1): 35-38.

Muthukrishnan, S., Gill, B.S., Swegle, M. and Chandra, G.R. (1984). Structural genes for barley α-amylases are located on chromosomes-1 and -6. J.Biol.Chem. **259**: 13637-13639.

Narziss, I., Rusitzna, P. and Stippler, K. Effect of the drying process on the development of malt enzymes and several other groups of components. European Brewery Convention Proceedings of the 14th Congress, Salzburg, (1973), Elsevier, Amsterdam.

Nelson, J.C. (1997). QGENE: Software for marker based genomic analysis and breeding. Mol.Breed. **3:** 229-235

Netsvetaev, V.P. (1993). The location of ß-amylase (*Bmy*1) on chromosome 4 of barley. Tsitologiya I Genetika **27**(5): 74-78

Nevo, E., Beiles, A. and Zohary, D. (1986). Genetic resources of wild barley in the near East: Structure, evolution and application in breeding. Biol.J.Linn.Soc. **27**: 355-380.

Nevo, E., Zohary, D., Brown, A.H.D. and Haber, M. (1979a). Genetic diversity and environmental associations of wild barley, *Hordeum spontaneum*, in Israel. Evolution **33**(3): 815-833.

Nevo, E., Brown, A.H.D. and Zohary, D. (1979b). Genetic diversity in the wild progenitor of barley in Israel. Experentia. **35**: 1027-1029.

Nielsen, G. and Johansen, H.B. (1986) Proposal for the identification of barley varieties based on the genotypes for two hordein and 39 isoenzyme loci of 47 reference varieties. Euphytica. **35**: 717-728.

Nielsen, G., Johansen, H., Jensen, J. and Hejgaard, J. (1983). Localization on barley chromosome 4 of genes coding for beta-amylase (*Bmy1*) and protein Z (*Paz1*). Barley Genetics Newsletter **13**: 55-57.

Niku-Paavola, M.L., Skakoun, A., Nummi, M. and Daussant, J. (1973). The polymorphism of barley ß-amylase. Biochim.Biophys.Acta. **322**: 181-184.

Norris, K. and Lewis, L. (1985). Application of a commercial barley ß-amylase in brewing. A.S.B.C. **43**: 96-101.

Okada, Y., Yoshigi, N., Sahara, H. and Koshino, S. (1995). Increase in thermostability of recombinant barley ß-amylase by random mutagenesis. Biosci.Biotech.Biochem. **59**(6): 1152-1153.

Olsen, O., Borriss, R., Simon, O. and Thomsen, K.K. (1991). Hybrid *Bacillus* (1→3,1→4)-ß-glucanases: Engineering thermostable enzymes by construction of hybrid genes. Mol.Gen.Genet. **225**: 177-185.

Paris, M., Jones, M.G.K., and Eglinton, J.K. (2002). Genotyping single nucleotide polymorphisms for selection of barley beta-amylase alleles. P.Mol.Biol.Rep. **20**(2): 149-159.

Peitsch, M.C. (1995). Protein modelling by e-mail. Bio/Technology **13**: 658-660.

Peitsch, M.C. (1996). ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. Biochem.Soc.Trans. **24**: 274-279.

Petersen, L., Ostergard, H. and Giese, H. (1994). Genetic diversity among wild and cultivated barley as revealed by RFLP. Theor.Appl.Genet. **89**: 676-681.

Powling, A., Islam, A.K.M.R. and Shepherd, K.W. (1981). Isozymes in wheat-barley hybrid derivative lines. Biochem.Genet. **19**: 235-232.

Ragot, F., Guinard, J.X., Shoemaker, C.F. and Lewis, M.J. (1989). The contribution of dextrins to beer sensory properties, Part I: Mouthfeel. J.Inst.Brew. **95**: 427-430.

Ramachandran, G.N., Ramakrishnan, and V.Sasisekharan, V. (1963). Stereochemistry of polypeptide chain conformations. J.Mol.Biol. **7**: 95-99.

Ramamoorthy, J., Chong, D.K. and Chinoppa, C.C. (1994). Comparative assessment of genetic diversity in wild and cultivated barley using ribosomal DNA spacer length variants. **42**: 115-123.

Rasmussen, S.K. (1993). A gene coding for a new plant serpin. Biochim.Biophys.Acta **1172**: 151-154.

Rasmussen, S.K., Dahl, S.W., Norgard, A. and Hejgaard, J. (1996). A recombinant wheat serpin with inhibitory activity. Plant Molec.Biol. **30**: 673-677.

Rasmussen, S.K., Hopp, H.E., Brandt, A., Svendsen, I. and Hejgaard, J. (1984). A cDNA clone for protein Z, a major barley endosperm albumen. Carlsberg Res.Commun. **49**: 385-390.

Ray, R.R., Jana, S.C. and Nanda, G. (1994). Biochemical approaches to increasing thermostability of ß-amylase from *Bacillus megaterium* B6. FEBS Letters **356:** 30-32.

Richardson, J.S. and Richardson, D.C. (1988). Amino-acid preferences for specific locations at the ends of alpha-helices. **240:** 1648-1652.

Rogers, J.C. (1985). Two barley α-amylase gene families are regulated differently in aleurone cells. J.Biol.Chem. **260**: 3731-3738

Rogers, J.C. and Milliman, C. (1983). Isolation and sequence analysis of a barley α-amylase cDNA clone. J.Biol.Chem. **258**(13): 8169-8174.

Runkel, U.D. (1975). Malt Kilning and its Influence on Malt and Beer Quality. Eur.Brew.Conv. Monograph II., Zeist.

Saghai-Maroof, M.A., Allard, R.W. and Zhang, Q. (1990). Genetic diversity and ecogeographical differentiation among ribosomal DNA alleles in wild and cultivated barley. Proc.Natl.Acad.Sci.USA. **87**: 8486-8490.

Sali, D., Bycroft, M. and Fersht, A.R. (1988). Stabilization of protein-structure by interaction of alpha-helix dipole with a charged side-chain. Nature **335**: 740-743.

Sallans, H.R. and Anderson, J.A. (1940). Varietal differences in barleys and malts II. Saccharifying activites of barleys and malts and the correlations between them. Can.J.Res. **16**: 405-416.

Sandegren, E. and Klang, N. (1950). On barley amylase and proteinase. J.Inst.Brew. **61**: 313-318.

Schulze, A.J., Huber, R., Bode, W. and Engh, R.A. (1994). Structural aspects of serpin inhibition. FEBS Lett. **344**: 117-124.

Schur, F. (1985). Beurteilung der Sudhausarbeit anhand des Starkeabbaugrades. Brauwelt **125**: 1195-1199.

Schuster, R. (1988). Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid chromatography. Journal of Chromatography **431**: 271-384.

Shen-Miller, J., Kreis, M., Shewry, P.R. and Springall, D.R. (1991). Spatial distribution of ß-amylase in germinating barley seeds based on the avidin-biotin-peroxidase complex method. Protoplasma **163**: 162-173.

Shewry, P.R., Parmar, S., Buxton, B., Gale, M.D., Liu, C.J., Hejgaard, J. and Kreis, M. (1988). Multiple molecular forms of ß-amylase in seeds and vegetative tissues of barley. Planta **167**: 127-134.

Sidenius, U., Olsen, K., Svensson, B. and Christenson, U. (1995). Stopped flow kinetics of barley α-amylase/subtilisin inhibitor and the high pI barley α-amylase. FEBS Lett. **361**: 250-254.

Simon, P. and Jones, R.L. (1988). Synthesis and secretion of catalytically active barley α-amylase isoforms by *Xenopus* oocytes injected with barley mRNAs. Eur.J.Cell Biol. **47**:213-221

Sissons, M.J., Lance, R.C.M. and Sparrow, D.H.B. (1992a). Studies on limit dextrinase in barley I. Purification of malt limit dextrinase and production of monospecific antibodies. J.Cer.Sci. **16**: 107-116.

Sissons, M.J., Lance, R.C.M. and Sparrow, D.H.B. (1992b). Studies on limit dextrinase in barley II. Application of an ELISA and immunoblotting to studies of genetic variation and malting effects. J.Cer.Sci. **16**: 117-128.

Sissons, M.J., Lance, R.C.M. and Sparrow, D.H.B. (1993). Studies on limit dextrinase in barley III. Limit dextrinase in developing kernels. J.Cer.Sci. **17**: 19-24.

Sissons, M.J. and MacGregor, A.W. (1994). Hydrolysis of barley starch granules by α-glucosidases from malt. J.Cer.Sci. **19**(2): 161-169.

Sjoholm, K., Macri, L.J. and MacGregor, A.W. (1995). Is there a role for limit dextrinase in mashing? Proceedings of the 25th European Brewing Convention, Brussels.

Slakeski, N., Baulcombe, D.C., Devos, K.M., Ahluwalia, B., Doan, D.N.P. and Fincher, G.B. (1990). Structure and tissue-specific regulation of genes encoding barley (1□3,1□4)-ß-glucan endohydrolases. Mol.Gen.Genet. **224**: 437-449.

Slakeski, N. and Fincher, G.B. (1992a). Barley (1→3)(1→4)-ß-glucanase isoenzyme EI gene expression is mediated by auxin and gibberellic acid. FEBS Lett. **306**: 98-102.

Slakeski, N. and Fincher, G.B. (1992b). Developmental regulation of (1→3)(1→4)-ß-glucanase expression in barley. Plant Physiol. **99**: 1226-1231.

Sogaard, M., Olsen, F.L. and Svensson, B. (1991). C-terminal processing of barley α-amylase1 in malt, aleurone protoplasts, and yeast. Proc.Natl.Acad.Sci.USA. **88**: 8140-8144.

Sopanen, T. and Lauriere, C. (1989). Release and activity of bound ß-amylase in a germinating barley grain. Plant Physiol. **89**: 244-249.

Sorensen, S.B., Bech, L.M., Muldberg, M., Beenfeldt, T., and Breddam, K. (1993). Barley lipid transfer protein 1 is involved in beer foam formation. M.B.A.A.Tech.Quarterly **30:** 135-145, 1993.

Sorensen, M.B., Cameron-Mills, V. and Brandt, A. (1989). Transcriptional and posttranscriptional regulation of gene expression in developing barley endosperm. Mol.Gen.Genet. **217**: 195-201.

Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996). Hordein promoter methylation and transcriptional activity in wild type and mutant barley endosperm. Mol.Gen.Genet. **250**: 750-760.

Sorensen, S.B., Svendsen, I. and Breddam, K. (1989). Primary structure of carboxypeptidase II from malted barley. Carlsberg Res.Commun. **54**: 193-202.

Stenholm, K. and Home, S. (1999). A new approach to limit dextrinase and its role in mashing. J.Inst.Brew. **105**(4): 205-210.

Stewart, R.J., Garrett, T.P.J., Varghese, J.N., Hoj, P.B. and Fincher, G.B. (1996). Protein engineering of enzymes for improved malting quality of barley. V International Oat Conference and VII International Barley Genetics Symposium, Saskatoon, University Extension Press. pp 7-19.

Stuart, I.M. and Fincher, G.B. (1983). Immunological determination of (1→3),(1→4)-ß-D-glucan endohydrolase development in germinating barley (*Hordeum vulgare*). FEBS Lett. **155**: 201-204.

Stuart, I.M., Loi, L. and Fincher, G.B. (1988). Varietal and environmental variations in (1→3,1→4) ß-glucan levels and (1→3,1→4)-ß-glucanase potential in barley: Relationships to malting quality. J.Cer.Sci. **7**: 61-71.

Svensson, B. (1988). Regional distant sequence homology between amylases, α-glucosidase and transglucanosylase. FEBS Letters **230**: 72-76.

Sun, Z. and Henson, C.A. (1991). A quantitative assessment of the importance of barley seed α-amylase, ß-amylase, debranching enzyme, and α-glucosidase in starch degradation. Arch.Biochem.Biophys. **284**: 298-305.

Svensson, B., Mundy, J., Gibson, R.M. and Svendsen, I. (1985). Partial amino acid sequences of α-amylase isozymes from barley malt. Carlsberg Res.Commun. **50**: 15-22.

Takano, T., Kiribuchi, C. and Takeda, G. (1988). Genetic analysis on banding patterns and activity of α-amylase isozymes in barley (*Hordeum vulgare* L.). Japan.J.Breed. **38**: 65-71.

Takano, T. and Takeda, G. (1985). Polymorphism for α-amylase in germinating seed and malt of barley varieties detected by isoelectric-focusing gel electrophoresis. Japa.J.Breed. **35**: 9-16.

Tanksley, S.D. and Nelson, J.C. (1996). Advanced backcross QTL analysis: A method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. Theor.Appl.Genet. **92** (2): 191-203.

Thompson, D.M., Powell, W. and Forster, B.P. (1990). Use of isoelectric focussing in barley varietal identification. Ann.Appl.Biol. **117**: 625-631.

Tsiboli, P., Konstantinidis, G., Skendros, Y., Katsani, A. and Cholipapadopoulou, T. (1997). Identification of post-translational modified amino acids. Amino Acids. **13**(1): 13-23.

Varghese, J.N., Garrett, T.P.J., Colman, P.M., Chen, L., Hoj, P.B. and Fincher, G.B. (1994). Three-dimensional structures of two plant ß-glucan endohydrolases with distinct substrate specificities. Proc.Natl.Acad.Sci.USA **91**(7): 2785-2789.

Visuri, K. and Nummi, M. (1972). Purification and characterisation of crystalline ß-amylase from barley. Eur.J.Biochem. **28**: 555-565.

Vriend, G. (1990). WHAT IF: A molecular modeling and drug design program. J.Mol.Graph. **8:** 52-56.

Wittmann, R, and Eichner, K. (1989). Detection of Maillard products in malts, beers, and brewing couleurs. Zeitschrift Feur Lebensmittel-Untersuchung und –Forschung. **188**(3): 212-220.

Wolf, N. (1991). Complete nucleotide sequence of a *Hordeum vulgare* gene encoding (1→3,1→4)–β–D–glucanase isoenzyme II. Plant Physiol. **96**(4): 1382-1384.

Wolf, N. (1992). Structure of the genes encoding *Hordeum vulgare* (1→3,1→4)-ß-D-glucanase isoenzymes I and II and functional analysis of their promoters in barley aleurone protoplasts. Mol.Gen.Genet. **234**: 33-42.

Woodward, J.R. and Fincher, G.B. (1982a). Purification and chemical properties of two (1→3,1→4)-ß-glucan endohydrolases from germinating barley. Eur.J.Biochem. **121**: 663-669.

Woodward, J.R. and Fincher, G.B. (1982b). Substrate specificities and kinetic properties of two (1→3,1→4)-ß-D-glucan endo-hydrolases from germinating barley (*Hordeum vulgare*). Carbohydr.Res. **106**: 111-122.

Yamashita, H., Fujino, S., Aoyagi, S., Tsumura, Y., Hayase, F. and Kato, H. (1989). Characterisation and mechanisms of formation of frozen beer precipitates. J.Am.Soc.Brew.Chem.: 77-81.

Yoshigi, N., Okada, Y., Maeba, H., Sahara, H. and Tamaki, T. (1995). Construction of a plasmid used for the expression of a sevenfold-mutant barley ß-amylase with increased thermostability in *Escherichia coli* and properties of the sevenfold-mutant ß-amylase. J.Biochem. **118**: 562-567.

Yoshigi, N., Okada, Y., Sahara, H. and Koshino, S. (1994). Expression in *Escherichia coli* of cDNA encoding barley ß-amylase and properties of recombinant ß-amylase. Biosci.Biotech.Biochem. **58**(6): 1080-1086.

Yoshigi, N., Sahara, H. and Koshino, S. (1995). Role of the C-terminal region of ß-amylase from barley. J.Biochem. **117**(1): 63-67.

Zhang, N.Y. and Jones, B.L. (1995). Characterisation of germinating barley endoproteolytic enzymes by two-dimesional electrophoresis. J.Cer.Sci. **21**(2): 145-153.

Zohary, D. (1969). The Progenitors of Wheat and Barley in Relation to Domestication and Agricultural Dispersal in the Old World. In: The Domestication and Exploitation of Plants and Animals. P.J. Ucko and G.W. Dimbleby (eds), Gerald Duckworth and Co. Ltd., London. p 47-66.

**Appendix I:** ß-amylase, Protein Z, and (1→3,1→4)-ß-glucanase isoenzyme EI screening results for *H. spontaneum* accessions from Israel.

| Number | Site in Israel | CPI # | Site/Family | B-Amylase | Protein-Z | EBG EI |
|---|---|---|---|---|---|---|
| | | | | | | |
| | **Coastal** | | | | | |
| 1 | Ackhziv | 77127 | 24/5 | SD1 | F | wt |
| 2 | Ackhziv | 77127 | 24/7 | SD2 | F | wt |
| 3 | Ackhziv | 77127 | 24/36 | SD1 | A | wt |
| 4 | Ackhziv | 77127 | 24/44 | SD1 | B | wt |
| 5 | Ackhziv | 77127 | 24/45 | SD2 | B | wt |
| 6 | Ackhziv | 77127 | 24/46 | SD2 | B | wt |
| 7 | Ackhziv | 77127 | 24/50 | SD1 | B | wt |
| 8 | Ackhziv | 77127 | 24/55 | SD2 | B | wt |
| 9 | Atlit | 77129 | 25/2 | SD1 | F | wt |
| 10 | Atlit | 77129 | 25/27 | SD1 | F | D13 |
| 11 | Atlit | 77129 | 25/28 | SD2 | G | D13 |
| 12 | Atlit | 77129 | 25/31 | SD1 | F | D13 |
| 13 | Atlit | 77129 | 25/37 | SD1 | F | nb |
| 14 | Atlit | 77129 | 25/44 | SD2 | A | wt |
| 15 | Atlit | 77129 | 25/49 | SD1 | F | wt |
| 16 | Atlit | 77129 | 25/50 | SD2 | F | wt |
| 17 | Atlit | 77129 | 25/54 | SD1 | F | D13 |
| 18 | Atlit | 77129 | 25/55 | SD2 | F | wt |
| 19 | Caesarea | 77132 | 26/4 | SD2 | F | wt |
| 20 | Caesarea | 77132 | 26/25 | SD2 | F | wt |
| 21 | Caesarea | 77132 | 26/38 | SD2 | B | wt |
| 22 | Caesarea | 77132 | 26/44 | SD2 | B | wt |
| 23 | Herzliyya | 77134 | 27/14 | SD2 | C | D13 |
| 24 | Herzliyya | 77134 | 27/19 | SD1 | F | D13 |
| 25 | Herzliyya | 77134 | 27/24 | SD1 | F | D13 |
| | | | | | | |
| | **Mountains** | | | | | |
| 26 | Bargiyyora | 71283 | 13/5 | SD2 | F | nb |
| 27 | Bargiyyora | 71283 | 13/8 | SD5 | B | wt |
| 28 | Bargiyyora | 71283 | 13/10 | SD5 | B | wt |
| 29 | Bargiyyora | 71283 | 13/20 | SD5 | B | wt |
| 30 | Bargiyyora | 71283 | 13/25 | SD5 | B | wt |
| 31 | Bargiyyora | 71283 | 13/27 | SD2 | F | wt |
| 32 | Bargiyyora | 71283 | 13/31 | SD2 | F | nb |
| 33 | Bargiyyora | 71283 | 13/32 | SD2 | F | nb |
| 34 | Bargiyyora | 71283 | 13/42 | SD5 | F | wt |
| 35 | Bargiyyora | 71283 | 13/48 | SD5 | F | nb |
| 36 | Damon | 71284 | 11/13 | SD2 | C | D13 |
| 37 | Damon | 71284 | 11/22 | SD2 | C | wt |
| 38 | Damon | 71284 | 11/23 | SD2 | C | nb |
| 39 | Damon | 71284 | 11/37 | SD2 | F | wt |
| 40 | Damon | 71284 | 11/48 | SD2 | F | wt |

| Number | Site in Israel | CPI # | Site/Family | B-Amylase | Protein-Z | EBG EI |
|---|---|---|---|---|---|---|
| | | | | | | |
| 41 | Damon | 71284 | 11/52 | SD2 | F | wt |
| 42 | Eyzariya | 77130 | 15/19 | SD2 | C | wt |
| 43 | Eyzariya | 77130 | 15/20 | SD2 | B | wt |
| 44 | Eyzariya | 77130 | 15/21 | SD2 | D | wt |
| 45 | Eyzariya | 77130 | 15/25 | SD2 | B | wt |
| 46 | Eyzariya | 77130 | 15/30 | SD2 | B | nb |
| 47 | Eyzariya | 77130 | 15/32 | SD2 | B | nb |
| 48 | Eyzariya | 77130 | 15/33 | SD2 | C | wt |
| 49 | Eyzariya | 77130 | 15/35 | SD2 | B | wt |
| 50 | Eyzariya | 77130 | 15/41 | SD2 | B | wt |
| 51 | Mt Hermon | 77133 | 1/9 | SD1 | B | wt |
| 52 | Mt Hermon | 77133 | 1/16 | SD1 | B | D13 |
| 53 | Maalot | 77136 | 10/17 | SD2 | F | wt |
| 54 | Maalot | 77136 | 10/29 | SD2 | B | wt |
| 55 | Mt Meron | 77138 | 9/20 | SD2 | B | wt |
| 56 | Mt Meron | 77138 | 9/26 | SD2 | B | wt |
| 57 | Rosh Pinna | 77140 | 5/21 | SD2 | C | wt |
| 58 | Rosh Pinna | 77140 | 5/36 | SD2 | C | wt |
| 59 | Zafat | 77140 | 5/53 | SD2 | C | wt |
| 60 | Talpuyyot | 77144 | 14/2 | SD2 | C | nb |
| 61 | Talpuyyot | 77144 | 14/4 | SD2 | C | wt |
| 62 | Talpuyyot | 77144 | 14/7 | SD2 | C | wt |
| 63 | Talpuyyot | 77144 | 14/11 | SD2 | C | wt |
| 64 | Talpuyyot | 77144 | 14/12 | SD2 | C | wt |
| 65 | Talpuyyot | 77144 | 14/17 | SD2 | C | wt |
| 66 | Talpuyyot | 77144 | 14/25 | SD2 | C | wt |
| 67 | Talpuyyot | 77144 | 14/28 | SD2 | C | wt |
| 68 | Talpuyyot | 77144 | 14/31 | SD2 | B | wt |
| 69 | Tel Hay A | 77145 | 4/2 | SD1 | C | wt |
| 70 | Tel Hay A | 77145 | 4/19 | SD1 | C | wt |
| 71 | Tel Hay A | 77145 | 44 | SD1 | B | wt |
| | | | | | | |
| | **Jordan Valley** | | | | | |
| 72 | Afiq | 77128 | 3/1 | SD2 | C | wt |
| 73 | Afiq | 77128 | 3/5 | SD2 | C | wt |
| 74 | Afiq | 77128 | 3/19 | SD2 | C | wt |
| 75 | Afiq | 77128 | 3/24 | SD2 | C | wt |
| 76 | Afiq | 77128 | 3/28 | SD2 | C | wt |
| 77 | Afiq | 77128 | 3/41 | SD2 | C | wt |
| 78 | Beit shean | 77131 | 21/7 | SD2 | F | wt |
| 79 | Beit shean | 77131 | 21/34 | SD2 | C | wt |
| 80 | Beit shean | 77131 | 21/48b | SD2 | C | wt |
| 81 | Beit shean | 77131 | 21/48a | SD2 | C | wt |
| 82 | Beit shean | 77131 | 21/51 | SD2 | C | wt |
| 83 | Mehola | 77137 | 22/7 | SD2 | C | wt |

| Number | Site in Israel | CPI # | Site/Family | B-Amylase | Protein-Z | EBG EI |
|---|---|---|---|---|---|---|
| | | | | | | |
| 84 | Mehola | 77137 | 22/11 | SD2 | C | wt |
| 85 | Mehola | 77137 | 22/26 | SD2 | C | wt |
| 86 | Mehola | 77137 | 22/31 | SD2 | C | wt |
| 87 | Mehola | 77137 | 22/53 | SD2 | C | wt |
| 88 | Tabigha | 77143 | 7/9 | SD1 | F | wt |
| 89 | Tabigha | 77143 | 7/13 | SD2 | C | wt |
| 90 | Tabigha | 77143 | 7/18 | SD2 | F | wt |
| 91 | Tabigha | 77143 | 7/19 | SD2 | F | wt |
| 92 | Tabigha | 77143 | 7/25 | SD1 | F | wt |
| 93 | Tabigha | 77143 | 7/31 | SD1 | C | wt |
| 94 | Tabigha | 77143 | 7/48 | SD1 | F | nb |
| | | | | | | |
| | **Desert** | | | | | |
| 95 | Bor mashash | 71285 | 17/12 | SD1 | B | wt |
| 96 | Bor mashash | 71285 | 17/18 | SD1 | B | wt |
| 97 | Bor mashash | 71285 | 17/27 | SD1 | B | wt |
| 98 | Bor mashash | 71285 | 17/44 | SD1 | B | wt |
| 99 | Bor mashash | 71285 | 17/45 | SD1 | B | wt |
| 100 | Bor mashash | 71285 | 17/50 | SD1 | B | wt |
| 101 | Rivivim | 77139 | 18/28 | SD1 | B | wt |
| 102 | Rivivim | 77139 | 18/30 | SD1 | B | wt |
| 103 | Rivivim | 77139 | 18/35 | SD1 | B | wt |
| 104 | Sede Boker | 77141 | 20/13 | SD1 | B | wt |
| 105 | Sede Boker | 77141 | 20/14 | SD1 | B | wt |
| 106 | Sede Boker | 77141 | 20/21 | SD1 | B | wt |
| 107 | Sede Boker | 77141 | 20/27 | SD1 | B | wt |
| 108 | Sede Boker | 77141 | 20/29 | SD1 | B | wt |
| 109 | Sede Boker | 77141 | 20/36 | SD1 | B | wt |
| 110 | Sede Boker | 77141 | 20/41 | SD1 | B | wt |
| 111 | Tel Shoqet | 77146 | 16/32 | SD4 | E | wt |
| 112 | Tel Shoqet | 77146 | 16/33 | SD3 | B | wt |
| 113 | Wadi Qilt | 77135 | 23/1 | SD2 | B | wt |
| 114 | Wadi Qilt | 77135 | 23/7 | SD2 | B | wt |
| 115 | Wadi Qilt | 77135 | 23/10 | SD2 | C | wt |
| 116 | Wadi Qilt | 77135 | 23/23 | SD2 | C | wt |
| 117 | Wadi Qilt | 77135 | 23/31 | SD2 | B | wt |
| 118 | Wadi Qilt | 77135 | 23/40 | SD2 | A | wt |
| 119 | Wadi Qilt | 77135 | 23/54 | SD2 | C | wt |
| 120 | Wadi Qilt | 77135 | 23/55 | SD2 | C | wt |
| 121 | Yeruham | 77147 | 19/5 | SD1 | D | wt |
| 122 | Yeruham | 77147 | 19/19 | SD1 | D | wt |
| 123 | Yeruham | 77147 | 19/41 | SD1 | D | nb |
| 124 | Yeruham | 122138 | | SD1 | C | nb |

Wt designated the wild type allele, D13 designates the allelic form of (1→3,1→4)-ß-glucanase isoenzyme EI described by MacLeod *et al.* (1991).

**Appendix II:** ß-amylase, protein Z, and (1→3,1→4)-ß-glucanase isoenzyme EI screening results for *H. spontaneum* accessions from Iran, Turkey and Morocco.

| Number | Site | CPI # | ß-Amylase | Protein Z | EBG EI |
|---|---|---|---|---|---|
| | | | | | |
| 125 | Iran | 109827 | SD1 | C | wt |
| 126 | Iran | 109828 | SD1 | A | wt |
| 127 | Iran | 109829 | SD2 | C | wt |
| 128 | Iran | 109830 | SD2 | C | wt |
| 129 | Iran | 109831 | SD2 | C | wt |
| 130 | Iran | 109832 | SD2 | C | wt |
| 131 | Iran | 109833 | SD2 | C | wt |
| 132 | Iran | 109834 | SD1 | C | wt |
| 133 | Iran | 109835 | SD1 | B | wt |
| 134 | Iran | 109836 | SD1 | C | wt |
| 135 | Iran | 109837 | SD1 | C | wt |
| 136 | Iran | 109838 | SD1 | C | wt |
| 137 | Iran | 109839 | SD1 | B | wt |
| 138 | Iran | 109840 | SD1 | C | wt |
| 139 | Iran | 109841 | SD1 | C | wt |
| 140 | Iran | 109842 | SD2 | A | wt |
| 141 | Iran | 109843 | SD1 | C | wt |
| 142 | Iran | 109844 | SD1 | C | wt |
| 143 | Iran | 114925 | SD1 | C | wt |
| 144 | Iran | 114926 | SD1 | C | wt |
| 145 | Turkey | 109846 | SD1 | C | wt |
| 146 | Turkey | 109850 | SD1 | C | wt |
| 147 | Turkey | 109851 | SD2 | C | wt |
| 148 | Turkey | 109853 | SD1 | C | nb |
| 149 | Turkey | 109855 | SD1 | C | wt |
| 150 | Turkey | 109856 | SD1 | C | wt |
| 151 | Turkey | 109857 | SD1 | C | wt |
| 152 | Turkey | 109858 | SD1 | C | wt |
| 153 | Turkey | 109860 | SD1 | C | wt |
| 154 | Turkey | 109861 | SD2 | C | wt |
| 158 | Morocco | 91870 | SD2 | C | wt |
| 159 | Unknown | 137/2 | SD1 | | |
| 160 | Unknown | 138/1 | SD1 | | |
| 161 | Unknown | 138/2 | SD3 | | |
| 162 | Unknown | 149/2 | SD3 | | |
| 163 | Unknown | 149/3 | SD3 | | |
| 164 | Unknown | 149/4 | SD3 | | |
| 165 | Unknown | 149/5 | SD3 | | |
| 166 | Unknown | 149/6 | SD3 | | |
| 167 | Unknown | 149/7 | SD3 | | |
| 168 | Unknown | 149/8 | SD3 | | |
| 169 | Unknown | 149/9 | SD3 | | |
| 170 | Unknown | 149/10 | SD3 | | |

| 171 | Unknown | 149/11 | SD3 | | |
|---|---|---|---|---|---|
| **Number** | **Site** | **CPI #** | **ß-Amylase** | **Protein Z** | **EBG EI** |
| | | | | | |
| 172 | Unknown | 149/12 | SD3 | | |
| 173 | Unknown | 149/13 | SD3 | | |
| 174 | Unknown | 149/14 | SD3 | | |
| 175 | Unknown | 149/15 | SD3 | | |
| 176 | Unknown | 149/16 | SD3 | | |
| 177 | Unknown | 149/17 | SD3 | | |
| 178 | Unknown | 149/18 | SD3 | | |
| 179 | Unknown | 149/19 | SD3 | | |
| 180 | Unknown | 149/20 | SD3 | | |
| 181 | Unknown | 149/21 | SD3 | | |
| 182 | Unknown | 149/22 | SD3 | | |
| 183 | Unknown | 149/23 | SD3 | | |
| 184 | Unknown | 149/24 | SD3 | | |
| 185 | Unknown | 150/1 | SD1 | | |
| 186 | Unknown | 150/2 | SD1 | | |
| 187 | Unknown | 150/3 | SD1 | | |
| 188 | Unknown | 150/4 | SD1 | | |
| 189 | Unknown | 150/5 | SD2 | | |
| 190 | Unknown | 150/6 | SD1 | | |
| 191 | Unknown | 151 | SD2 | | |
| 192 | Unknown | 154 | SD2 | | |
| 193 | Unknown | 161 | SD1 | | |
| 194 | Unknown | 170 | SD2 | | |
| 195 | Unknown | 177/1 | SD2 | | |
| 196 | Unknown | 177/2 | SD2 | | |
| 197 | Unknown | 177/3 | SD2 | | |
| 198 | Unknown | 177/4 | SD1 | | |
| 199 | Unknown | 177/5 | SD1 | | |
| 200 | Unknown | 177/6 | SD2 | | |
| 201 | Unknown | 177/7 | SD2 | | |
| 202 | Unknown | 177/8 | SD3 | | |
| 203 | Unknown | 177/9 | SD2 | | |
| 204 | Unknown | 177/10 | SD2 | | |
| 205 | Unknown | 177/11 | SD2 | | |

*wt designates the wild type allele, nb designates no banding pattern was detected.