A STUDY OF ENDO-β-MANNANASE IN BARLEY (HORDEUM VULGARE)

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ABSTRACT

Endo-β-mannanase is an endohydrolase enzyme responsible for the breakdown of mannan-containing polysaccharides common in the cell walls of many plants. The action of endo-β-mannanase in barley, its optimum temperature and pH for action, temporal and spatial localization, activity in the presence of hormones and sugars and its effect on the seed's mechanical strength were assayed. The development of a spectrophotometric assay for endo-β-mannanase detection was also trialed.

The optimum temperature and pH for these experiments were found to be 37°C and pH 7. Using these parameters, the endo-β-mannanase enzyme was found to be initially localized in the seed coat and moved through to the endosperm over time. The detected level of enzyme activity increased in the presence of gibberellic acid and glucose, or decreased when abscisic acid was added. Similar results were seen when the embryo was removed and the endosperm and seed coat were incubated in hormone- and sugar-containing media. The presence of exogenous endo-β-mannanase did not affect the mechanical strength of the seed but there was a strong correlation between increasing endo-β-mannanase activity and decreasing mechanical strength over time. The spectrophotometric assay for quantifying endo-β-mannanase in extracts showed promise but did not reach fruition due to unexplained sources of variation.

The localization and regulation of endo-β-mannanase in barley were similar to those seen in other plants, such as tomato, lettuce and coffee. These findings have biotechnological applications within the brewery industry. By increasing the mobilization of reserves such as mannan, it is thought that the seedling can utilize this secondary carbohydrate source instead of, or at least supplementing, glucose which was mobilized from starch. This will theoretically reduce the starch and glucose lost during the malting period leaving a higher sugar content free for fermentation.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ABA	Abscisic Acid
BSA	Bovine Serum Albumin
dH_2O	Distilled Water
GA	Gibberellic Acid
GH	Glycosyl Hydrolase
IAA	Indole Acetic Acid
LBG	Locust Bean Gum
nkat	Nano katals
MOPS	Morpholinopropanesulfonic acid
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	Phenylmethylsulfonyl Fluoride
SDS	Sodium Dodecyl Sulphate

Chapter 1

INTRODUCTION

1.1. General Introduction

Nearly all biochemical reactions in plants are carried out by enzymes, catalytic proteins present in all organisms. Enzymes play an integral part in plant growth and development. Enzymes are present at all stages of plant life. There are seed enzymes responsible for mobilization of carbohydrate reserves within the seed to fuel the growth and development of the seedling. Other enzymes are found in the mitochondria, the 'power stations' of the cell, and the chloroplasts, the light harnessing organelles. Enzymes in these organelles carry out the essential catalytic steps for the production of adenosine triphosphate (ATP), the main energy source for many cellular functions, while others are responsible for harnessing light in the chloroplasts and converting carbon dioxide to the photosynthetic product glucose (da Silva et al., 2007).

Enzymes, hormones and sugar signals involved in seed germination are of considerable interest as they give us insight into the growth and development of plants. Of particular interest are the enzymes and signal molecules that are active in the mobilization of the carbohydrates present in the starchy endosperm (Buckeridge et al., 2000). The presence of sugars (Gibson, 2005) and hormones (da Silva et al., 2004) are common signals which: induce the production of enzymes within the seed; promote growth of the plant; initiate flowering; initiate senescence; and a wide range of other biological activities (Kucera et al., 2005).

The use of enzymes in industry and biotechnology is widely known and the art of brewing is a prime example of enzymes being used in a large scale process. Brewing, the production of alcoholic beverages from starch sources, most predominantly barley (Hornsey, 1999), is an application that has been pursued by man for thousands of years, from ancient Egyptians (Samuel, 1996) and Mesopotamians

(Hartman et al., 1950) to the modern day. Nowadays, brewing is a worldwide industry with revenues on the order of USD$300 billion (Beer Global Industry, 2006). Technology plays a large part in the evolution of brewing practices, and today's sophisticated multistage systems are substantially more advanced than the basic bowls of barley and water mixture described by Xenophon in the 5th century BC (Unger, 2004). As in any industry, there is constant focus on ways to increase yield or improve efficiency from precursor grain stock. One way to do this is to manipulate the enzymes present in the barley grain.

The main enzyme responsible for the breakdown of galactomannans and glucomannans in barley cell walls is endo-β-mannanase (Buckeridge et al., 2000). Mannan-containing polysaccharides are present in only low amounts, yet they are still a structurally important cell wall component (Hrmova et al., 2006). While the role and mechanism of mannanase in breaking down cell walls have been thoroughly researched (Buckeridge et al., 2000) there has not been notable research to date regarding whether mannans can play a significant role in supplementing starch glucose as food sources, nor whether accelerated seed softening due to mannan breakdown could result in less sugar "lost" to seed growth instead of remaining available to be fermented.

The germination of the barley grain during the production of malt also requires detailed understanding of changes to grain components and the behaviour of the enzymes that mediate those changes. Better understanding of the properties of barley endo-β-mannanase will contribute to the elucidation of the biological role of grain glucomannans and galactomannans to improved malting performance.

It is also hypothesized that barley endo-β-mannanase will have analogous properties to those from other vascular plants, and possibly those from microbial sources. Published knowledge about these putative analogues can be used to guide investigations into the properties of barley endo-β-mannanase.

1.2. *Hordeum vulgare L.* (Barley)

H. vulgare, or barley, is a monocotyledonous cereal crop from the grass family Poaceae (Table 1.1) that originated in the Eastern Mediterranean region (Anderson et

al., 1995). Barley is grown world wide and has preference for moderate temperature levels: Schelling et al. identified the temperature range of 14-18ºC to result in optimum grain yield. This means it can be grown as a summer crop in tropical regions, or as a winter crop in temperate regions.

There are several species of barley showing a variety of morphological and genetic differences, as well as many different cultivars within each species (Gramene, 2006a). While fertile flowers are produced by all species of barley, some cultivars may not be fertile, such as Sengenta™ barley (Pyne Gould Guinness, 2006). This ensures buyers for a second season, as the growers have to buy more seeds of this cultivar as they cannot harvest and sow seeds from their crop. While this is the case with some European barley, New Zealand cultivars grow and reproduce in the normal fashion, with only a slight decrease in productivity over the generations (Pyne Gould Guinness, 2006).

Table 1.1. – The taxonomy of common barley *Hordeum vulgare* from kingdom to species level of the species studied in this thesis (Gramene, 2006a). Dash and Fairview are cultivars of the common barley *H. vulgare*, and represent a standard for malting and feedstock barley cultivars.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae
Genus	*Hordeum*
Species	*vulgare*
Common Name	**Barley**
Cultivar	Dash
Cultivar	Fairview

1.2.1. Life Cycle

The life cycle of barley can be broken up into eight divisions: germination, seedling establishment, leaf production, tillering, stem elongation, pollination, kernel development and maturity (Anderson et al., 1995).

Germination begins in favorable temperatures (1-2° C minimum) when moisture is taken up (imbibed) into the seed. The primary root (radicle) then emerges and

grows downward, anchoring the seed, as well as harvesting water and nutrients from the surrounding soil. After radicle emergence, the shoot, protected by a coleoptile, penetrates the seed coat and grows upwards. Once the seedling has emerged from the soil, it produces its first true leaf. The seedling continues to develop leaves, until the emergence of the final leaf (Anderson et al., 1995).

Tillering, the occurrence of shoots originating at the base of the leaf occurs after approximately three leaves have been developed. These tillers can put down roots, adding to the total nodal root system, and tillers can form heads in favorable conditions. The ability of the barley plant to form tillers is an important method of adapting to changing environmental conditions. For example, if there is an abundance of soil nutrients, the plant will form more tillers to take advantage of the nutrients and thus produce more seeds (Anderson et al. 1995).

During the above stages of development, the plant apex has been below the soil to protect it from adverse weather conditions and mechanical damage. Three to four weeks after the seedling first emerges, the stem begins to elongate, pushing the plant apex above the soil. During this elongation the barley head is developing rapidly, but is not easily visible until the 'boot' stage, five to six weeks after seedling emergence (Anderson et al., 1995).

Six to seven weeks after seedling emergence, pollination occurs in the head of the barley. This pollination begins in the centre of the head and moves towards the tip and base. Following pollination, the kernels begin to develop. The first stages in kernel development are the 'watery ripe' and 'milk' stages. These stages last for about 10 days and determine the number of cells that will be available for the starch storage in the mature seed. As the kernels begin to store starch, they are growing quickly and have a semi-solid endosperm in what is often termed the 'soft dough' stage. When the kernel reaches maturity it starts to lose water, forming the 'hard dough' texture characteristic of mature kernels. At this final stage, the kernel has reached physiological maturity and will not grow any larger or accumulate any more starch (Anderson et al., 1995)

1.2.2 Seed Structure

A barley kernel essentially consists of three parts: the seed coat, the endosperm and the embryo (Figure 1.1). The seed coat consists of three elements: the husk, the pericarp and the testa. The husk is the outermost layer and is an extra protective layer that consists of coarse dead cells arranged in a honeycomb design (Hornsey, 1999). The actual seed coat consists of the fused pericarp and testa, both of which function to protect the seed from mechanical damage and harsh environmental conditions such as frost, wind, hail, high temperatures or high levels of sunlight.

The endosperm consists primarily of thin walled cells filled with starch grains, and is usually non-living in the mature kernel (Taiz et al., 2006). Starch is the primary source of carbohydrate reserves in cereal crops and is digested by enzymes into metabolically useable sugars upon contact with favorable germination conditions (Berger, 1999).

Lying between the seed coat and the endosperm is the aleurone layer, which is technically the outermost layer of the endosperm. However, the aleurone layer is cytologically and biochemically different from the starchy centre. This layer consists of cells that have thick primary cell walls and contain many protein bodies which are a mixed solution of phytic acid and salts. The aleurone cells receive hormonal signals from the embryo which trigger enzyme synthesis. The aleurone layer synthesizes enzymes that disperse into the starchy endosperm and progressively digest and soften it, starting at the proximal end of the seed (Bamforth, 1998). The digested starch, now in the form of metabolically usable products, is used by the embryo for growth and development until it is able to photosynthesize and produce glucose (Bewley, 1997)

The embryo consists of the plant embryo proper, along with its specialized absorptive organ called the scutellum. The function of the scutellum is to absorb the solubilized food reserves from the endosperm and pass it to the growing embryo (Taiz et al., 2006). The embryo produces the hormones that stimulate the synthesis of enzymes in the aleurone layers, which mobilize the carbohydrate reserves present in the starch endosperm (Jones, 2005). The embryo proper consists of the cotyledon (seed leaf), radicle (embryonic root), plumule (embryonic shoot) and the coleoptile (protects the emerging seedling). Nutrients obtained by the radicle add to the nutrients from the starchy endosperm, allowing the seedling to grow large enough to break

through the soil. Upon exposure to sunlight, the seedling begins photosynthesizing its own carbohydrates (Hornsey, 1999)

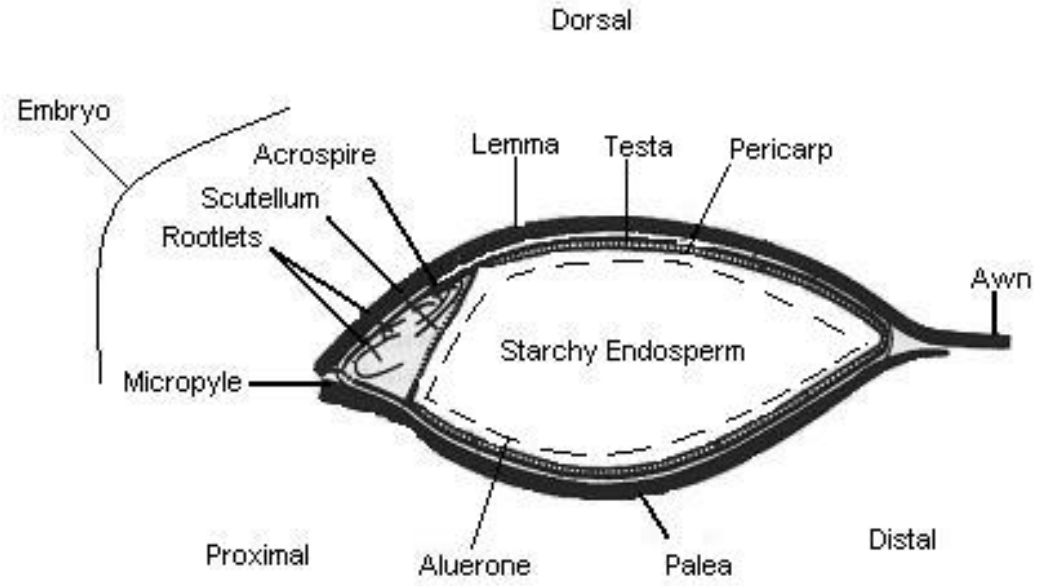

Figure 1.1. – A longitudinal section of a barley kernel showing the three major areas of the seed: endosperm, seed coat (husk) and embryo. (Image adapted from Hornsey, 1999)

1.2.3. Enzymes in Barley

Barley, like all plants, has a range of enzymes that are required for carrying out a wide variety of tasks within the plant and its seeds. Enzymes located in the seed for mobilization of carbohydrate reserves are well studied in many plants, particularly amylases, which are responsible for the breakdown of starch (Juge et al., 2002). Amylases are by far the most abundant enzymes in the seed. The endo-acting α-amylase form is the most abundant and is highly specific to the α-1,4 bond from the 1’ carbon of glucose to the 4’ carbon of an adjacent glucose (Juge et al., 2002).

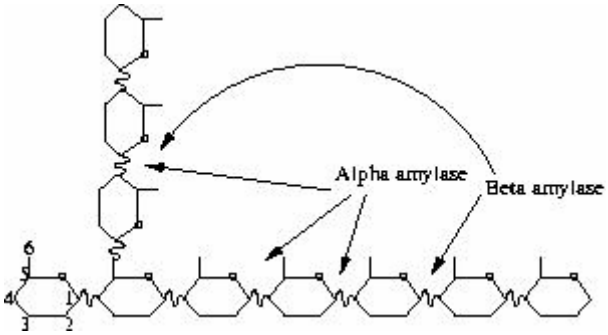

Figure 1.2. - Diagram shows the sites of attack on a stylized starch molecule for the α– and β–amylases in barley. The numbering system of glucose is shown on the first residue. (Wikibooks, 2006)

While amylases are an important class of enzyme, there are a variety of other enzymes, such as pectinases, hemicellulases, galactosidases and mannanases, which are all crucial to the function of the seed during germination and initial reserve mobilization. The enzymes mentioned above all target structural polysaccharides such as starch, cellulose, hemicellulose, glucomannans, galactomannans and other structural compounds in the cell wall. As the seed continues to germinate and grow, the actions of these enzymes weaken the cell wall, allowing radicle protrusion as well as releasing a range of soluble sugars for use by the embryo (Buckeridge et al., 2000).

1.2.4. Commercial Uses – Feed Stock

Harvested barley has two primary uses: feed stock and malting (Doak, 2003). Many cultivars of barley are used specifically as feed stocks for both animal and human consumption. Barley for human consumption is almost always processed in some manner. Whole-grain, crushed, rolled, flaked, pelleted or powdered barley are common ingredients in many breads, porridges, soups and flours found on supermarket shelves throughout the world (Bamforth et al., 1993).

Barley for animal consumption is also processed, although it is not often as refined as it is for human consumption. Crushed barley grains are staple food for pigs and chickens, as well as the whole plant being used for green feed (grazing fodder), pasture, roughage and bedding (Gramene, 2006b).

Barley, like most cereal grains, is high in dietary fibre, which is an essential part of a balanced diet for both animals and humans (Napolitano et al., 2006). The ability

to provide nutritional food for both humans and other animals makes barley production of economic importance, as 150,000 hectares of New Zealand land are planted in cereal grains of which barley is a major crop (NZGSTA, 2007).

1.2.5. Commercial Uses – Malting

The second most common use for barley in New Zealand is in malt production for use in the brewing industry, the bulk of which is grown in Canterbury in the Ellesmere and Ashley counties (Farming Arable, 2007). Barley is an essential ingredient in the production of beer. Historically, other cereal crops such as wheat, rye and spelt have been used as the malted grain. However, it soon became evident that beer made from barley was easier to produce and superior in quality compared to beer produced using other cereal crops. In 1516, the Bavarian Dukes Wilhelm IV and Ludwig X instigated the German Purity Law which stated that beer could only include barley, hops, yeast and water (Hornsey, 1999). Some breweries still adhere to this law, although many include other additives such as fruit extracts to provide a unique flavour. Regardless of ingredients, the method of brewing remains remarkably similar between breweries (Hornsey 1999).

1.2.5.1. Malting

Grains of appropriate malting quality, that is moisture content no higher than 20%, are steeped in water to increase moisture content to a level at which germination begins – typically 42-46%. During steeping, it is important to ensure that the grains are not in too little water as this produces weak embryo development and poor modification of the endosperm. It is also important to ensure that the grain is not in too much water, as this can lead to overmodification of the endosperm or even kill the embryo. To prevent grains being exposed to too little or too much liquid there are intermittent periods of drainage and aeration through the steeping grains, which also disperse carbon dioxide and promote germination (Hornsey, 1999).

Upon coleorhiza protrusion, the grains are germinated and moved to the germination floor. The grains are kept at ~19°C and metabolic heat is dispersed by convection and raking the grains. The raking of the grain beds also prevents the

rootlets becoming matted. The grains are allowed to germinate until the acrospires (the sprout at the end of the seed as it is germinating) are about three-quarters of the way along the dorsal side of the grain. Once the acrospires reach the designated point, the grains are moved into the kilning process (Hornsey, 1999)

1.2.5.2. Kilning and Milling

The kilning stage involves driving off water using heat until the moisture level in the grain is below 5%. At this moisture level, metabolic activity stops and the product is stabilized (Hornsey, 1999). Kilning must be carried out carefully: the grain must be heated to allow for the rapid evaporation of moisture, but not heated to a high enough temperature that the enzymes produced by the embryo are denatured. The kilning temperature is also a key parameter in determining the flavour of the final product. During germination, proteins and carbohydrates break down to form amino acids and sugars, which combine to form coloured melanoidins during kilning. Melanoidins are brown-pigmented and flavour-active compounds. They can produce a wide range of flavours and aromas in the beer, such as off flavours (bitter, burnt), off aromas (burnt, onion, solvent, rancid, sweaty, cabbage) or positive flavours (malty, bread crust-like, caramel, coffee, roasted) and positive aromas, (bready, cracker, fine malt) (Scandrett, 1997). High kilning temperatures are most commonly used to produce ales and particularly dark brews such as stout as well, since they produce burnt and smoky flavours. Malt kilned at lower temperatures is used more often in lager beers, as the grains are less modified and therefore contain fewer amino acids and sugars for a smoother flavour (Bamforth, 1998).

1.2.5.3. Fermenting

The final stage of interest is the fermenting process. After kilning, the malt, now called the wort, has to be mixed with hops (the dried ripe flowers of twining vine *Humulus lupulus L.*), boiled and cooled. The hopped wort is run into a fermentation vessel and pitched with brewers' yeast (*Saccharomyces cerevisiae* L.) (Hornsey, 1999). The wort is a primarily anaerobic environment, which facilitates anaerobic respiration in the yeast. Anaerobic respiration involves the intake of a sugar, e.g. glucose, which is converted into alcohol and carbon dioxide gas (Ishtar et al., 2007).

$C_6H_{12}O_6 \rightarrow 2(CH_3CH_2OH) + 2CO_2$

Glucose → 2(Ethyl Alcohol) + 2(Carbon Dioxide)

Figure 1.3. The above equation is a simplified version of the fermentation process (Hardwick, 1995)

The fermentation process begins with a lag phase of 8-10 hours in which the yeast is adjusting to its new environment in the hopped wort and is not noticeably reproducing or respiring. The lag phase is followed by a short phase of accelerating growth, which leads to a phase of exponential growth as the yeast buds and reproduces rapidly. During this logarithmic period, the yeast density is expected to increase four- to six-fold. It is during this stage that yeast growth is at its highest level and rapidly producing ethanol and carbon dioxide. During the logarithmic phase a large amount of respiratory heat is produced and it is necessary for the temperature to be regulated. If the temperature is not regulated, alcohols such as propanol, isoamyl alcohols and isobutyl alcohols are produced, which cause unusual and undesirable flavours (Hornsey, 1999)

Logarithmic growth usually continues for 48-60 hours, at which point the growth rate decreases and the yeast enters a stationary phase. During the stationary phase there are still cells reproducing but they are equalled by the number of cells being killed by the alcohol concentration of the wort (Hornsey, 1999). Figure 1.4 shows a diagrammatic representation of the growth of yeast in a fermentation system.

There are numerous post-fermentation steps involved in the brewing of beer to extract the beer from the wort, impart flavours and allow for the storage of beer in kegs, casks, bottles and cans. However, the malting step is of key interest to this research as understanding the activity of endo-β-mannanase may affect and possibly improve the end product of the fermentation process.

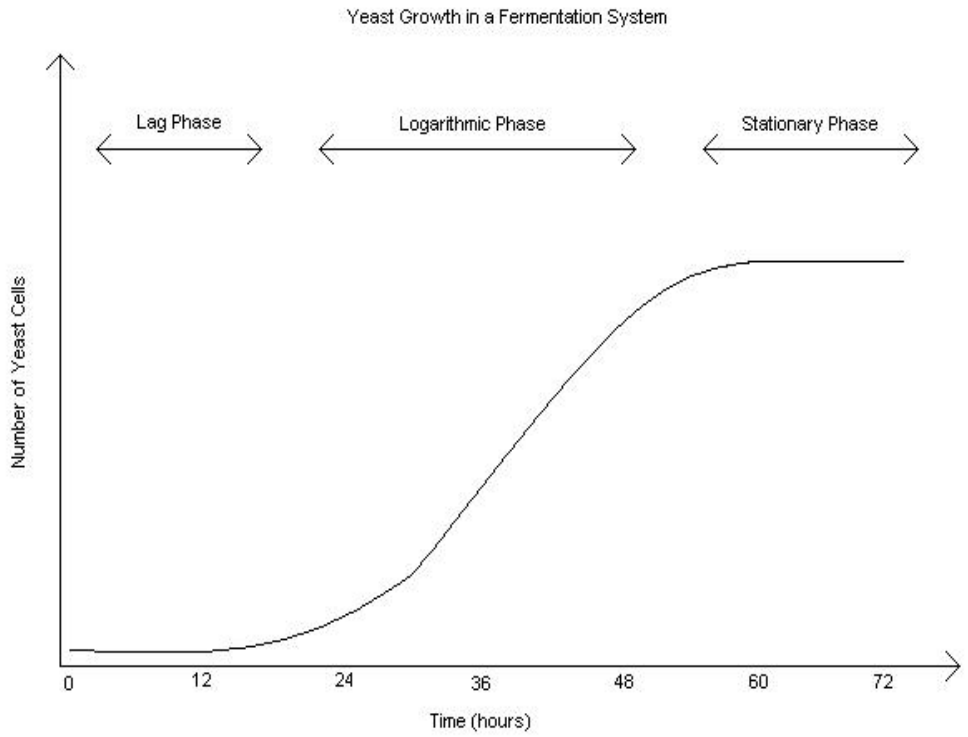

Figure 1.4. A graphical representation of the growth of *S. cerevisiae* in a fermentation system over approximately 72 hours. There is no quantitative cell count on the y axis as this graph is to show the pattern of growth only, and is not representing actual quantitative data (Hardwick, 1995).

1.3. Endo-β-1,4-Mannanase

The endohydrolase enzyme endo-β-mannanase and its action in barley are the focus of this research, as the action of this enzyme on the galactomannans and glucomannans found in the cell wall during germination will lead to a more detailed understanding of changes to grain components. Better understanding of the properties of barley endo-β-mannanase may contribute to the elucidation of the biological role of grain galactomannans and to improved malting performance.

As an endohydrolase, this enzyme hydrolyzes the internal linkages between adjacent mannan residues in a mannan chain. This enzyme has been well studied in tomato *(Lycopersicon esculentum* L.) (Bewley et al., 1997), coffee (*Coffea arabica* L.) (Marraccini et al., 2001), carrot (*Daucus carota* L.) (Homrichhausen et. al., 2003) and some fungi (Sabini et al., 2000).

Recently, endo-β-mannanase has also been found to act as a transglycosylase in tomato. Mannan transglycosylases are cell wall enzymes that are able to transfer part of a mannan polysaccharide backbone to a mannan oligosaccharide. N-terminal sequencing has shown that mannan transglycosylase is exactly the same as the tomato endo-β-mannanase LeMAN4a. When LeMAN4a was expressed in *Escherichia coli* it was shown to carry out both functions as of a mannanase and a transglycosylase. Therefore, endo-β-mannanase has two functions which are separated only by the presence of the appropriate substrates (Schroder et al., 2006). This finding has led to the proposition that endo-β-mannanase should be renamed mannan transglycosylase/hydrolase in keeping with the nomenclature used for xyloglucan endotransglucosylase/hydrolase (Schroder et al., 2006).

Sections 1.3.1 to 1.3.3, relating to the classification, molecular structure and catalytic activity of endo-β-mannanase are based on research carried out on the tomato protein LeMAN4a. While the molecular structure is not exactly the same as that of endo-β-mannanase from a barley source, the active site contains many conserved regions common to all mannanases. Hrmova et al. (2006) have shown that although there is only 54% sequence similarity between tomato and barley endo-β-mannanase, there was 95% certainty that the tomato enzyme was a good model for the barley enzyme (Hrmova et al., 2006).

1.3.1. Classification

Glycosyl hydrolases (GH) are a large group of enzymes that hydrolyze the bonds between two or more carbohydrates. The nomenclature of glycosyl hydrolases is based on their molecular mechanisms and does not reflect the structural features of the particular enzyme. The classification into families is based on amino acid sequence, which, in turn, relates enzymes into families with similarly folded tertiary structures. Therefore, the GH family classification relates enzymes based on their structure, regardless of their substrate specificity, as well as show possible evolutionary relationships between the enzymes (Henrissat, 2007).

Endo-β-mannanase is an endohydrolase, which is a member of glycosyl hydrolase families 5 (GH5) and 26-β (GH26-β). As an endohydrolase it hydrolyzes

the internal bonds of a polysaccharide, in this case mannan-containing polysaccharides (Figure 1.5).

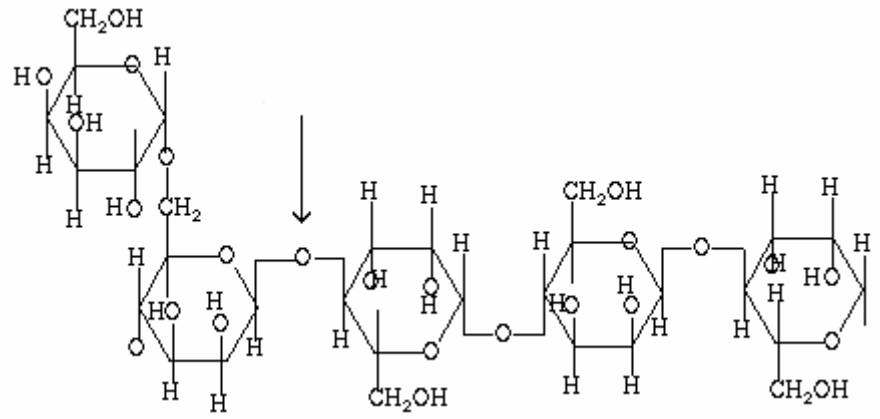

Figure 1.5. – Diagram of a mannan chain. Mannans are 6-carbon sugars which form long polysaccharide chains. The mannans are held together by β-(1,4) bonds (arrow) which connect carbon 1 of one mannan to carbon 4 of an adjacent mannan. It is this bond that is the target of endo-β-mannanase (Bewley, 1997)

Endo-β-mannanase belongs to clan GH-A, a higher order of grouping, which contains both families GH5 and GH26. GH5 and GH26 also contain endo-glucanases, exo-1,3-glycanases and mannan endo-1,4-β-mannosidases. Therefore, in keeping with this classification system, these enzymes all share a similar molecular mechanism and amino acid sequence (Henrissat, 2007).

1.3.2. Catalytic Reaction of Endo-β-Mannanase

Endo-β-mannanase randomly hydrolyzes internal 1,4-β-D-mannopyranosyl linkages in mannans. In all characterized glycosyl hydrolases, subsites exist for the binding of multiple sugar groups. These are numbered -4, -3, -2, -1, +1, and +2, from the non-reducing end to the reducing end of the polysaccharide (Davies et al., 1998). The reducing end of a polysaccharide is the end which has an anomeric carbon (a carbon atom that is attached to two oxygen via single bonds) that is not involved in a glycosidic bond with an alcohol group, usually an alcohol group on another carbohydrate.

The polysaccharide chain is cleaved between the mannosyl residues occupying the -1 and +1 subsites. Family GH5 and family GH26 β-mannanases catalyze

hydrolysis via a 'retaining mechanism', which means there is net retention of the configuration of the anomeric carbon atom of the -1 mannosyl group.

Two key catalytic residues have been identified: an acid and a nucleophile. The oxygen atom at the -1 and +1 mannosyl residue is protonated by the acid/base and the nucleophile forms a covalent bond with the C1' atom and the -1 mannosyl residue releasing the reducing end, resulting in the inversion of the C1' anomeric carbon atom. The acid/base residue then deprotonates the water molecule, and the resulting hydroxide ion attacks the glycoside-enzyme ester linkage, regenerating the nucleophile, reinverting the anomeric C1' atom, and releasing the non-reducing end from the substrate (Bourgault et al., 2005). Figure 1.6 shows the proposed catalytic mechanism for LeMAN4a.

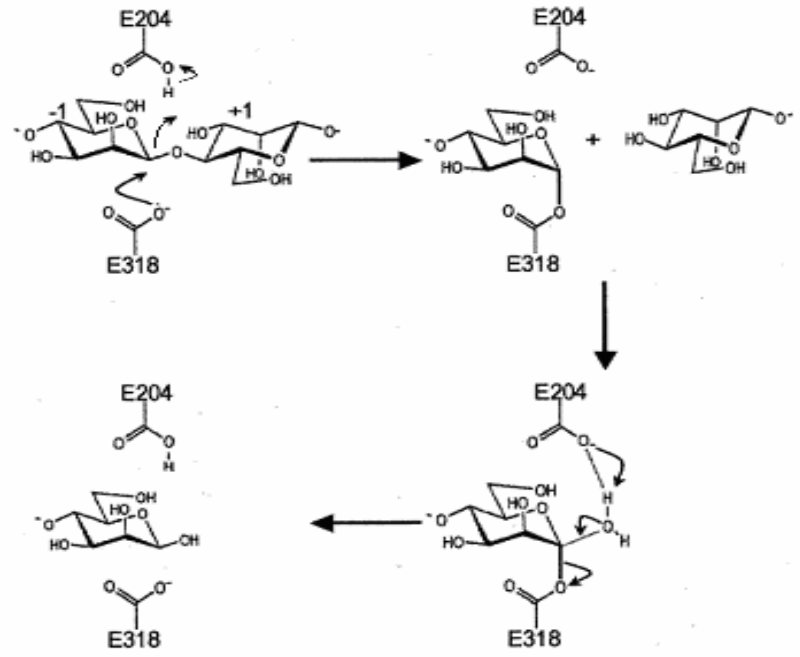

Figure 1.6. This mechanism has been proposed for the catalytic mechanism of LeMAN4a (Bourgault et al., 2005). The catalytic steps are described in section 1.3.2.

At the time of this research the catalytic mechanism of barley endo-β-mannanase had not yet been published. Recently Hrmova et al. (2006) have discovered that barley endo-β-mannanase contains a cleft analogous to that found in tomato endo-β-mannanase. This is consistent with a substrate-binding site corresponding to six or seven (1,4)-β-D-mannosyl residues. The putative catalytic

amino acid residues, Glu179 and Glu298, are located close to the centre of the cleft. The catalytic nucleophile Glu298 and catalytic acid/base Glu179 are 0.5–0.6 nm apart, at the ends of β-strands 4 and 7 respectively. These arrangements are typical of retaining polysaccharide endohydrolases (Hrmova, 2006).

1.3.3. Molecular Structure

The structure of endo-β-mannanase has been elucidated in some plants, such as tomato (LeMAN4a), fungi e.g *Trichoderma reesei* (TrMAN) (Sabini et al., 2000) and bacteria e.g *Cellulomonas fimi* (Le Nours et al., 2005). Tomato endo-β-mannanase LeMAN4a is considered to have a molecular structure typical of most mannanases, and adopts the canonical $(\beta/\alpha)_8$ fold also known as a triosephophate isomerase barrel, or TIM barrel (Madan Babu, 2007).

The TIM barrel is the most common tertiary protein structure observed in high resolution protein crystal structures, with 10% of all known enzymes containing this domain. The TIM barrel is characterized by a central barrel formed by parallel β−strands surrounded by seven or eight α-helices which shield the barrel from solvents.

The β-strands of the barrel form a network of hydrogen bonds with the adjacent strands and are all oriented in the same direction. The overall twist associated with the strands causes the first and the eighth strand to register in parallel and are held in place by hydrogen bonds causing the closure of the barrel (Figure 1.7) (Madan Babu, 2007).

The endo-β-mannanase protein has a roughly V-shaped groove that binds mannan, as shown via its crystal structure (Bewley et al., 1997). This fold contains a secondary structure, consisting of β sheets and α helices that are conserved in all mannanases. However, there are additional β strands and α helices present in the LeMAN4 protein, just as there are other additions in endo-β-mannanase from other sources (Bourgault et al., 2005).

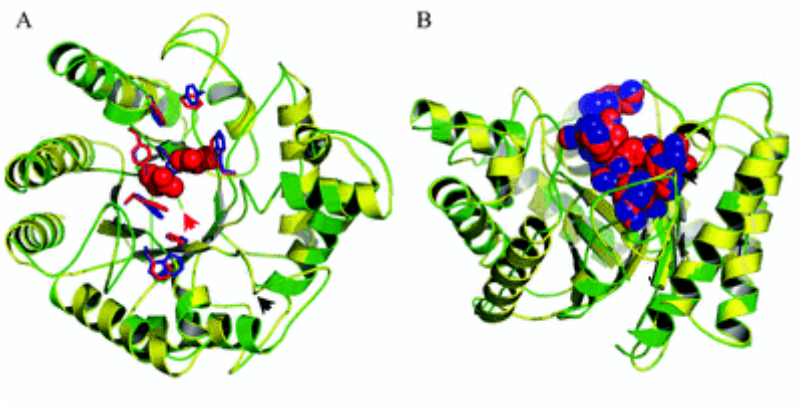

Figure 1.7. – (A) Superposition of the modelled HvMAN1 (yellow) and the template tomato endo-β-mannanase (green). (B) The deep cleft represents the substrate-binding cleft and its geometry is conserved in both structures. The conserved catalytic and substrate-binding amino acid residues of the modelled barley HvMAN1 (red spheres) and the tomato 1RH9 (blue spheres) structures are shown (Hrmova et al., 2006).

In the crystal structure of this enzyme, leucine residue L398 is very important for catalytic activity. Deletion of this residue results in exposure of hydrophobic residues, and leads to a great reduction in the enzyme's activity. The L398 residue is the penultimate residue in the LeMAN4a mannanase from tomato fruit and is found in the same position in the seed mannanases LeMAN1 and LeMAN2. This residue is also conserved in coffee (*Coffea arabica* L.*)* and lettuce (*L. sativa* L.). Although this residue is not found in the same location in coffee and lettuce mannanases, its removal results in a decrease in enzyme activity, just as with the tomato mannanase (Bourgault et al., 2002).

At the time of writing this thesis, the sequence and structure of barley endo-β-mannanase had not yet been elucidated. However, Hrmova et al. have now isolated, purified and sequenced the barley endo-β-mannanase gene HvMAN1. Although HvMAN1 shares only 54% sequence similarity with the tomato enzyme, there was 95% certainty that the template sequence of tomato mannanase was optimal for molecular modeling of barley mannanase (Hrmova et al., 2006).

The 3D model of the barley HvMAN1 showed that the structure adopts a $(\beta/\alpha)_8$ TIM barrel fold and has a deep substrate-binding cleft, where catalytic and substrate-binding amino acid residues are positioned. The morphology of the substrate-binding clefts and of the tomato and the barley endo-β-mannanase are highly conserved (Hrmova et al., 2006). Therefore the assumption that the tomato endo-β-mannanase

would make a good model for barley endo-β-mannanase has been validated retrospectively.

1.3.4. Detection of Endo-β-Mannanase

There are a variety of methods, such as viscometry, spectrophotometry and gel diffusion, that have been used in the past to detect the presence of endo-β-mannanase in an extract (Downie et al., 1994). Each of these assays' methods have advantages and disadvantages, which were assessed before commencing this research.

The viscometry assay assumes that galactomannan substrates suspended in buffer form viscous solutions, particularly at higher concentrations of substrate. The viscometry assay then measures changes in flow rate of viscous galactomannan solutions over time, relying on the action of the endo-β-mannanase to cleave the internal bonds in the galactomannan chains. As the enzyme cleaves the mannan bonds, the viscosity of the solution decreases and the time taken for a specific volume to drain from a pipette also decreases. It is this change in flow rate, when compared to a standard curve, which can be used to quantitatively determine the activity of the enzyme in solution (Tampion, 1972). While this assay is able to detect very low levels of activity, it has the disadvantage of being very laborious and leading to low throughput. Also, because the enzyme activity is being expressed in arbitrary units there is no direct connection between the measured values and the quantity of substrate which has been hydrolyzed (Downie et al., 1994).

Spectrophotometry is another common method to determine the activity of endo-β-mannanase, either measuring an increase in reducing units over time or the increase of an ethanol-soluble dye released from high M_r coloured substrates (Downie et al., 1994). The ethanol-soluble dye method involves dyeing a galactomannan substrate with Remazol Brilliant Blue then adding the enzyme to the dyed product. After a specific incubation period, the soluble dye is separated from the insoluble dyed substrate by filtration, and the amount of dyestuff liberated in the separated solution is determined spectrophotometrically (McCleary, 1978). However, the Remazol Brilliant Blue which is most often used to colour the substrate may interfere with the enzyme's ability to bind with its substrate, resulting in an underestimation of enzyme activity (Downie et al., 1994).

Another type of spectrophotometric assay measures the increase in reducing units over time. An enzyme extract sample is mixed with a galactomannan substrate and allowed to react for a set amount of time. During this time, any endo-β-mannanase present in the extract hydrolyzes the mannan chains into smaller mannose fragments. These mannose fragments are reducing sugars and will reduce an oxidizing agent, often 3,5-dinitrosalicylic acid. The mannose fragments react to produce 3-amino-5-nitrosalicylic acid which is a dark red-brown colour. This colour is measured spectrophotometrically (McCleary, 1988). The darker the colour, the more reducing sugars are present, and therefore the greater the enzyme activity. The major disadvantage with this assay is that it yields nonlinear results and is prone to interference from other enzymes found in crude extracts, which may also yield reducing sugars (Downie et al., 1994).

More recently, the most common method of determining mannanase activity in a crude extract has been the Congo red dye diffusion assay. It is possible to do many assays simultaneously, is quantitative and there is no interference by other enzymes. The Congo red dye gel diffusion assay relies on the specificity of Congo red dye in binding to a range of mannan polysaccharides. The galactomannan substrate is suspended in a 0.7% agar gel and extracts are inoculated into wells bored into the gel. As the extract in the wells diffuses out through the gel, the galactomannan substrate is hydrolyzed. Congo red dye binds to intact mannan chains and not to the hydrolyzed mannose fragments. This creates a clear zone, the diameter of which is proportional to the enzyme activity present. This assay can be used to quantify endo-β-mannanase activity from a wide range of sources, is extremely sensitive and produces linear results (Downie et al., 1994). These advantages, as well as the ability to perform many assays simultaneously, made this method of quantification ideal for this research.

1.3.5. *Aspergillus niger* (Teigh) Control

A. niger is one of the most common fungi of the genus *Aspergillus* and is the cause of black mould on fruits and vegetables. *A. niger* is cultured for industrial production of many substances and various strains are used for the manufacturing of citric and gluconic acid. These fungal products have been accepted for human intake by the World Health Organization (WHO, 1974).

A. niger is also used for the industrial production of enzymes such as pectinases, glucoamylase and α-galactosidases. Endo-β-mannanase is also produced by *A. niger*, which is isolated and purified by companies such as Megazyme. The *A. niger* endo-β-mannanase produces a single band on SDS gel electrophoresis (MW = 48,000) and a single major band on isoelectric focusing (pI = 3.6). It has a pH optimum of 3.0, a pH stability of 3.0-8.0, a temperature optimum of 60°C and a stability range of up to 70°C (Megazyme, 2007).

The large range of pH and temperature stability, as well as the availability of the *Aspergillus* enzyme, made it ideal to use as a positive control enzyme for this research.

1.3.6. Isozymes and Regulation of Endo-β-Mannanase in Other Plants

Tomato endo-β-mannanase has been well studied and there are two isoforms of endo-β-mannanase expressed in the seed endosperm, one before and one after radicle protrusion (Dirk, 1995).

The onset of germination requires the presence of favourable conditions. In the presence of these conditions the embryo produces gibberellic acid (GA) which overrides the nascent level of abscisic acid (ABA), which controls seed dormancy. GA stimulates the aleurone layer to begin the production of hydrolytic enzymes, e.g. endo-β-mannanase and α-amylase, and the seed begins to germinate (Gomez-Cadenas et al., 2001). In this pre-germinative stage, before the emergence of the radicle, mannanase isoform LeMAN2 is produced. LeMAN2 and LeMAN1 – the post germinative isoform of tomato endo-β-mannanase – have similar amino acid sequences as shown by Nonogaki (2000), yet they are encoded by different genes, which was confirmed by southern hybridization (Nonogaki et al., 2000). The post-germinative form of endo-β-mannanase is of interest in this research, as it is the post germinative form that will be most active during the malting process as malting, by definition, occurs in a germinated seed (Hornsey, 1999). For this reason endo-β-mannanase activity is only assayed in germinated seeds throughout this research.

Mannanase activity appears initially in the endosperm cap of the tomato seed and increases in production in the remaining parts of the endosperm following the radicle emergence (Bradford et al., 2003). LeMAN2 is the mannanase isoform involved in tissue weakening and the beginning of germination. LeMAN2 is expressed exclusively in the micropylar region and is responsible for the weakening of the endosperm cap to allow protrusion of the radicle. In the strictest sense, germination only includes the events up until the time of radicle protrusion from the seed, so the other isoform of mannanase detected in tomato seeds after radicle protrusion is the post-germinative isoform (Nonogaki et al., 2000).

LeMAN1 is a mannanase isoform involved in the reserve mobilization of stored mannans and which only occurs in the endosperm after germination. The expression of LeMAN1 and LeMAN2 are separated spatially as one is produced in the endosperm and the other in the micropylar region, as well as separated temporally since they are expressed at different germinative stages (Homrichhausen et al., 2003).

While tomato is the most commonly studied source of endo-β-mannanase, other plants such as rice, coffee, lettuce, and to a lesser extent, fenugreek (Gong et al., 2005), carob (Kontos et al., 1996), carrot (Homrichhausen et al., 2003), cucumber (Ramakrishma et al., 2005) and more recently barley (Hrmova, 2006) have all also been studied. Rice in particular has been used in studies to elucidate the action of hormones on the production of endo-β-mannanase in intact grains and half grains (grains with the embryo-containing half excised) (Wang et al., 2005).

Rice (*Oryza sativa* L.) exhibits an increase in endo-β-mannanase activity, mostly after the completion of germination, with the largest sustained increase in the peripheral regions of the endosperm. Like most seeds, the aleurone layer of the rice seed, being the only living region of the endosperm, is presumed to be the site of synthesis and secretion of the enzyme into the non-living starchy endosperm. In the intact grain, gibberellic acid (GA) causes an increase in endo-β-mannanase activity, while abscisic acid (ABA) causes a large decrease; this inhibition is overcome when GA is supplied along with ABA. Incubating half-grains in the presence of GA results in a large increase in enzyme activity; or in ABA reduces the amount of activity compared to the water controls. GA is capable of reversing the inhibitory effect of ABA with respect to endo-β-mannanase activity (Wang et al., 2005).

Another common plant for studying endo-β-mannanase activity is coffee (*Coffea arabica*). Coffee has been used to study the effects of endo-β-mannanase on endosperm cap weakening (da Silva et al., 2004). These studies showed there were two steps in endosperm cap weakening: an increase in cellulase activity, followed by an increase in endo-β-mannanase activity. ABA inhibited the second step of endosperm cap weakening, presumably by inhibiting the activities of endo-β-mannanase isoforms. The increase in the activities of endo-β-mannanase and cellulase coincided with the decrease in the force required to puncture the endosperm and with the appearance of porosity in the cell walls. Tissue printing showed that endo-β-mannanase activity was spatially regulated within the endosperm. Activity was initiated in the endosperm cap whereas later, during germination, it could also be detected in the remainder of the endosperm (da Silva et al., 2004).

Similar experiments have been carried out in lettuce (*Lactuca sativa*) (Nonogaki et al., 1999). The development of endo-β-mannanase activity and the changes in the enzyme content were followed during and after germination of lettuce seeds. Endo-β-mannanase activity was not detected before germination and began to develop immediately after radicle protrusion. The development of the enzyme activity occurred specifically in the endosperm tissue, and activity staining of native-PAGE gels revealed that three isoforms of the enzyme were present. The changes in the activity of endo-β-mannanase in the endosperm during seedling growth were due to the accumulation of the enzyme protein. Tissue prints showed that the activity initially developed in the endosperm region near the embryonic axis and then spread over the endosperm tissue. These results indicated that endo-β-mannanase production in lettuce endosperm was carried out in a spatially and temporally regulated manner (Nonogaki et al., 1999).

Previously the barley endo-β-mannanase enzyme has not been studied in extensive detail. Studies have looked at the presence of endo-β-mannanase isozymes in cereal grains, including barley (Dirk et al., 1995) and commented that barley contains appreciable levels of endo-β-mannanase (Downie et al., 1994). Recently, an extensive barley endo-β-mannanase study was carried out by the Hrmova group. The recent research by Hrmova et al. showed that mannanase does not break mannan-containing polysaccharides into free mannose (man), but most commonly into mannobiose (man2) and mannotriose (man3). This finding means that there is little

free mannan to act as a usable carbohydrate source for the barley seedling. Therefore the hypothesis that optimizing endo-β-mannanase activity to increase the available carbohydrate for the seedling and hence preserve starch and glucose for the fermentation process in brewing has been proven unlikely. However the hypothesis that increasing the endo-β-mannanase activity to break down the seed cell walls to allow for greater movement of other enzymes and therefore decrease the time the malting process takes is still valid.

Barley endo-β-mannanase (HvMAN1 enzyme) has a molecular mass of 43 kDA compared to an A. *niger* endo-β-mannanase enzyme which ahs a molecular mass of 48 kDA (Hrmova et al., 2006).

There are myriad other studies on different plants, and on bacterial and fungal sources of endo-β-mannanase (Table 1.2). Even though many of these plants are structurally quite different from each other, and have different isoforms of endo-β-mannanase, the localization and regulation of the enzyme appears to be quite similar.

Table 1.2. Summary table of some of the organisms in which endo-β-mannanase has been studied and the major findings of each study.

Species	Findings	Reference
Cellulomonas fimi	Characterization	Nours et al., 2005
Trichoderma reesei	Digestion of mannan crystals	Sabini et al., 2000
Thermotoga neapolitana	Purification and characterization	Duffad et al., 1997
Aspergillus fumigatus	Purification and characterization	Puchart et al., 2004
Lycopersicon esculentum L. (Tomato)	Germination-specific endo-β-mannanase gene is expressed in micropylar endosperm cap in tomato seeds	Nonogaki et al., 2000
Lycopersicon esculentum	3-D structure	Bourgault et al., 2005
Lycopersicon esculentum	Cloning of cDNA	Bewley et al., 1997
Coffea arabica (Coffee)	Molecular and	Marraccini et al., 2001

	biochemical characterization	
Mytilus edulis (Blue mussel)	3-D crystal structure and enzyme characterization	Larsson et al., 2006
Daucus carota L. (Carrot)	Activity is associated with completion of embryogenesis	Homrichhausen et al., 2003

1.3.7. Mannanases in Biotechnology

Mannanases from a wide range of other plants are already used in biotechnology. The use of mannanases and other hemicelluloses, particularly xylanase, are being investigated for potential use in the bleaching of pulp for paper production (Khanongnuch et al., 1998). During pulping, hemicelluloses and lignin are dissolved and partially degraded during the heating process. In subsequent phases, the pH drops because of the discharge of xylan side groups, and xylan precipitates with re-adsorption of lignin on top of the cellulosic microfibrils. Lignin is coloured during pulping which results in the cellulosic fibres being darkly stained. Usually, one or more bleaching sequences are needed to remove the dark colour caused by the deposition of lignin (Khandeparkar et al., 2007). Currently, harsh chlorinated chemicals are used to bleach pulp, but it is likely these will be banned sometime in the future due to the negative impact they have on the environment (Khanongnuch et al., 1998). There are oxygen-based bleaching processes which are more environmentally friendly. They are, however, not as efficient as chlorine-based treatments. The addition of mannanases and xylanases to increase the bleachability of the pulp by cleaving and solubilising the precipitated xylan and lignin may allow the more environmentally-friendly oxygen treatments to work more effectively. The use of hemicellulases in pulp bleaching is not currently viable as the chlorine-based method is too effective to be replaced. However, the environmental concerns around the chlorine compounds may result in a bio-bleaching method as a more acceptable alternative (Khanongnuch et at., 1998).

Another biotechnological application of mannanases is in the cleanup of coffee waste. In 1995, Mexico alone produced 600 tonnes of coffee waste, which is mainly composed of water and sugar (Regaldo et al., 2000). The sugar in the coffee waste can be fermented by bacteria and acidified, resulting in a bad smell. The water leaking from the pulp can be highly acidic; hence, treating coffee waste is of environmental importance. Current common methods of waste treatment and disposal involve composting and drying for use as animal feed. Another new biotechnological use of coffee waste is in solid substrate fermentation. Due to the high proportion of galactomannan present in coffee waste, it makes an ideal substrate for fermentation by *A. niger* for the commercial production of endo-β-mannanase.

Solid substrate fermentation produced a high level of endo-β-mannanase which could be purified and commercialized. Mannanases are commonly used in fruit and vegetable maceration, wine and juice clarification, oil extraction from legume seeds and to reduce the viscosity of coffee extracts (Regaldo et al., 2000). Therefore, using coffee waste as a substrate for endo-β-mannanase production is a very cost effective system, which also removes the raw coffee waste from the environment.

The brewery industry has used barley in malting for centuries, waiting until the seeds have achieved a certain 'softness' via germination before using the grains in further processes (Hornsey, 1999). With the onset of germination, there is a mobilization of carbohydrate reserves, most importantly starch (Buckeridge et al., 2000). In the time taken for the grain to achieve the desired softness, up to 5% of the starch in the seed has been metabolized by the growing embryo (Bamforth, 1952). In this malting stage there could be a possible, yet untested, biotechnological application for endo-β-mannanase to facilitate grain softening and reduce starch loss. There was a possibility that by increasing the concentration or level of activity of endo-β-mannanase there would have been an alternative carbohydrate source available for use by the embryo. This would have left more glucose available for use by yeast during fermentation. Another possible outcome of increasing endo-β-mannanase concentration or activity in barley would be to speed up the process of cell wall degradation. This would cause the cell wall to be degraded in a shorter time than usual and enzymes such as amylase will be more easily dispersed through the endosperm, reducing the length of time needed to malt the grain. The structure of the endosperm greatly affects the quality of malting barley (Holopainen et al., 2005). Therefore, by

modifying the endosperm by increasing the concentration or activity of endo-β-mannanase we may see an increase in the quality of the malting barley and ultimately in the quality of the beer. There are numerous ways to increase the enzyme activity, such as introducing an external hormone which up-regulates enzyme production (Kucera et al., 2005), selectively breeding for grains which exhibit high enzyme activities or high enzyme concentrations (Gepts, 2002), or, theoretically, by genetic modification of the barley grain to enhance or replace the endo-β-mannanase gene with the gene of a different plant so it produces more of, or a more active form of endo-β-mannanase.

In biotechnology there are always avenues open to apply naturally-occurring reactions to a commercial purpose. In this case, the possible application of natural germinative enzymes to aid the malting process was investigated.

1.4. Aims and Objectives

1. To find the pH and optimum temperature for barley endo-β-mannanase activity. The pH and optimum temperature are different for different sources of the enzyme, i.e. barley enzyme will have different specifications than tomato fruit enzyme (Bewley et al., 2000). Therefore, it is necessary to know barley mannanase's pH and temperature profiles to observe the maximum activity (Section 3.1).
2. To ascertain the localization of the enzyme, both temporally and spatially, within the seed. It will be informative to know in which part of the seed the enzyme is being produced and how long after initial imbibition the enzyme's activity is at its highest (Section 3.3).
3. To observe the effect of a protease inhibitor cocktail on the level of detectable enzyme activity. Due to the harsh mechanical extraction procedure, proteases are released which may degrade endo-β-mannanase, resulting in a lower observed enzyme activity (Section 3.4).
4. To elucidate the action of sugars and hormone in promoting, inhibiting and regulating endo-β-mannanase activity. The action of these compounds on other enzymes has been well documented, particularly in the production of amylase enzymes (Gibson, 2005). The actions of glucose, sucrose, gibberellic acid (GA),

indole acetic acid (IAA) and abscisic acid (ABA) on the detected level of endo-β-mannanase activity will be observed (Section 3.5).

5. Once the effect of those hormones and sugars has been determined, seeds which have had their embryos excised will be sowed on hormone- or sugar-containing media and assayed via the Congo red dye assay. The results are to be compared and contrasted with the results of similar studies on amylases (Section 3.6).

6. To determine whether a relationship between seed softening and endo-β-mannanase production is present. Correlating the force required to compress the barley by 3mm with the enzyme activity (nkat) per milligram of fresh weight of seed will show whether such a relationship exists (Section 3.7).

7. To develop a new way to detect and quantify the presence and activity of endo-β-mannanase. This is to be achieved by developing a spectrophotometric assay which should be faster, more accurate and use fewer materials than the standard gel diffusion assay (Section 3.8).

The overall aim of this research is to gain a more detailed understanding of the changes to the barley grain components, such as mannans, and the behaviour of the enzyme that mediates these changes. A better understanding of the properties of barley endo-β-mannanase will contribute to the possible biotechnological application this enzyme may have within the brewery industry.

Chapter 2

METHODS AND MATERIALS

2.1. *Hordeum vulgare* Cultivars Dash and Fairview

The cultivars of barley selected for this work were Dash and Fairview. These cultivars were chosen as they were readily available and are commonly used feed and malting varieties. Dash was a feed stock variety donated by Pyne Gould Guinness (PGG) and Wrightsons Grains Ltd of Christchurch, and Fairview was a malting variety donated by the International Malting Company of Ashburton.

20 seeds from each cultivar were imbibed over 2 petri dishes on agar consisting of 1.0% phytagel and 20 mL of water. These were incubated at 26°C without light for periods of time that varied with different experiments (Downie et al., 1994). 10 germinated seeds from each cultivar were used in the subsequent experiments.

2.2. Enzyme Extraction Procedure

In the first enzyme extractions, ten germinated seeds of each variety were chosen at random and removed after 24 h of imbibition. The seeds' fresh weights were recorded and the seeds transferred to a mortar. Mortar and pestle with liquid nitrogen were used to grind the seeds into a powder. Once ground to a powder, 1 mL of extraction buffer (Appendix 1.3) per 200 mg of fresh weight was added to the mortar and the seeds further ground into a slurry. The slurry was transferred into Eppendorf tubes and stored on ice until all extractions were carried out. The barley slurries were centrifuged in an Eppendorf 5530 centrifuge at 4°C for 10 minutes at 14,000 *g*. After centrifugation, the

resulting supernatant was decanted off into a new Eppendorf tube and stored at -20°C until required for testing.

2.3. Assay for Detection and Quantification of Endo-β-Mannanase

2.3.1. Gel Diffusion Plates

The Congo red dye gel diffusion assay was the prime assay used throughout this research. This assay, developed by Downie et al. relies on the specificity of Congo red dye binding to mannan chains but not to mannan that has been hydrolyzed by the action of the enzyme (Downie et al., 1994).

A 0.1% (w/v) galactomannan substrate, in this case locust bean gum, was suspended in a pH 7 McIllvaine buffer (Appendix 1.1) by heating the solution to 60°C with stirring on a magnetic hotplate stirrer. The substrate and buffer solution was stirred for two hours at approximately 60-80°C, and then left to stir for a further 12 hours at room temperature as per the method outlined by Downie et al.

Once the substrate was completely dissolved in the buffer, 0.7% (w/v) of phytagel was added as a solidifying agent. The phytagel was fully dissolved by heating the solution, and then 20 mL aliquots were poured into clean Petri dishes and left for ~30 minutes to set. Once set, 4 mm diameter wells were made using a cork borer and the plugs removed with a scalpel.

Enzyme extracts of unknown concentration were inoculated into the wells by pipetting 10 μL of extract into the well. This was then incubated for 24 hours at 37°C. After incubation, the plates were calibrated to pH 9 to match the pH of the Congo red dye solution. 10 mL of 0.1 M K_2HPO_4 (pH 9) was added to each plate and the plates were placed on an orbital shaker for 30 minutes to allow the pH of the plate to increase. After 30 minutes, the pH 9 solution was drained off and 10 mL of 1% (w/v) Congo red dye (Appendix 1.4) was added (Downie et al., 1994). The plates were placed on the orbital shaker for a further 20 minutes to allow the dye to stain the regions of the plate containing mannan chains.

After 15 minutes, the Congo red dye solution was drained off and a 1 M solution of NaCl was used to wash the plates. Approximately 10 mL of NaCl solution was poured on each plate, agitated on the orbital shaker for 5 minutes then drained. This step was repeated as many times as necessary for the liquid in the wells to clear.

Once the plates were stained and sufficiently washed they were scanned using a Microtek ScanMaker X 12USL and analyzed using Adobe Photoshop version 7.0.1. The diameter of the clearing zone was measured in pixels in two directions using a measuring tool in Photoshop and the pixels were converted into millimetres. The average of the measured diameters was then converted to enzyme activity (nkat) using the equation derived by assaying a serial dilution of commercial enzyme, in a similar manner to the method followed by Downie et al. (1994).

2.3.2. Serial Dilution

To quantitatively assay the enzyme activity in an unknown sample, a series of enzyme dilutions of known concentrations must be assayed on the plates. A series dilution was made using Megazyme endo-β-mannanase from an *Aspergillus niger* source. The commercial enzyme preparation was suspended in 3.2 M ammonium sulfate which was dialysed before use. 1 mL of *Aspergillus* enzyme was pipetted into dialysis tubing and floated in a pH 7 citrate/phosphate buffer with stirring for 24 hours. The dialysed enzyme was then diluted to 0.75, 0.075, 0.0075 and 0.00075 nkat in pH 7 McIllvaine buffer. It was assumed that the volume of enzyme did not change and the enzyme activity did not fall during dialysis. This was achieved by setting up a serial dilution transferring 0.1 mL of enzyme into 0.9 mL of buffer and repeating this step until the desired dilutions were achieved. The enzyme activity of the commercial enzyme was assumed to be as stated by the manufacturer (Megazyme, 2007).

These dilutions were inoculated into the wells of the gel diffusion plates and incubated at 37°C for 24 hours. These plates were calibrated, stained, washed and analyzed as described in 2.3.1. The clearing zone diameters were measured and recorded in a Microsoft Excel spreadsheet. The data were then imported into Minitab and a regression analysis performed to find an equation relating enzyme activity to the clearing

zone diameter in millimetres. This was also the method used by Downie et al. (1994) to form their equation for relating enzyme activity and clearing zone.

2.4. Temperature and pH

As the pH and optimum temperature for endo-β-mannanase activity varies depending on the source of the enzyme, it was necessary to find the pH and optimum temperature for the barley enzyme (Montiel et al., 2002). Two extracts, one of each barley cultivar from 10 seeds imbibed for 24 hours, were used to determine the respective pH and temperature optima.

Citrate phosphate buffers (Appendix 1.1) were made up with pH 3, 5 and 7 and used to make gel diffusion plates. The barley extract was inoculated into these plates and incubated at a series of temperatures: 7, 17, 27, 37 and 50°C. After 24 hours of incubation, the plates were stained, washed, analyzed, and the results recorded and graphed in Microsoft Excel. The pH and temperature optima ascertained from these results were chosen to be the standard pH and temperature for carrying out all further assays with barley extracts.

2.5. Enzyme Localization

Just as the optimum pH and temperature for enzyme activity are important, it is also of interest to know how long after the onset of germination the enzyme activity peaks and in which part of the seed the enzyme is most prevalent.

100 seeds of each variety were imbibed and left to incubate for 24 hours at 26°C without light. After 24 hours, 10 seeds of each variety were removed with forceps and the remaining plate was resealed and placed back into the incubator for a further 24 hours. The seeds were then cut longitudinally along the crease with a scalpel. The embryos were excised from the seeds using a scalpel, and collectively weighed on a balance. The starchy endosperm was scraped away from the seed coat using a scalpel and the seed coat and endosperm were weighed separately.

Each of the three separated seed parts were then weighed separately and ground to a powder with liquid nitrogen. 1 mL of buffer per 200 mg of seed fresh weight was added and the powdered seed was further ground to slurry with extraction buffer, centrifuged and the supernatant collected. The supernatant, the enzyme-containing extract, was inoculated into wells on the gel diffusion plates and incubated at the temperature determined in section 2.4 for 24 hours. The plates were then stained, scanned and analyzed.

This procedure was carried out for 7 days, each time removing 10 seeds of each variety and replacing the remaining seeds in the incubator. Finally, a time line of enzyme activity (nkat), ranging from 24 hours after being initially imbibed to 168 hours later at 24 hour intervals, was created. The results were collated and expressed as a graph in Microsoft Excel.

2.6. Protein Quantification

A method to determine the concentration of protein in the sample was required to load approximately equal amounts of protein into the lanes of a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). A Biuret assay was initially tried but had very large blank errors, so a Folin-Lowry assay was undertaken instead (Zhou and Regenstein, 2006).

2.6.1. Folin-Lowry Assay

This method, known as the Folin-Lowry assay, is based on the procedure first done by Lowry in 1951 (Campbell and Reece, 2001). 2 mL of Folin-Lowry reagent (Appendix 2.1) was vorticed with 50 μL of sample extract and left to stand at room temperature for 10 minutes. 0.2 mL of phenol reagent (Appendix 2.1) was added to each sample tube, vorticed immediately and left to stand for a further 30 minutes at room temperature. The samples were measured at 600 nm in a BioRad SmartSpec spectrophotometer, which was first blanked with a control. This assay was set up in triplicate with a control tube consisting of 0.1 mL of water in place of sample extract.

To equate absorbance at 600 nm with protein concentration, a standard curve was required. In place of the sample extract a series dilution of known concentration of bovine serum albumin (BSA) was assayed to create a standard curve. The standard curve was created using concentrations 0 μg/μL to 100 μg/μL of BSA, in 10 μg/μL increments. The equation of the curve is judged to be an accurate equation for determining the protein concentration in an unknown sample by reading the absorbance (Lowry, 1951). Any absorbance outside the upper limit of the standard curve, i.e. above 100 μg/μL, may not fit the equation of the standard curve's line and was diluted.

2.7. Protease Inhibitor Cocktail

A protease inhibitor cocktail created in the laboratory was added to the extraction solutions to prevent the suspected proteolysis of endo-β-mannanase during the extraction procedure. A solution of protease inhibitor (Appendix 2.2) and extraction buffer was made and used in place of the standard extraction buffer for the 'plus inhibitor' condition. The extraction, gel diffusion assay and analysis of the plates were carried out as per section 2.3.

2.8. Hormone and Sugar Regulation

The test for hormone and sugar regulation or interference was undertaken using the following method, with only the addition of a sugar or hormone varying. Four aliquots of 40 mL dH_2O were poured and designated as additive concentrations 1, 2, 3 and control (containing no additive). These buffer solutions were turned into a gel with 1.0% Phytagel agar and 20 mL aliquots poured into Petri dishes. 15 seeds were germinated on each plate and incubated at 26°C without light. Samples of three seeds were taken at 1, 2, 3, 4 and 5 days after initial incubation. These seeds were then broken up with liquid nitrogen in a mortar, ground to slurry, centrifuged and the gel diffusion assay carried out as per section 2.3.

2.8.1. Glucose

Three concentrations of glucose were used: 10, 20 and 30 mM. 0.072 g, 0.144 g and 0.216 g of glucose respectively were dissolved in 40 mL of dH_2O to achieve these concentrations. Osmotic control plates were poured containing 30 mM of mannitol.

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2.8.2. Sucrose

Three concentrations of sucrose were used: 10, 20 and 30 mM. 0.136 g, 0.272 g and 0.408 g of sucrose respectively were dissolved in 40 mL of dH_2O to achieve these concentrations. Osmotic control plates were poured containing 30 mM of sorbitol.

2.8.3. Abscisic Acid

Three concentrations of abscisic acid were used: 142, 286 and 505 μM. 1.51, 3.02 and 4.54 mg of abscisic acid respectively were dissolved in 40 mL of dH_2O to achieve these concentrations. A control plate containing no abscisic acid was also poured.

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2.8.4. Gibberellic Acid

Three concentrations of gibberellic acid were used: 83, 167 and 245 μM. 1.15, 2.31 and 3.40 mg of gibberellic acid respectively were dissolved in 40 mL of dH_2O to achieve these concentrations. A control plate containing no gibberellic acid was also poured.

2.8.5. Indole Acetic Acid

Three concentrations of indole acetic acid were used: 314, 642 and 970 μM. 2.2, 4.5 and 6.8 mg of abscisic acid respectively were dissolved in 40 mL of dH_2O to achieve these concentrations. A control plate containing no indole acetic acid was also poured.

2.9. Excision of the Embryo

20 dry seeds of each variety were cut transversely and the embryo separated from the remainder of the seed. 10 isolated endosperms were then placed in 2 mL of dH_2O and incubated for 72 hours at 26°C without light. The remaining 10 seeds were placed in 2 mL of dH_2O with 167 μM gibberellic acid added, then incubated for 72 hours at 26°C without light.

After 72 hours incubation, both conditions were removed from the incubators and 5 seeds from each had enzymes extracted as per section 2.2. 10 μL of enzyme extract was inoculated into the wells on a locust bean gum (LBG) agar plate and incubated at 37°C without light for 24 hours.

The liquid that the seeds were suspended in was also tested for the presence of endo-β-mannanase. 10 μL of imbibition water from each of the two conditions was pipetted into the wells of two separate LBG agar plates and incubated in the same conditions as the enzyme extract plates.

After 24 hours incubation the plates were stained, scanned and analyzed as per section 2.3. This experiment was repeated with 286 μM ABA, 642 μM IAA, 20 mM glucose and 20 mM sucrose in 2 mL of dH_2O.

2.10. Mechanical Strength

The mechanical strength of barley seeds of both varieties was tested on an Instron 4400 extensometer and analysed using TPA v5.0. Mechanical strength testing was broken up into two categories: with or without exogenous endo-β mannanase, and time after the seeds were first imbibed.

2.10.1. Addition of Exogenous Endo-β-Mannanase

1 mL of dialysed commercial endo-β-mannanase was added to 5 mL of dH_2O and used to imbibe 30 seeds of each variety. As a control, 30 seeds of each variety were imbibed with 6 mL of dH_2O. These seeds were placed in a 26°C incubator for 48 hours. After 48 hours, 20 germinated seeds of each variety were removed and had any protruding roots and shoots removed with a scalpel. Each seed had its length, width and height measure with callipers. These measurements were recorded and each seed sealed in a bag with a number referencing it to its measurements.

Each individual seed had its mechanical strength tested on the Instron 4400 using a flat head and a descending speed of 30 m/min and a compression of 3 mm. The modulus of the curve generated by the programme TPA v5.0 was taken from the point of contact to the fracture point of the seed, and the results imported from an XML file into Microsoft Excel.

The average of the modulus was calculated using the descriptive statistics function of Microsoft Excel. As well as the average of the modulus, the change in modulus respective to the weight, height, length and width of the seeds were also examined.

2.10.2. Time after Initial Imbibition

The second experiment was to examine the change in mechanical strength of barley seeds over different germination time and relate this to the change in enzyme activity from the results of section 2.5.

30 seeds of each variety were imbibed with 6 mL of dH_2O and incubated for 96 hours. Each day for the subsequent three days, a further 30 seeds of each variety were imbibed and incubated at 26°C. At the end of this time there were 4 sets of seeds which, when harvested on the same day, represented 24, 48, 72 and 96 hours after sowing in dH_2O agar plates.

20 seeds from each condition and of each variety were removed from their plates, had any roots and shoots removed and were measured and bagged in the same manner as

those seeds in section 2.10.1. The mechanical strength of each seed was measured on the Instron 4400 using the same parameters, program and method of analysis as those seeds in section 2.10.1.

2.11. Spectrophotometric Assay

Three substrate concentrations were trialed for the development of the spectrophotometric assay: 0.1%, 0.5% and 1.0% (w/v) locust bean gum in pH 7 citrate/phosphate buffer. 1.95 mL of buffer/substrate solution was pipetted into a test tube, and 20 μL of enzyme extract was added and the solution vorticed for 5 seconds. Enzyme extractions were made as per section 2.2.

The substrate and enzyme solution was then incubated at 37°C for one hour. During this time a 1% Congo red dye solution was prepared by first leaving a 3 mL aliquot of pH 9 K_2HPO_4 in a lidded vial in a heated oven for 10 minutes. Once the K_2HPO_4 was warm (~40°C) the Congo red dye was dissolved then filtered through a Millex® GP Filter Unit (0.22 μm) attached to a syringe.

After 1 h incubation at 37°C, the enzyme was heat inactivated by placing the tubes in a boiling water bath for 5 minutes. Following heat inactivation, 50 μL of the filtered Congo red dye solution was added to the tubes, vorticed and left to stand at room temperature for 15 minutes.

50 μL of dyed substrate solution was then added to 950 μL of pH 7 citrate/phosphate buffer to dilute the colour to a level readable by a spectrophotometer. These diluted solutions were read by a BioRad SmartSpec spectrophotometer and the absorbance spectrum found. The peaks of the absorbance spectrum were then used as the wavelengths for the quantitative enzyme assay.

Using the same method of preparation as above, a serial dilution of *Aspergillus* endo-β-mannanase was used to create a standard curve for use in quantifying enzyme activity (nkat) in sample extracts. It was assumed that the activity of the commercial enzyme was as the manufacturer claimed. After heat inactivation and dilution, the series

dilution was placed in the spectrophotometer and the absorbance at the wavelengths determined in the absorbance spectra recorded.

The resulting absorbance values of the triplicate samples were averaged and plotted using the Microsoft Office graphing tools.

2.12. Data Analysis

Regression analysis and t-tests were performed using Minitab (release 14) and significance was calculated to a minimum of 95% confidence. Standard deviations and means were calculated using the descriptive statistics function of Microsoft Excel 2003, and the bar and line graphs created using the Microsoft Excel 2003 graph tool.

Chapter 3

RESULTS

The breakdown of mannan-containing polysaccharides in the grains of barley by endo-β-mannanase is one of many essential processes during germination. By better understanding the conditions under which barley endo-β-mannanase is optimized and, conversely, those in which it is inhibited, as well as the way in which this enzyme works, we may be able to improve biotechnological processes, such as malting, which utilize the germination of barley. The following sections show the results gained to create a better understanding of this process.

3.1. pH and Temperature

Preliminary experiments were carried out to determine an appropriate pH and optimum temperature for measuring endo-β-mannanase activity in extracts from the seed of two barley cultivars, Dash and Fairview. Five different incubation temperatures were trailed: 7, 17, 27, 37 and 50°C. Endo-β-mannanase activity was measured using the gel diffusion assay (section 2.3.1). In this case, the clearing zone was measured in millimetres and not converted into nkatals per milligram of fresh weight (Figure 3.1).

At pH 7, for the extracts of both cultivars, the enzyme activities at almost all temperatures were significantly different from one another (t-test, P=0.05). There was, however, no significant difference between the enzyme activity from seeds incubated at 27° and 50°C in the extracts of cultivar Dash. The general trend of these data showed endo-β-mannanase to be most active, or most highly concentrated, when incubated at higher temperatures.

At all the incubation temperatures tested, higher endo-β-mannanase activity was detected at pH 7 than pH 5 conditions, and this pH was therefore selected for all further

incubations. At pH 7, the highest enzyme activity was observed from seeds incubated at 37°C. Therefore, all further endo-β-mannanase plate assays in this work were carried out at pH 7 and 37°C. While this temperature was found to give the highest activity of endo-β-mannanase and was used in laboratory-based experiments, it would not be applicable to a brewing situation, as malting most commonly takes place at 15°C (Roehr, 2001).

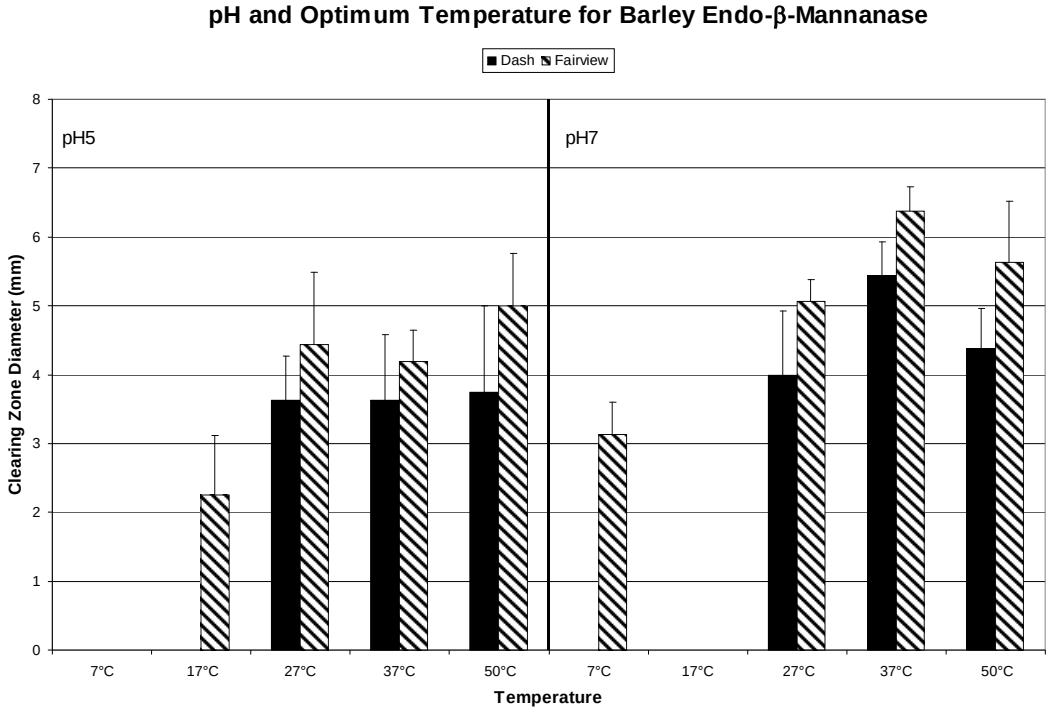

Figure 3.1. Endo-β-mannanase activity as determined by clearing zone diameter (mm) in barley seed extracts incubated in gels of varying pH and temperatures. The average value represents the mean of 6 replicates and the error bars indicate the standard deviation from the mean. The absent data points at 7°C in the pH 5 condition and at 17°C in the pH 7 condition are due to no detectable enzyme activities on the gel diffusion plates. This experiment was also carried out at pH 3, at which there was no detectable enzyme activity at all.

3.2. An Equation Relating Clearing Zone to Enzyme Activity

Knowing the pH and optimum temperature to assay endo-β-mannanase activity in a barley extract was the first step to being able to produce an equation relating clearing zone diameter from the gel diffusion assay to enzyme activity.

A stock solution of endo-β-mannanase of known concentration (*Aspergillus niger* enzyme Lot 008101 (Megazyme, 2007)) was used to prepare a series of diluted enzyme solutions. The stock solution was serially diluted to form four solutions of a known enzyme activity (0.75, 0.075, 0.0075 and 0.00075 nkat). It was assumed that the manufacturer's enzyme activity was correct as stated, and that a series dilution of this enzyme would yield the desired dilutions with no loss of activity.

This serial dilution was inoculated into 8 x pH 7 plates (2 plates for each dilution) with 4 wells in each plate (1 x control, 3 x enzymes) for a total of 6 replicates at each enzyme dilution. These were incubated for 24 hours at 37°C, stained with Congo red dye, and the clearing zones measured using Adobe Photoshop 7.0. An example of a typical serial dilution plate using this staining method is shown in Figure 3.2. Black rings were superimposed on the clearing zones to highlight the border between stained and non-stained gel as some contrast was lost during scanning the image into the computer.

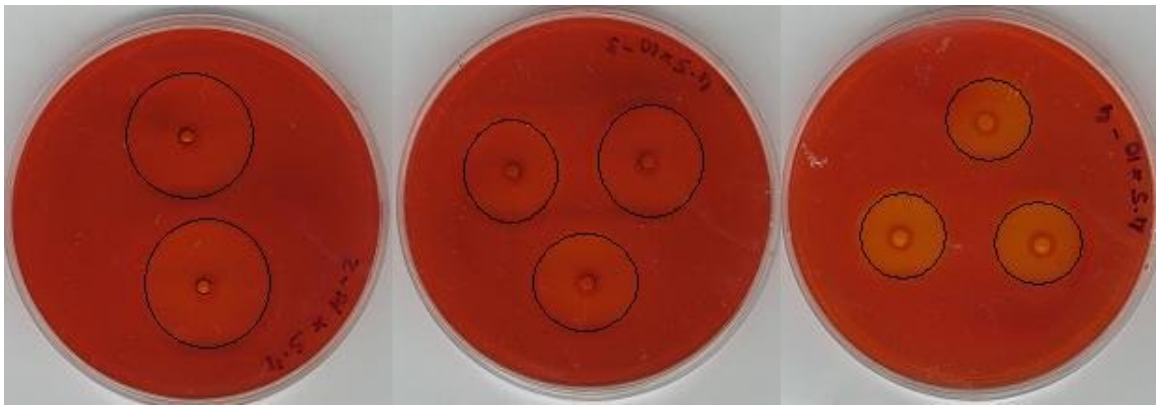

Figure 3.2. Appearance of gel diffusion assay plates. From left to right: 0.75 nkat, 0.075 nkat and 0.0075 nkat of the *Aspergillus niger* enzyme in each well of the appropriate plate.

The relationship between clearing zone and enzyme activity, displayed in Figure 3.3, is logarithmic and the R^2 value of 0.9942 shows the line of best fit to be a very close fit to experimental data. For future experiments using the Congo red dye gel diffusion assay at pH 7 and 37°C, this equation was used to calculate the enzyme activity as a function of clearing zone diameter.

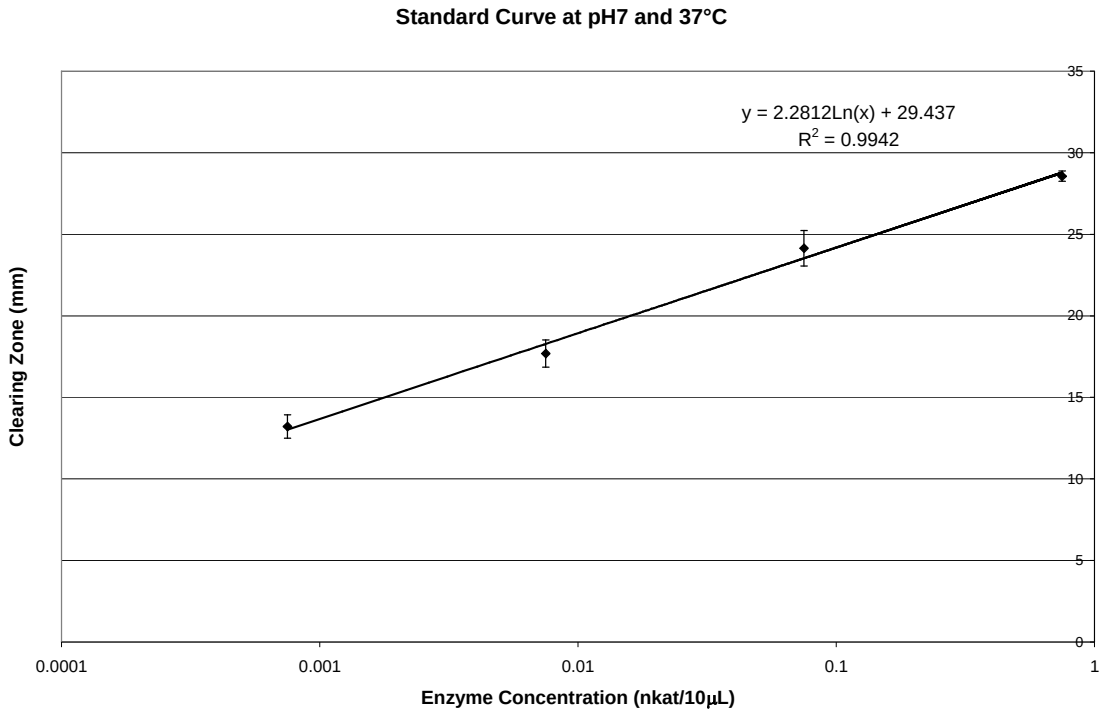

Figure 3.3. The equation y = 2.2812ln(x) + 29.437, represented by the line of best fit, formed by the relationship between clearing zone (mm) and enzyme activity (nkat) can be rearranged to form $x = e^{((y-29.437)/2.2812}$. The data points represent the mean of 15 replicates +/- the standard deviation.

The data were logarithmically transformed to create a straight line. The equation of the line of best fit was y = 2.2812ln(x) + 29.437, with y being the clearing zone (mm) and x being the enzyme activity (nkat). The clearing zone is the subject of the equation; however, it would be more useful to rearrange the equation to have enzyme activity as the subject. Rearrangement of the equation results in $x = e^{((y-29.437)/2.2812)}$. This format is more useful as the unknown quantity is now the subject of the equation.

3.3. Temporal and Spatial Localization of Endo-β-Mannanase

The temporal and spatial localization of endo-β-mannanase during barley seed germination was determined. The seeds, at varying stages of germination, were divided into their three parts (endosperm, embryo and seed coat) before they were assayed for endo-β-mannanase activity using the Congo red dye method (2.3.1).

The three seed parts showed different levels of enzyme activity, particularly the embryo, which had no detectable endo-β-mannanase activity (Figures 3.4 and 3.5). The seed coat and the endosperm both had detectable endo-β-mannanase activity. Initially, a higher level of enzyme activity was present in the seed coat of both varieties. The presence of endo-β-mannanase in both the endosperm and the seed coat corroborates evidence from literature that shows the aleurone layer to be the site of enzyme production (Ritchie, 2000).

In both Fairview and Dash cultivars there was a significantly greater enzyme activity (t-test, P=0.05) in the seed coat than in the endosperm in the first 2-3 days from sowing of the seeds. During germination, the level of detectable enzyme activity in the seed coat decreased almost proportionally to the increase in activity detected in the endosperm of both cultivars (Figures 3.4 and 3.5).

The enzyme, which is produced in the aleurone layer, is most likely being transported from its site of production, near the seed coat, to its destination in the endosperm. Therefore, the results here suggest the movement of the enzyme within the seed during germination (Ritchie, 2000).

Four days after the start of imbibition, the level of detectable endo-β-mannanase activity in the endosperm and the seed coat were no longer significantly different. After this point the detectable enzyme activity in the endosperm continued to increase and that in the seed coat decrease, until their levels were again significantly different. At day four the germination appeared to be at a midpoint with almost equal concentration of enzyme detected in the endosperm and the seed coat. After day four the detectable enzyme in the endosperm increased at a similar rate to the enzyme decreasing in the seed coat, suggesting that the bulk of the endo-β-mannanase produced by the aleurone layer has been secreted into the endosperm. This pattern of temporal and spatial changes in endo-β-mannanase was exhibited during imbibition and seed germination of both barley cultivars Dash and Fairview.

In further experiments, the embryo was not used as part of the extraction as it was shown to have no significant detectable activity. Moreover, the endosperm and the seed coat were left intact during harvest so as not to disrupt the aleurone layer and therefore retain as much viable enzyme as possible for enzyme assays.

As well as showing which parts of the seeds are most useful for enzyme extraction and measuring enzyme activity, the results here also indicate which point in germination to harvest the seed for optimum enzyme concentration. An extract prepared from seeds which had germinated for four days was chosen as these seeds gave a good balance between enzyme activity and ease of harvesting the endosperm. While there was a higher level of enzyme detected in later stages of germination, the embryo had almost entirely consumed the endosperm making endosperm harvest difficult.

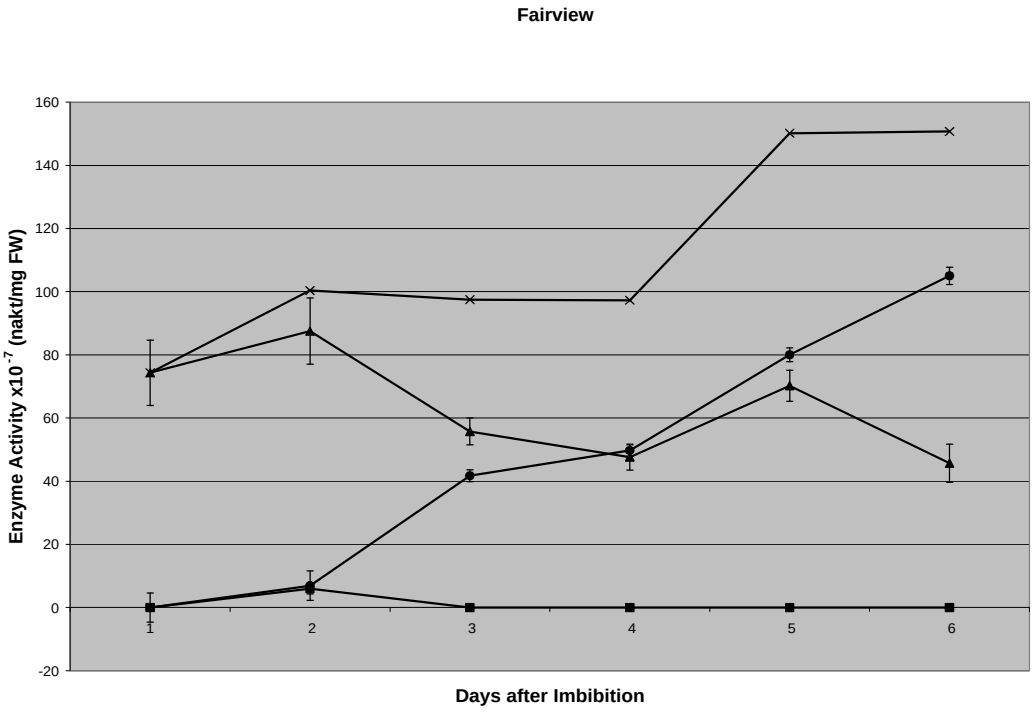

Figure 3.4. Changes in detectable endo-β-mannanase activity (nkatals per milligram of fresh weight of Fairview barley seeds) over a period of 6 days. Each point represents the mean value of 6 replicates and the error bars represent the standard deviation of those replicates. The fours lines on the graph represent total enzyme activity in the seed (crosses), and the enzyme activity in the three seed section; endosperm (circles), seed coat (triangles) and embryo (squares).

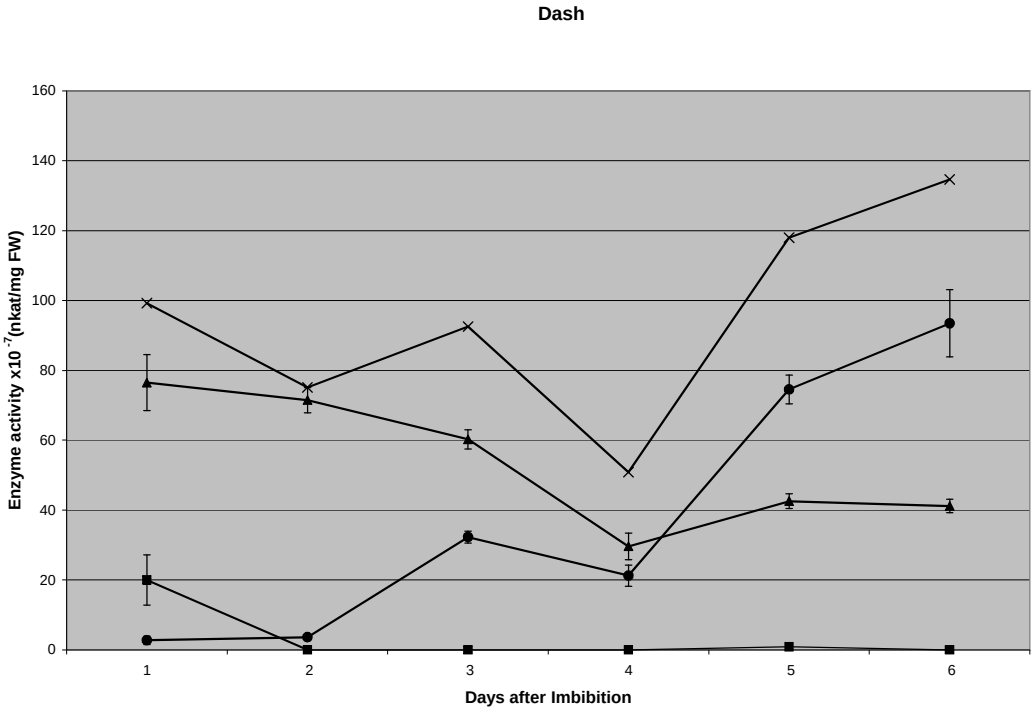

Figure 3.5. Changes in detectable endo-β-mannanase activity (nkatals per milligram of fresh weight of Dash barley seeds) over a period of 6 days. Each point represents the mean value of 6 replicates and the error bars represent the standard deviation of those replicates. The fours lines on the graph represent total enzyme activity in the seed (crosses), and the enzyme activity in the three seed section; endosperm (circles), seed coat (triangles) and embryo (squares).

The day 3-4 extracts showed relatively high activities, particularly considering that in further experiments the seed coat and endosperm were not separated. Also, by taking seeds that had been germinating longer, we were more likely to obtain consistently higher enzyme yield as the seeds from earlier germination times had quite varying results as indicated by the larger error bars.

3.4. Effect of Protease Inhibitor

Since the enzyme extracts were seen by SDS-PAGE to contain many proteins (data not shown) potentially including proteases, the addition of a protease inhibitor cocktail on the level of detectable enzyme activity was explored and the activity compared to an untreated extract. The addition of an inhibitor should increase the level of

detectable enzyme as it is being protected from degradation (Doonan, 2004). There was an extremely significant difference in the level of activity detected in the two extracts as no enzyme activity was detected in extracts containing the protease inhibitor. Interestingly, the presence of the protease inhibitor inhibited the action of the enzyme. The action of endo-β-mannanase is reliant on its ability to donate a hydrogen from a hydroxyl group to the oxygen on the β-(1, 4) bond of the substrate. One of the inhibitors in the cocktail inhibits the hydroxyl site, which is a common site in many proteases. So, while there may have been more intact enzyme in these extracts it was unable to function due to inhibition and was therefore undetectable using the Congo red dye gel diffusion assay. In further experiments no protease inhibitor was included.

3.5. Hormone and Sugar Regulation

The actions of two sugars, one monosaccharide and one disaccharide, and three plant growth regulators on the production of endo-β-mannanase and development of the barley seedling were studied. These compounds were chosen for both their natural and commercial abundance. It would not be economical to work with a rare or expensive substance that could not be economically or effectively upscaled for use in a brewery. They are also naturally occurring sugars and hormones, all of which have been extensively studied in other organisms (Kucera, 2005; da Silva, 2004).

The presence of fungal and bacterial contamination was noted throughout this experiment and it is unknown how much of an effect this contamination had on the results which were obtained. The presence of the contaminants could inhibit the action of the enzyme resulting in decreased level of activity being observed, or conversely, the production of endo-β-mannanase by the contaminants may show an inaccurately high level of enzyme activity. These experiments could not be repeated due to time constraints, however if they were repeated surface sterilization of the seeds in a hypochlorite solution would be advised.

3.5.1. Glucose

Qualitative observation of the seeds on the glucose plates showed that the seeds appeared to germinate slightly slower than those on the control plate, putting forth slightly shorter shoots and roots than those on the control plates of the same day. The addition of the monosaccharide sugar glucose to the germination medium caused an increase in the detectable activity of endo-β-mannanase in both Dash and Fairview seeds (Figures 3.7 and 3.8). After 24 hours of imbibition, there was no significant difference in the enzyme activity between the osmotic control and any of the glucose plates in either cultivar. However, after 48 hours there was a significant (t-test, P=0.05) increase in enzyme activity per milligram of fresh weight in all glucose treatments for Dash. For Fairview, this difference was only observed in the 10 mM glucose treatment. Later on, there was overall a significant increase in enzyme activity in the 10 and 20 mM glucose treatments for Dash, and the 10 and 30 mM glucose treatments for Fairview. The apparent decrease in activity in day three Dash extracts could be explained by the presence of fungal or bacterial contamination on the day three plates. This contamination may have inhibited enzyme production or activity on this plate. This may also be the cause of the decrease of activity seen in the day five Fairview 20 mM condition.

3.5.2. Sucrose

The seeds on plates containing sucrose appeared to germinate at much the same rate and the seedlings developed were of similar size to those on the control plate containing 30 mM sorbitol as an osmotic control. The addition of sucrose to the germination media caused an increase in the detectable activity of endo-β-mannanase in Fairview, but not in Dash (Figure 3.9 and Figure 3.10). Fairview showed small differences in detectable enzyme activity at any concentration of sucrose in comparison to the control for days 1, 2 and 3. However on day 4 there was a significantly higher level of activity detected in the 30 mM sucrose treatment than the control plate. On day 5 there was more enzyme activity detected in the control wells than in any other of the

experimental wells. This could again be due to contamination, or possibly the combined endogenous and exogenous sucrose levels had become inhibitive to enzyme production in the three test conditions. Dash results had quite large standard deviations. One day after sowing there was no significant difference between the control and the sucrose treatments, but by day two there was a significant increase in detectable endo-β-mannanase activity at all of the three sucrose concentrations in comparison to the control. On day 3, there was no significant difference in the enzyme activity among the 10 and 20 mM sucrose and the control treatments, but there was a lower activity in the 30 mM sucrose treatment than in the control. On days 4 and 5, there was no significant difference in the enzyme activity among the different treatments.

3.5.3. Abscisic Acid (ABA)

The seeds on plates containing ABA did not appear to germinate at all, whereas those on the control plate put forth roots and shoots as normal. This was not an unexpected result as previous studies have shown ABA to inhibit germination in many plants (Srivastava, 2002). The addition of ABA to the germination media caused mixed results in the detectable activity of endo-β-mannanase (Figures 3.11 and 3.12). The effect of ABA in the germination media had a much greater effect on the germination of the seed that it did on the level of detectable activity. In Fairview there were very few significant differences between the extracts from ABA containing plates and extracts from seeds on the control plate (with distilled water only). Where there were significant differences, the ABA extracts showed a significantly decreased activity level compared to the control. There was, however, a spike in activity in Fairview day four control seeds. This unusually large increase in activity is most likely caused by bacterial or fungal contamination. This activity spike is present in Figures 3.12, 3.14 and 3.16 as the same control plates were used for all three hormone assays. The results from the Dash extracts are quite varied: many show no significant difference due to the high level of variation in the replicates. In the first two days, slightly higher enzyme activities were detected in the extracts from the plates containing ABA. However, at day 5, a significantly higher

enzyme activity was detected in the extracts from the seeds on the control plates (t-test, P=0.05)

3.5.4. Gibberellic Acid (GA)

The germinated seeds on plates containing GA had much longer shoots than those on the control plate. There was a significant increase in detectable enzyme activity in extracts of germinated Dash barley seeds at almost all days and concentrations of GA (Figure 3.13). However, there was no difference between the control seeds incubated in plates containing distilled water and the GA treatment at day 1. Also, day 5 extracts from the 245 μM treatment show increased activity detected, but it was not statistically significant from that of the control. The overall trend of the Fairview data shows an increase in detectable enzyme activity in extracts from the GA-containing plates in comparison to the controls, with the exception of day four, which as previously stated was contaminated. While there were many significant increases in detectable activity in the extracts from GA plates, there were also some increases such as day 1 at 83 μM and 167 μM which were statistically insignificant. The statistically insignificant results do not detract from the overall trend of the data. In both cultivars the presence of GA in the germination media resulted in an overall increased level of detectable enzyme activity.

3.5.5. Indole Acetic Acid (IAA)

Qualitative observation of germinated seeds on plates containing IAA showed stunted root growth, but otherwise appeared to germinate normally in comparison to those seeds on the control plate. A significant stimulation effect (t-test, P=0.05) (Figure 3.15) in Dash was observed on day 2 and day 3, particularly resulting from the treatments with 314 μM or 642 μM IAA. However, the day 3 Dash result may be inaccurate as there were signs of bacterial or fungal contamination. In Fairview, however, there was a significant increase in all but the day 4 condition, which as previously alluded to was

probably due to contamination. However, the overall trend showed a significant increase in the detectable activity at all concentrations of IAA at almost all days in both cultivars.

In all treatments, there was variation in the control samples. This variation was likely the result of natural variations in the seeds. The seeds, while similar in size, were not uniform and quite probably had different compositions or amounts of structural polysaccharides, sugars or endosperm sizes. This natural variation within the seeds could also be contributing to the variation seen in the treatments.

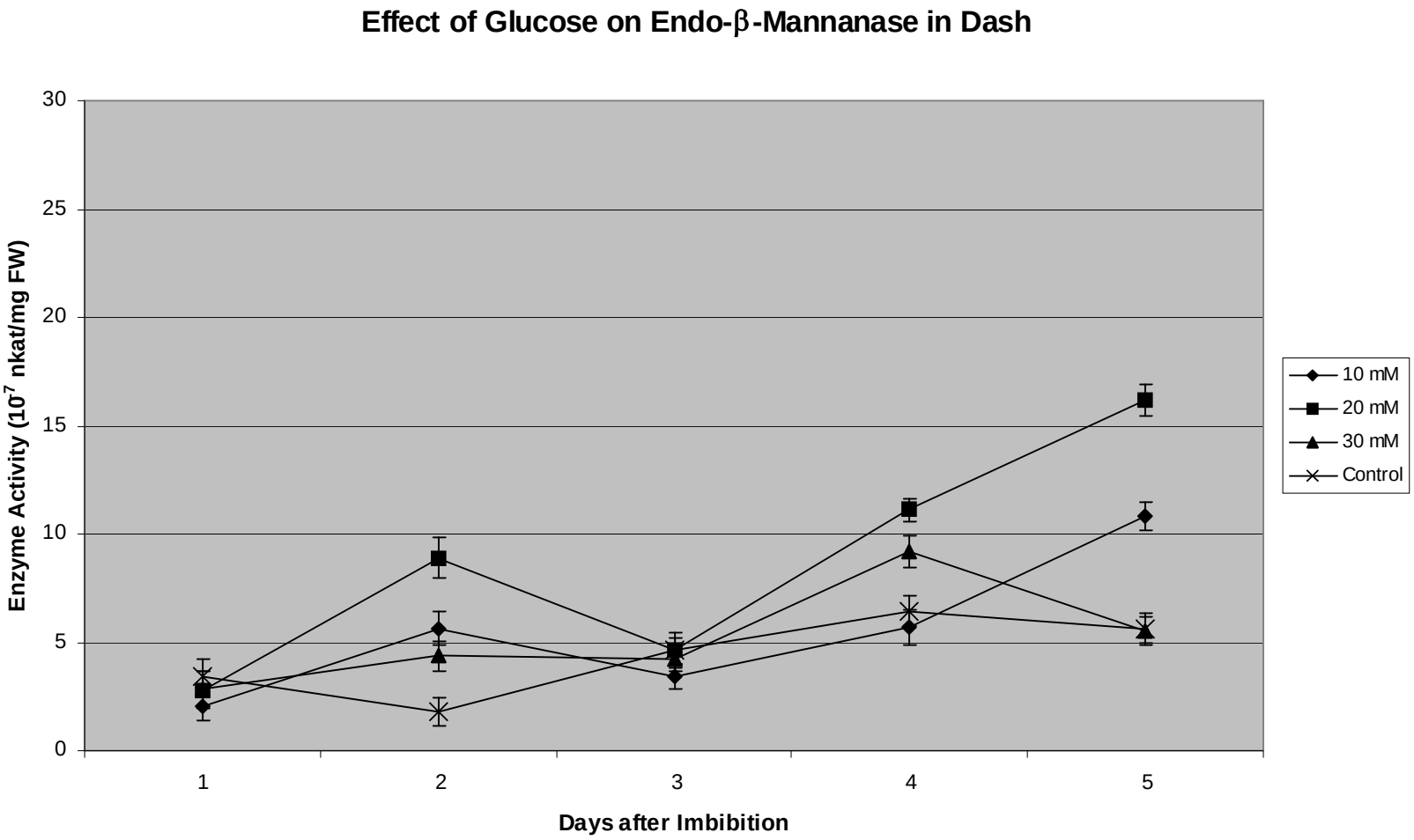

Figure 3.6. The effect of glucose at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Dash barley seeds compared to a control containing mannitol. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

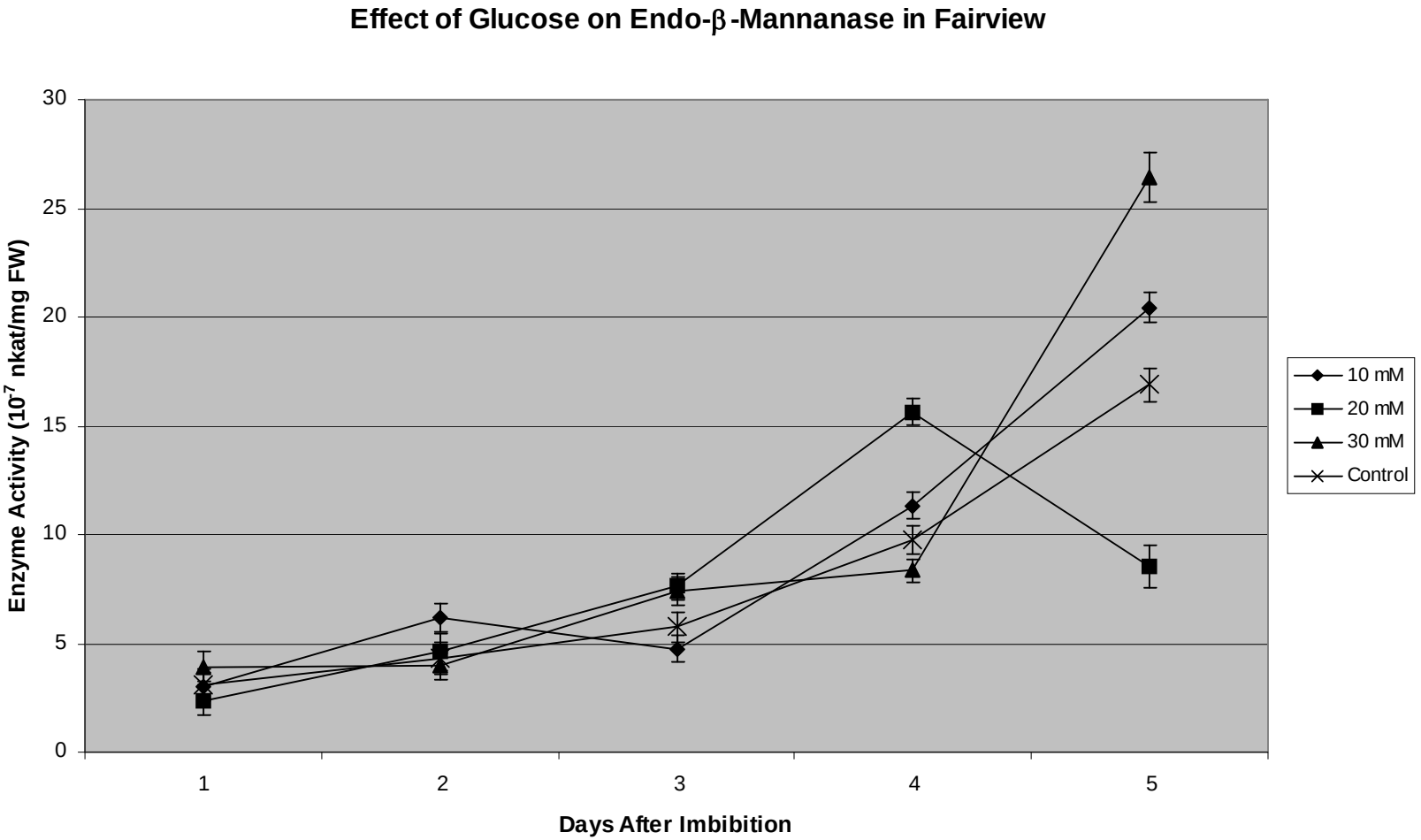

Figure 3.7. The effect of the monosaccharide glucose at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Fairview barley seed compared to a control containing mannitol. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

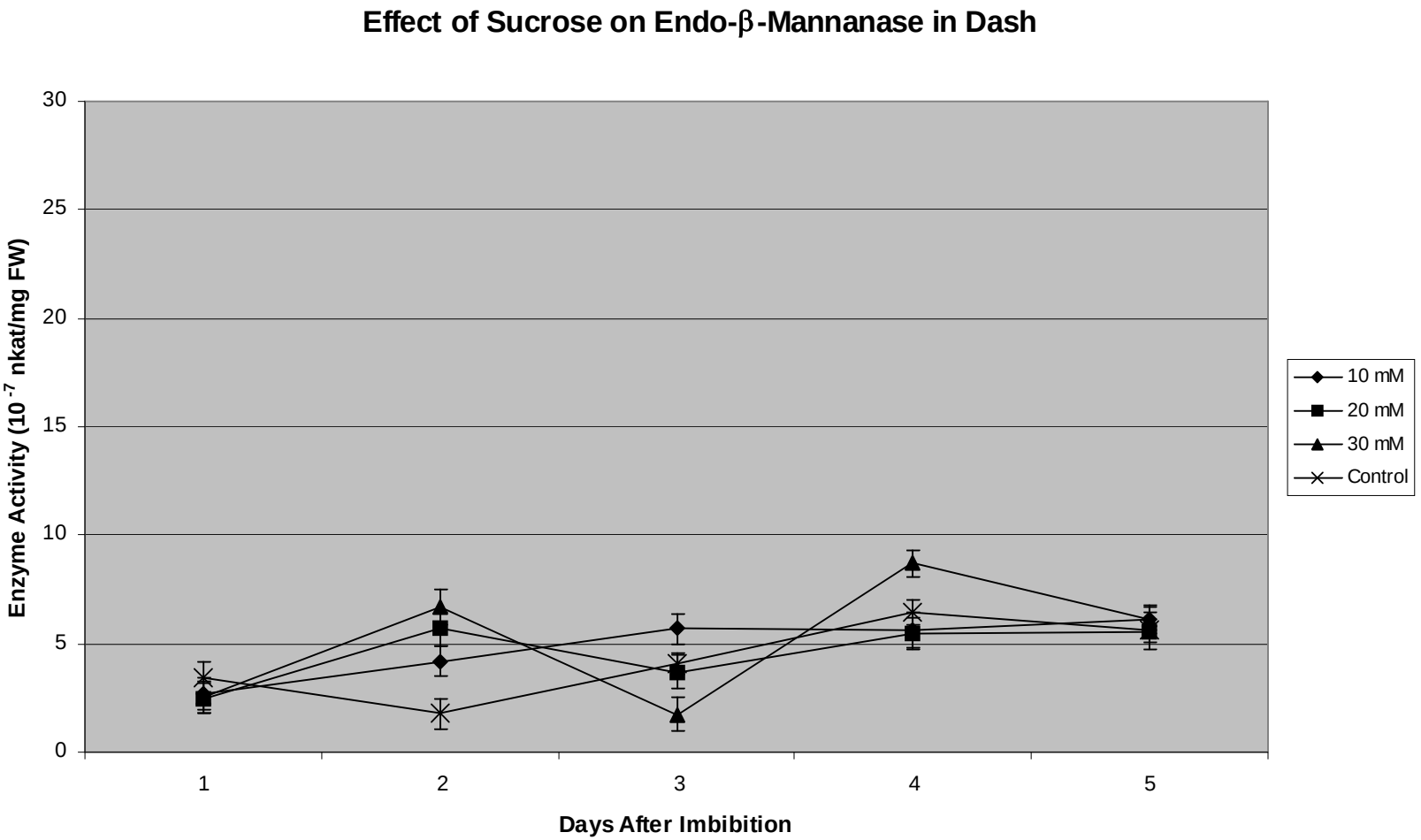

Figure 3.8. The effect of the disaccharide sucrose at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Dash barley seeds compared to a control containing sorbitol. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

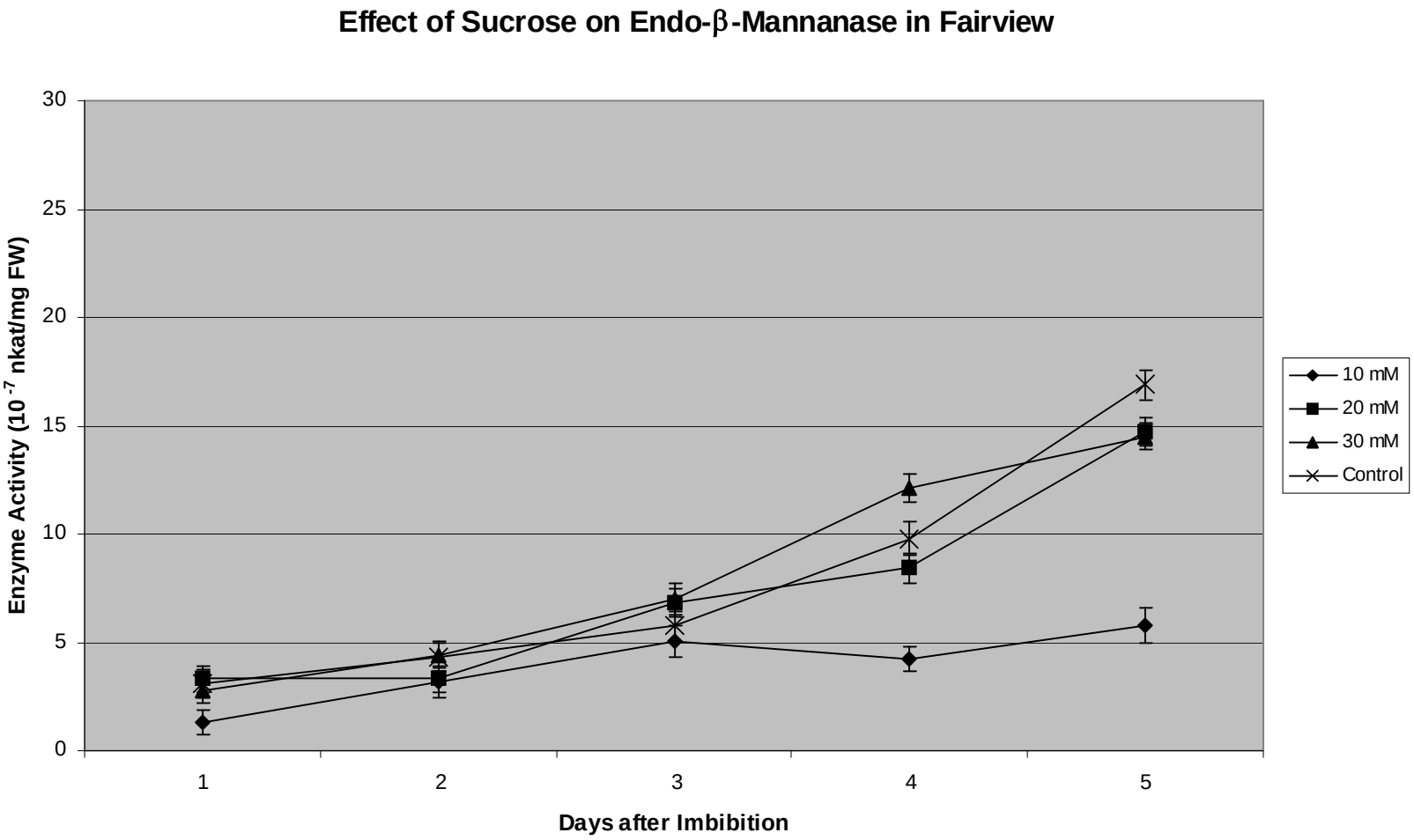

Figure 3.9. The effect of the disaccharide sucrose at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Fairview barley seeds compared to a control containing sorbitol. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

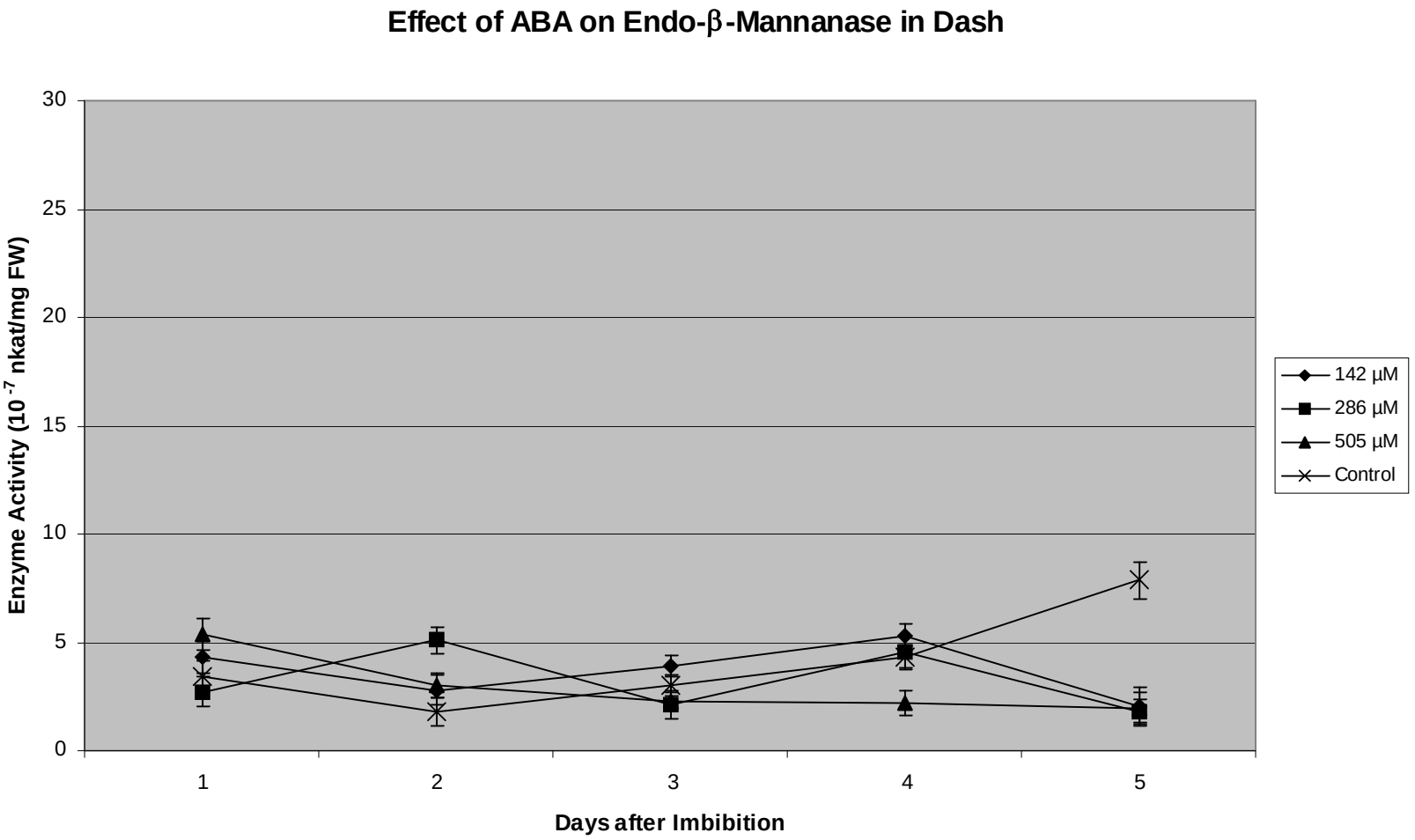

Figure 3.10. The effect of the hormone abscisic acid (ABA) at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Dash barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

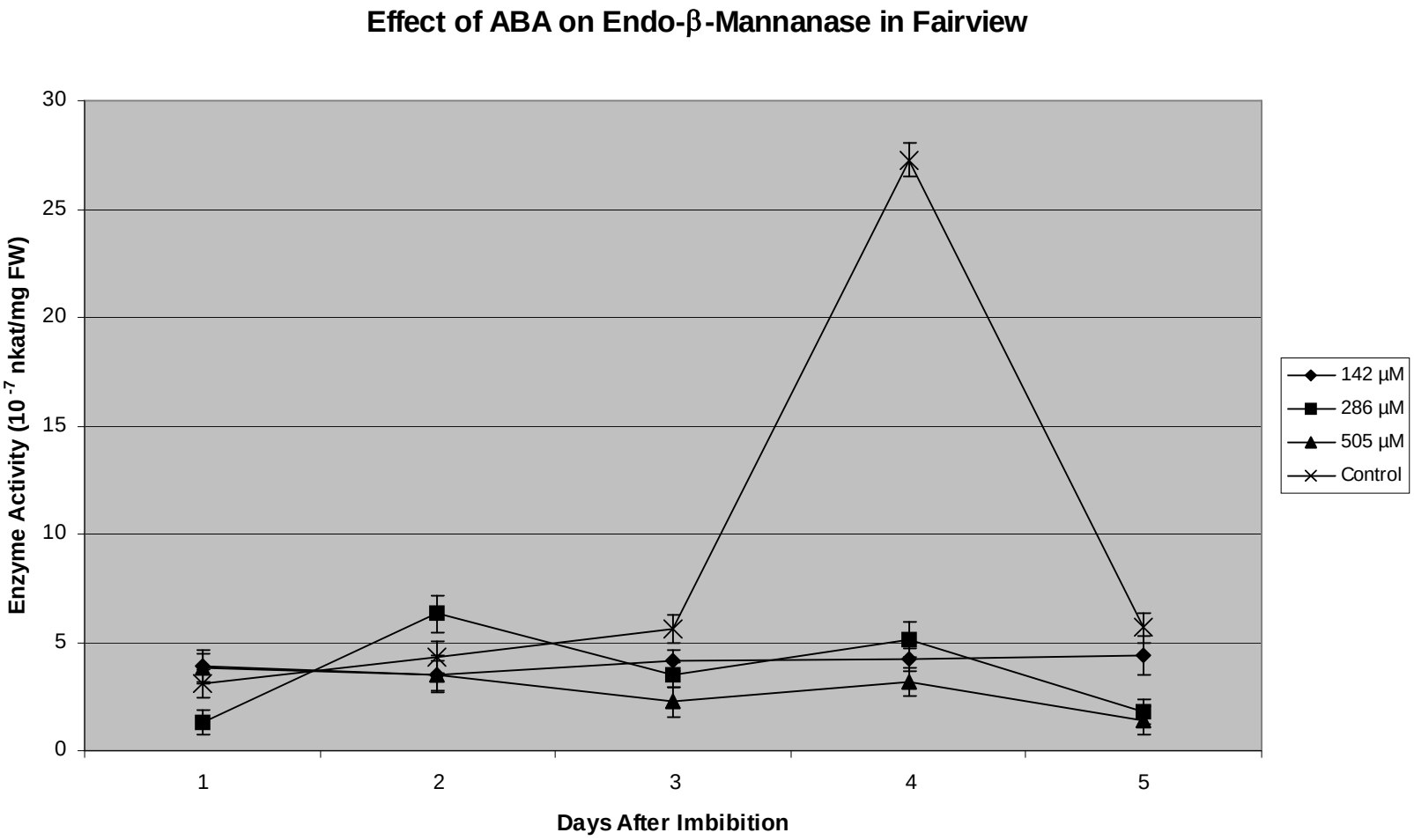

Figure 3.11. The effect of the hormone abscisic acid (ABA) three concentrations on the detectable activity of endo-β-mannanase in the extracts of Fairview barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

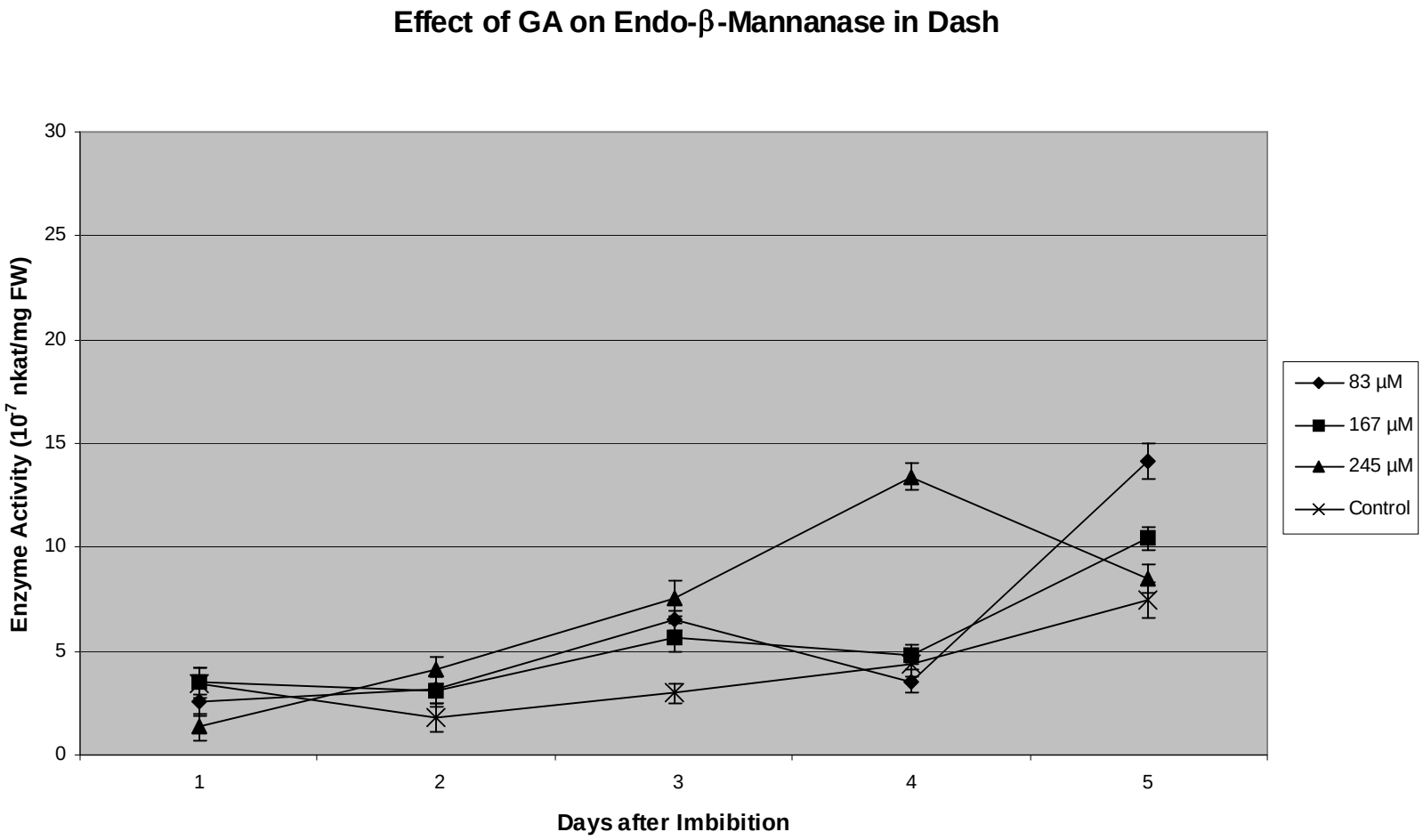

Figure 3.12. The effect of the hormone gibberellic acid (GA) at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Dash barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

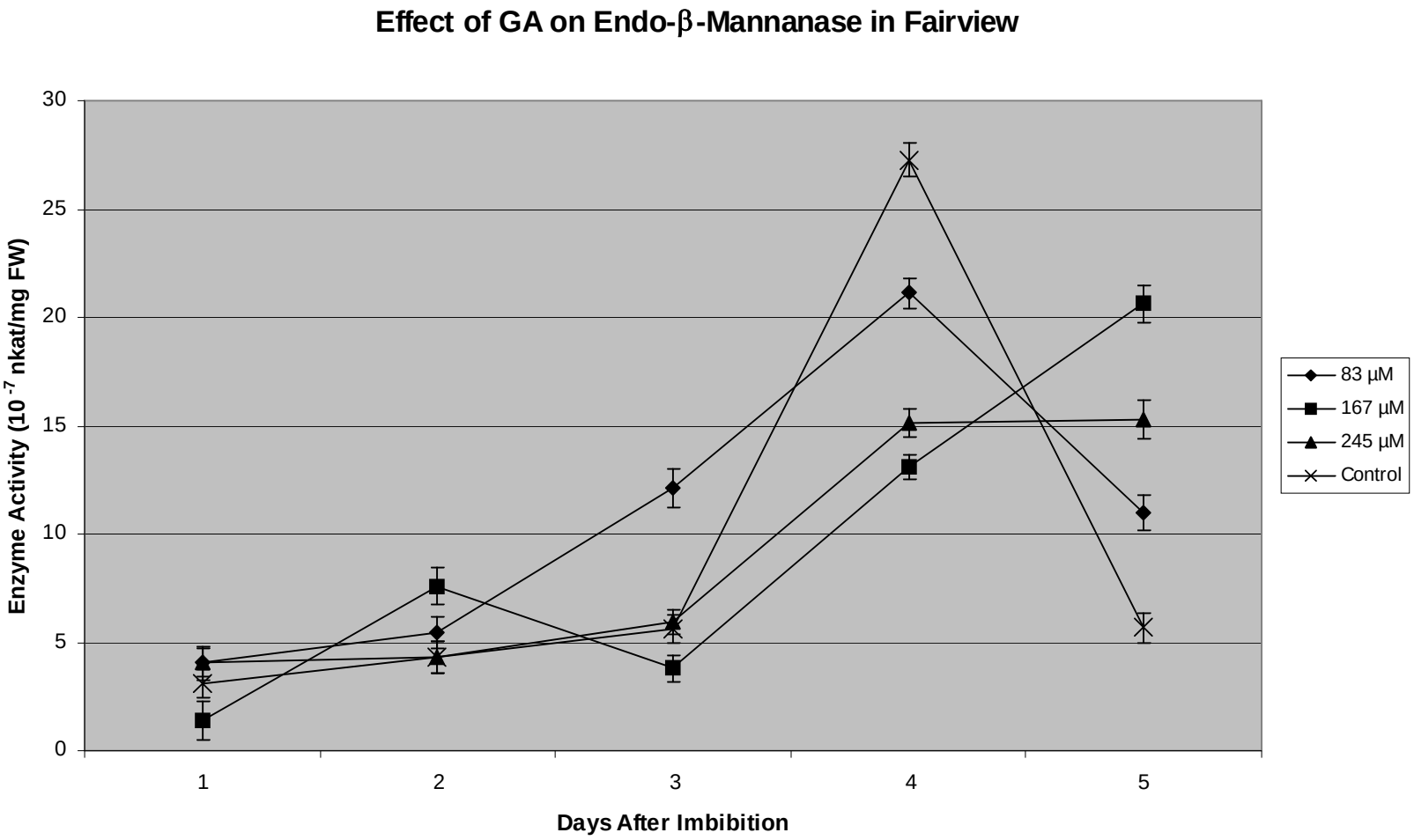

Figure 3.13. The effect of the hormone gibberellic acid (GA) at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Fairview barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

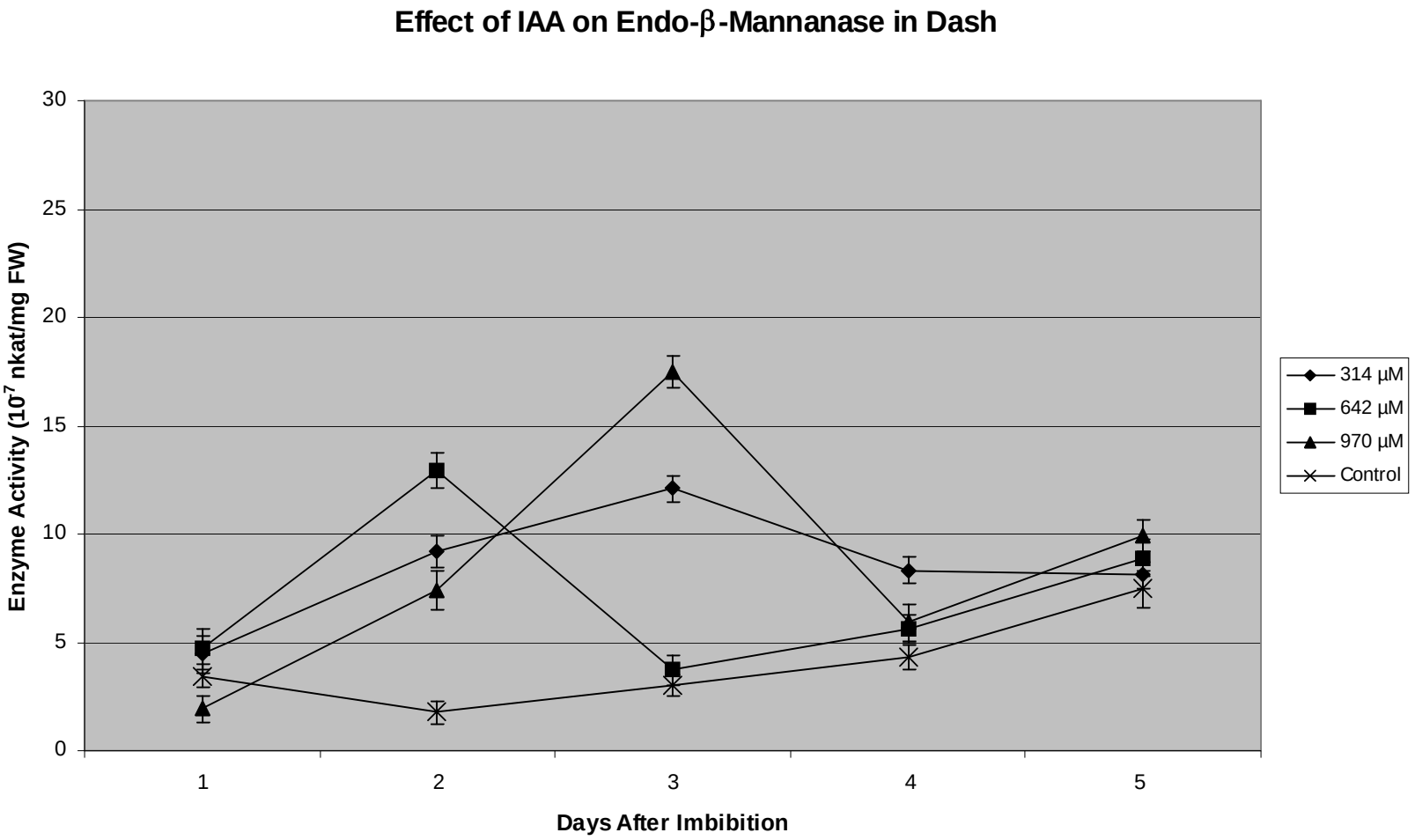

Figure 3.14. The effect of the hormone indole acetic acid (IAA) at three concentrations on the detectable activity of endo-β-mannanase in the extracts of Dash barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

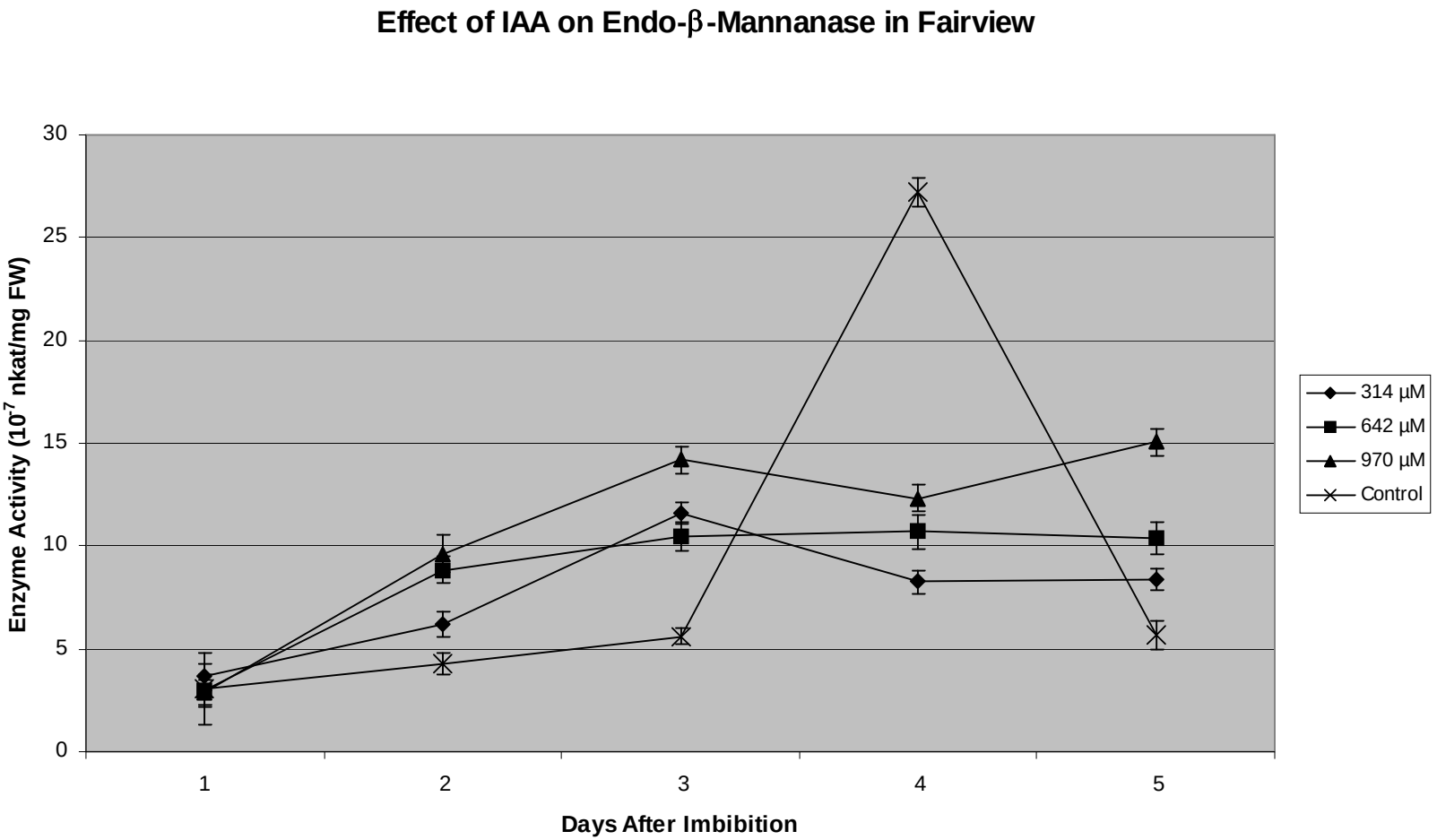

Figure 3.15. The effect of the hormone indole acetic acid (IAA) three concentrations on the detectable activity of endo-β-mannanase in the extracts of Fairview barley seeds compared to a control containing water. The points represent the mean value of 6 replicates and the error bars are the standard error of the mean.

3.6. Effects of Sugar and Hormone Treatments on Endo-β-Mannanase Activity in Isolated Endosperms

The endosperm was separated from the embryo of Dash and Fairview seeds. The isolated endosperms were incubated at 26°C for 72 hours before extracts were prepared from them for endo-β-mannanase assays. The incubation solution surrounding the endosperm was also tested for the presence of endo-β-mannanase activity.

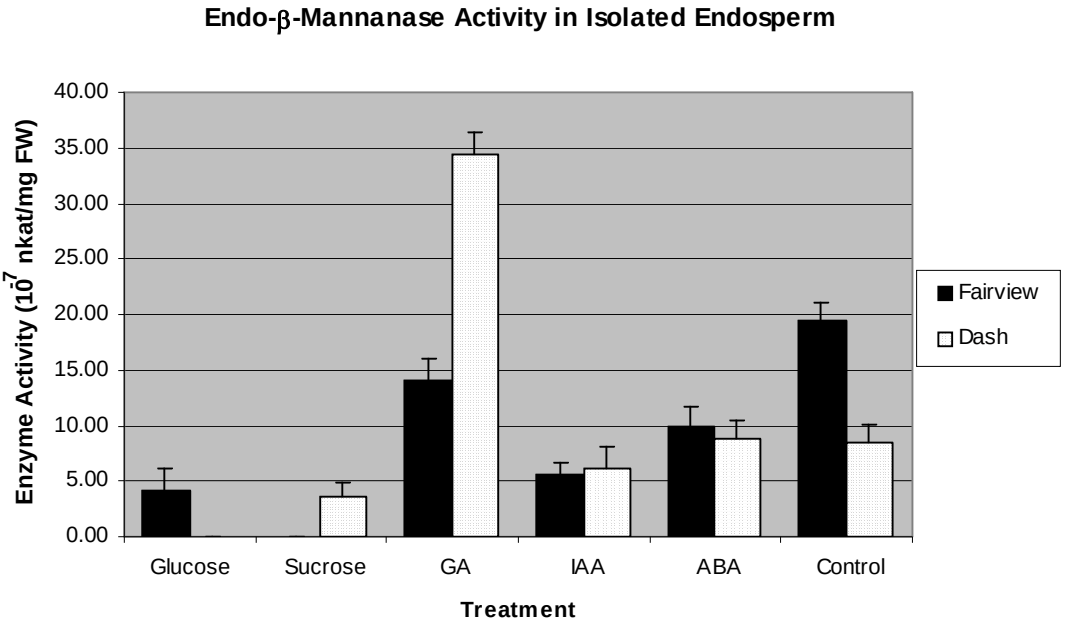

Figure 3.16. Endo-β-mannanase activity (in nanokatals per milligram of fresh weight) detected in the isolated endosperm of barley cultivars Fairview and Dash when incubated in different chemicals and distilled water (control) for 72 h at 26°C. The error bars are one standard deviation of the mean of six replicates. Concentrations used were: 20 mM Glucose, 20 mM Sucrose, 167 μM GA (Gibberellic Acid), 642 μM IAA (Indole Acetic Acid), 286 μM ABA (Abscisic Acid).

Response of cultivar Dash (Figure 3.17): a significantly higher level of detectable endo-β-mannanase activity in the isolated endosperms incubated with GA than in any other treatments (t-test, P=0.05). Isolated endosperms in the sucrose and glucose treatments showed significantly less activity than those incubated in water. The isolated endosperm after incubation in IAA or ABA showed no significant difference in the enzyme activity when compared to the control.

Response of cultivar Fairview (Figure 3.17): a significantly lower level of detectable endo-β-mannanase activity in all chemical treatments in comparison to the control.

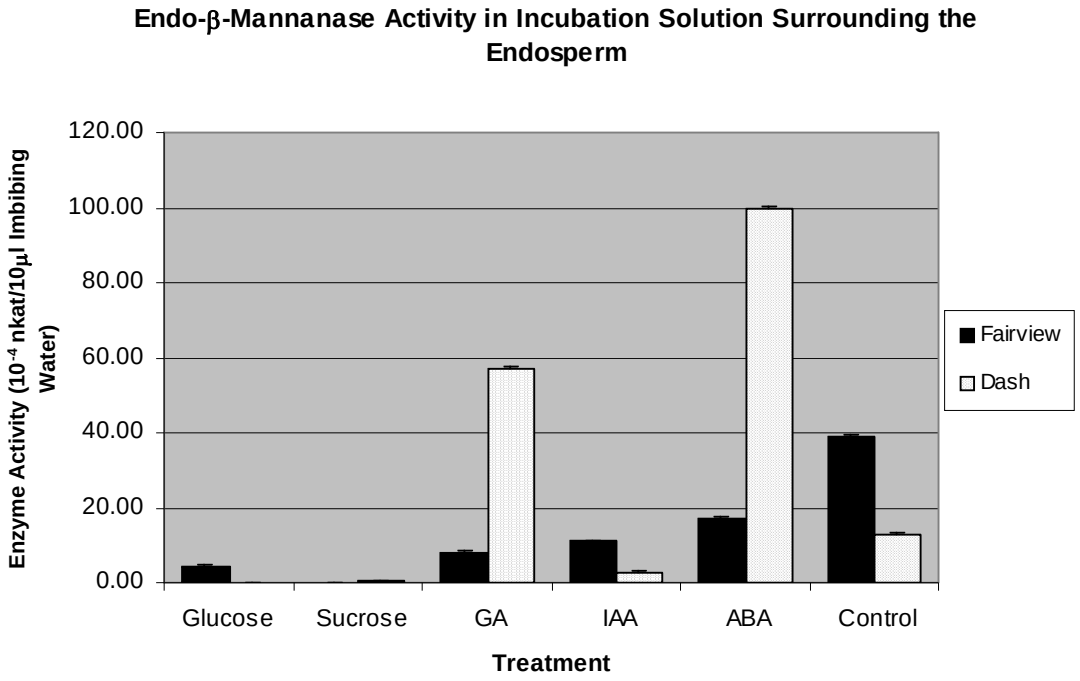

Figure 3.17. Endo-β-mannanase activity (in nano katals per 10 µL of imbibing liquid) detected in the solution used for incubation of the isolated endosperms of barley cultivars Fairview and Dash for 72h at 26°C. The error bars are one standard deviation of the mean of six replicates. Concentrations used were: 20 mM Glucose, 20 mM Sucrose, 167 µM GA (Gibberellic Acid), 642 µM IAA (Indole Acetic Acid), 286 µM ABA (Abscisic Acid).

There was significantly less endo-β-mannanase activity detected in the incubation solution from the chemical treatments of the isolated endosperms of Fairview barley when compared to the control.

There was significantly less enzyme activity detected in the glucose, sucrose and IAA conditions when compared to the control. However, the GA and ABA treatments had a very large increase in detectable activity. Of note in the GA and ABA conditions was the presence of fungal or bacterial contamination. The imbibing fluid was off-colored, no longer clear as it was initially, and the viscosity had increased dramatically compared to the water-like viscosity of the other conditions. Therefore, the significantly higher enzyme activity detected in the imbibing fluid of these conditions may not be indicative of the condition, but of the presence of an

endo-β-mannanase producing contaminant. This is also a likely explanation of the variation observed in earlier results.

3.7. Mechanical Strength

3.7.1. Effect of Exogenous Enzyme

The addition of an exogenous supply of endo-β-mannanase from *A. niger* did not result in any significant change in the mechanical strength of germinated barley seeds of either cultivar (t-test, P=0.05) when compared to seeds not treated with *A. niger* endo-β-mannanase (Figure 3.19), as measured on an Instron universal testing machine at the New Zealand Institute of Crop and Food Research using a self-devised method.

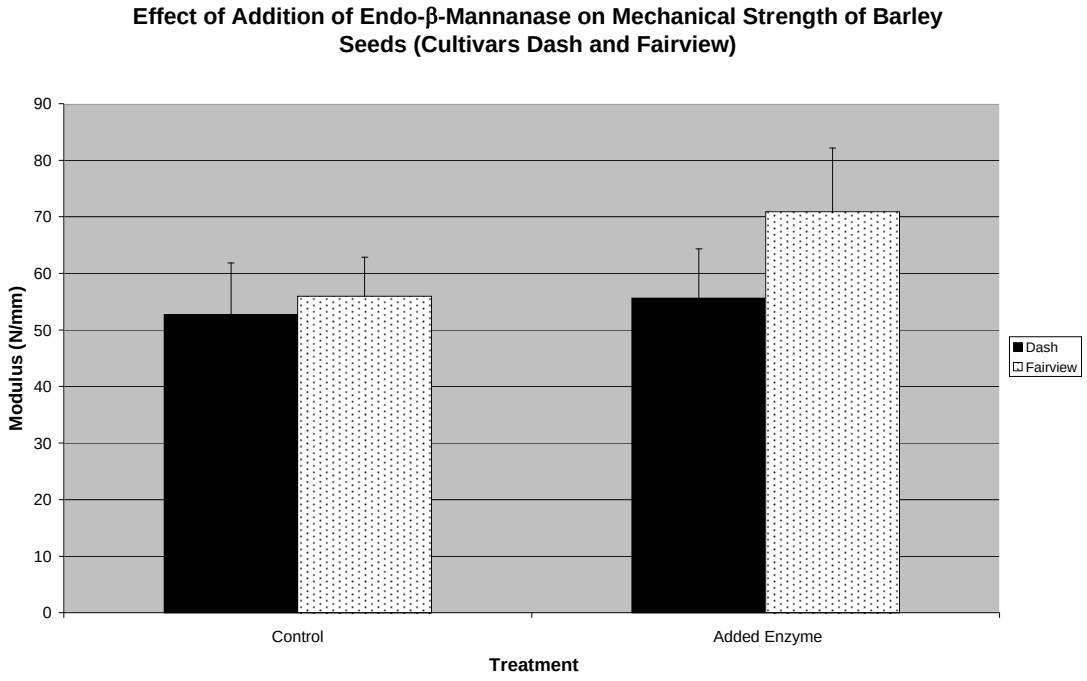

Figure 3.18. Effect of the addition of endo-β-mannanase from *Aspergillus niger* to barley seeds. The change in mechanical strength after 48 h of incubation with or without the enzyme solution is measured by the modulus: newtons of force required to crush the seed by one millimetre. The error bars represent standard error of the mean of twenty replicates.

The large variation in the experimental data results in very large error bars. When measured with callipers there were no significant differences in the length, width or height of the seeds used in each treatment (data not shown). Therefore the

large variation in these results is likely due to the natural variation and distribution of structural polysaccharides among the seeds. It is unlikely that the Instron and its accompanying software were the source of the variation as the machinery was well maintained and regularly serviced.

3.7.2. *Change in Mechanical Strength over Time*

Despite the very large variation in experimental data at day 1 and day 2, there was a significant decrease in mechanical strength of the barley seeds during germination (t-test, $p=0.05$) in both cultivars Dash and Fairview (Figures 3.20 and 3.21). There was a significant negative correlation between the mechanical strength of the seed and its endo-β-mannanase activity: as the level of detectable activity of endo-β-mannanase increases, the mechanical strength of the seed decreases.

The large variation in the experimental data is not a result of variation in the weight, height or width of the seed. There were no significant differences in any of these parameters within the 20 seeds used for each treatment. There appears to be a lower average mechanical strength observed in Fairview seeds when compared to Dash seeds. This correlates well with the initial results of section 3.1, which showed Fairview to have a higher level of detectable enzyme activity. However, the difference in the mechanical strengths of Dash and Fairview on day 1 is not statistically significant.

While the decrease in mechanical strength correlates with an increase in endo-β-mannanase activity, it does not follow that endo-β-mannanase is the sole cause of the seed weakening. There are many other enzymes targeting many other structural polysaccharides, which may be contributing to the observed decrease in mechanical strength over time.

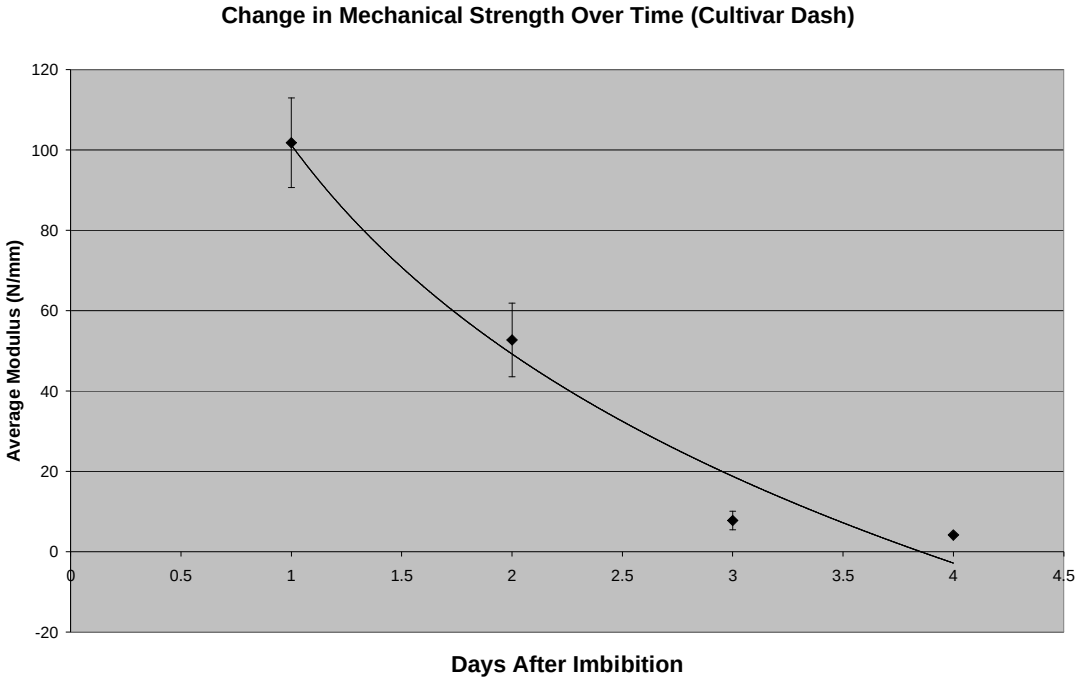

Figure 3.19. Change in mechanical strength of Dash barley seeds during germination, measured as the average force (N) to crush the seed by one millimetre. The error bars represent one standard error of the mean of 20 measurements. The last data point's error was too small to visually represent on this graph.

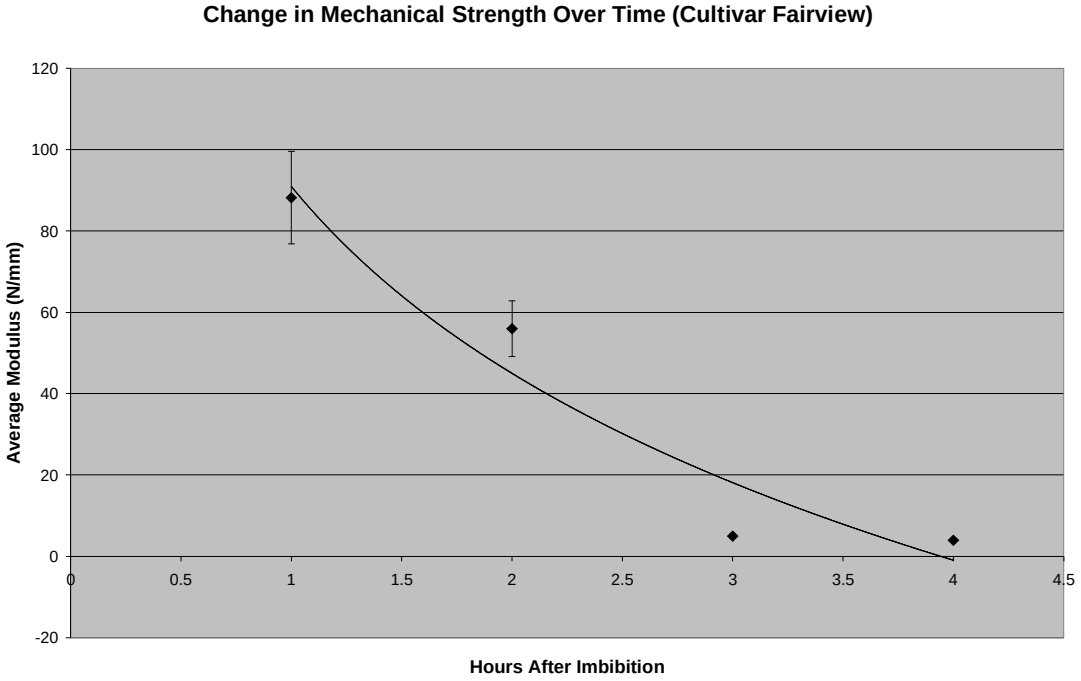

Figure 3.20. Change in mechanical strength of Fairview barley seeds during germination, measured as the average force (N) to crush the seed by one millimetre. The error bars represent standard error of the mean of 20 measurements. The 3 and 4 hour data points' errors were too small to visually represent on this graph.

3.8. Spectrophotometric Assay

The initial step in developing this assay was to determine an absorbance level at which to perform the spectrophotometric reading. A variety of solutions containing varying concentration of substrate, Congo red dye and buffer solution were run through a spectrophotometer and their absorbance spectra were compared to that of the same solutions with the addition of endo-β-mannanase (Figure 3.22). The spectra showed that there were peaks in absorbance at 350 nm and 490 nm, as well as a difference in absorbance between a blank consisting of buffer/substrate/dye and a solution of buffer/substrate/dye/enzyme. For reference, the buffer was McIllvaine buffer at pH 7, the substrate was 0.1% locust bean gum, the dye was 1% Congo red dye solution and the enzyme was a dilution of A. *niger* endo-β-mannanase. From the results of this absorbance spectrum, wavelengths of 350 nm and 490 nm were used for the development of a spectrophotometric assay of endo-β-mannanase activity.

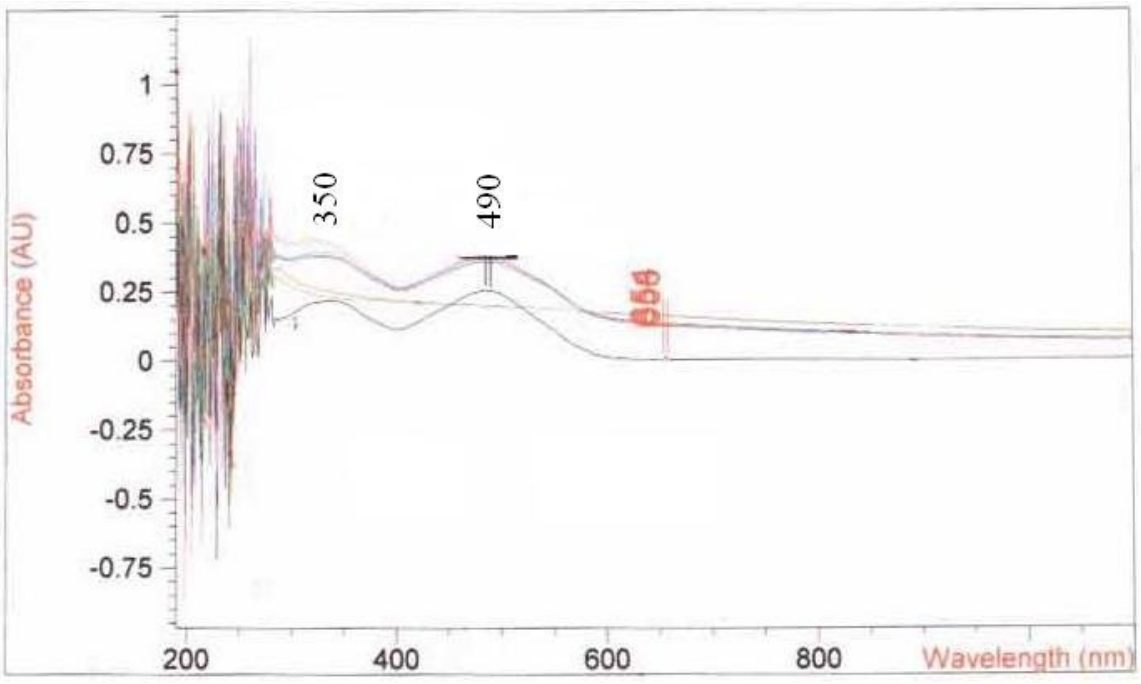

Figure 3.21. This absorbance spectrum shows two major peaks in absorbance at 350 nm and 490 nm for all solutions run except the distilled water blank (line between the peaked lines) which consists of dH_2O. The lowest of the lines represents a solution of buffer/substrate/dye, and the cluster of lines above it represent solutions of buffer/substrate/dye/enzyme (various dilutions).

A range of different concentrations of substrate was trialed ranging from 0.1% up to 5% (w/v) of locust bean gum. Observations regarding the effect of the enzyme

on the different solutions were made. These observations were purely qualitative as the aim was not to collect quantitative data but to select a substrate level that presents visible changes in either viscosity or colour during incubation with the enzyme.

The 0.1% and 0.2% substrate levels were low-viscosity solutions from the start and showed no visible change in viscosity during incubation. The 0.5% and 1.0% substrate solutions showed a noticeable change in viscosity, as well as a change in colour. The 2% and 5% solutions also showed a definite change in viscosity and colour. However, the solutions were so viscous that pipetting was very difficult and accurate amounts could not be reliably dispensed. With these qualitative observations in mind, 0.5% and 1.0% substrate concentrations were used for the next steps in developing this spectrophotometric assay.

The length of time to incubate the solution with the enzyme was also determined. Initially a 24 hour incubation time was trialed. However, after 24 hours incubation, the viscosity of all the solutions was the same, even with very small quantities of enzyme present. This is indicative of the enzyme hydrolyzing all of the substrate. 24 hour incubation was therefore deemed unsuitable as there would be no detectable differences in absorbance if all solutions contained completely hydrolyzed substrate. A one hour and a five hour incubation time were then trialed. The five hour incubation time had similar results to the 24 hour incubation and hence similarly unsuitable. The one hour incubation time showed large differences in viscosity, ranging from very viscous (control solution and very low enzyme concentrations) to solutions of similar viscosity to water (high enzyme concentration solutions). Therefore, in keeping with the gel diffusion assay, the substrate solution was incubated with enzyme added for 1 hour at 37°C.

The next parameter determined was the volume of 1% (w/v) Congo red dye used in the assay. Three different volumes of dye were trialed: 50 μL in 950 μL of buffer/substrate, 100 μL in 900 μL of buffer/substrate and 200 μL in 800 μL of buffer/substrate. The latter two concentrations produced very dark red solutions which required large dilution factors in order to be accurately read in the spectrophotometer. As well as requiring substantial dilution, these two dye concentrations also showed little to no variation in absorbance reading between controls and samples. 50 μL dye in 950 μL of buffer/substrate required only one dilution step to produce viable

spectrophotometer readings. The dye was left in the enzyme/substrate solution for 15 minutes, the same amount of time as in the gel diffusion assay staining step.

Once the enzyme was incubated it was transferred to the cuvettes and absorbance read at both 350 and 490 nm. There was a large amount of variation between replicates of the same enzyme concentration. With the aim of reducing variation, a second dilution step was introduced immediately before transferring the solution to the cuvette. It was hypothesized that the variation was a result of pipetting error caused by the viscosity of the control and low enzyme concentration solutions. This made the pipetting more accurate as the viscous solutions were now being easily drawn up and expelled by the pipette. A heat inactivation step was also introduced after enzyme incubation to reduce variation as it was thought that the reaction was continuing during the staining process and also in the cuvette.

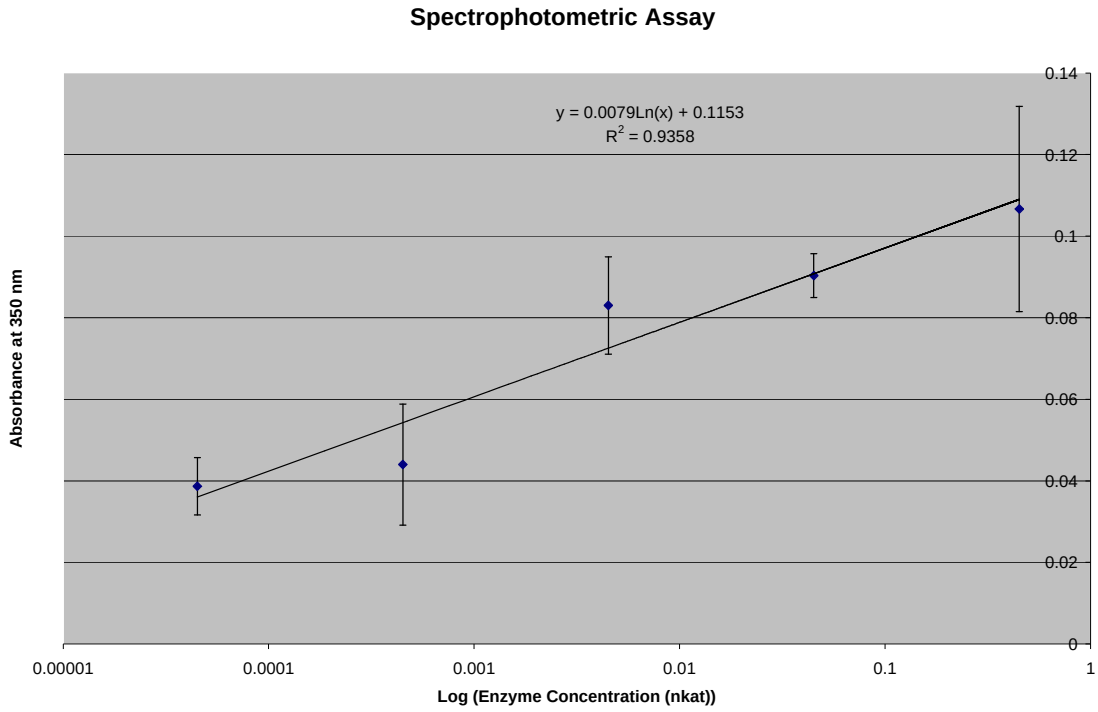

Figure 3.22. The logarithmic relationship between enzyme activity (nkat) and the absorbance at 350 nm. The points represent the mean of 3 replicates and the error bars are one standard error from the mean. The equation of the line of best fit and the R^2 value are inset.

The relationship between the enzyme activity and the absorbance at 350 nm was logarithmic (Figure 3.23). However, variation between replicates was still large and despite trying many different dilutions, incubations, dye concentrations, heat inactivation, pipetting and mixing methods the variation could not be reduced. There was a similar relationship when absorbance was measured at 490 nm but these

measurements also contained high variation. Figure 3.23 shows the best results obtained in the process of developing a spectrophotometric assay. However there were issues in replicating this result. So while it can be concluded that a spectrophotometric assay using Congo red dye is possible, there are numerous issues regarding replication and variation that would need to be addressed before this could be deemed an accurate method of endo-β-mannanase quantification.

While a spectrophotometric assay would have several advantages over a gel diffusion assay, they would still suffer from some of the same disadvantages, namely the lack of uniformity among the seeds being assayed. The natural variation among seeds, differences in percentage that germinate naturally, and differences in the seeds' internal composition as well as human error in preparation of extracts could lend a large source of error to even the most accurate assay. Future work in the development of this assay would need to reduce these sources of error in some way.

CHAPTER 4

DISCUSSION

It is useful to first summarize the major findings of this research before taking a closer look at the main points of interest and their possible biotechnological applications. The optimum assay conditions for measuring the activity of endo-β-mannanase from the seed tissue of two barley cultivars, herein Dash and Fairview, were pH 7 and 37°C.

The development of a new, faster, more accurate and more cost-effective spectrophotometric assay was promising after numerous attempts. However, due to time constraints other objectives were instead pursued.

Initially the localization of enzyme activity changes during seed germination was highest in the endosperm and seed coat regions, with very low to no detectable activity in extracts of the embryo. Later during germination, the starchy endosperm contained the most enzyme activity whilst the activity was virtually not detectable in the embryo.

The presence of sugars and hormones in the media in which the seeds were germinated had mixed effects. Glucose, gibberellic acid and sucrose all produced an increase in the detectable enzyme activity of barley seed extracts. Sucrose only produced this increase in Dash. Indole acetic acid and abscisic acid produced a decrease in detectable enzyme activity when compared to a control extract. The addition of sucrose only produced a decrease in detectable activity in Fairview.

Upon excision of the embryo, gibberellic acid promoted the production of endo-β-mannanase in Dash but not in Fairview. In the imbibition water for incubation of the isolated Dash endosperm, but not Fairview, high levels of endo-β-mannanase were also found. There was considerable variation within and between treatments in these assays, probably due to fungal and bacterial contaminants and natural seed variation as well as the effects of the treatments. This variation was particularly noticeable in the control treatments.

The addition of an exogenous supply of endo-β-mannanase made no significant difference to the mechanical strength of the germinated barley seed; however there was a strong inverse correlation between the lengths of time the seed had been imbibed and the mechanical strength. This also correlates well with an increase in endo-β-mannanase activity within the endosperm over time.

It is important to note that there was one unexpected source of variation that was not realized until the publication by Hrmova (2006). A single freeze/thaw cycle of the enzyme extract without the presence of BSA can result in loss of more than 80% of the enzyme's activity. Many of the extracts used in the assays for this thesis had been frozen prior to thawing and used on subsequent days. Therefore there will be a level of uncertainty in results as some extracts were assayed on the day of extraction while the majority of others were frozen and used the following day due to time constraints.

As there was no record of which samples were frozen and which were used fresh, it was not possible to correlate a decrease in activity with enzyme instability from freezing in the subsequent assays. Future experiments would take this enzyme instability into account and all assays would be done in the presence of BSA, which was shown to protect the enzyme (Hrmova et al., 2006), and assayed on the day of extraction.

4.1. Enzyme Localization

The data on the localization of the endo-β-mannanase within the barley seed and its changes during germination imply that in barley the site of enzyme production is the aleurone layer, which is a 4-cell thick layer of living endosperm cells located on the very outside layer of the endosperm underneath the seed coat (Bamforth, 1998).

During germination, the enzymes synthesized in the aleurone layer (Ritchie, 2000), including endo-β-mannanase, are secreted and have had time to diffuse into the starchy endosperm. As enzymes then degrade the cells closest to the aleurone layer, gaps or pores are created through which the enzymes can access the centre of the endosperm. This method of enzyme diffusion has been well documented in barley (Varner et al., 1972).

Three to four days after the start of imbibition, the level of the enzyme activity in the seed coat/aleurone layer region and starchy endosperm are approximately equal. This point of equilibrium is probably when aleurone cells begin to undergo programmed cell death (Palma et al., 2003).

It is likely that the aleurone layer was no longer producing many enzymes efficiently, which could account for the decrease in detectable endo-β-mannanase activity in the seed coat extract from day 3 onwards. This assumption is based on the observation that as time passed the physical structure of the seed was being deconstructed and utilized, and therefore the aleurone layer would not be functioning at the level it was when the seed was intact. Had the localization experiments been continued beyond day 5, we would eventually expect to see no detectable enzyme activity in the seed coat extract and the level of activity in the endosperm to plateau. Eventually the enzyme activity would become undetectable as the entire reserves in the endosperm are mobilized and consumed by the embryo. The enzymes, which are no longer required in what remains of the seed, are probably digested to free amino acids by hydrolytic enzymes. The free amino acids are reassembled by the ribosomes into new proteins which are needed by the plant in the next stage of its life cycle (Koning, 1994). At that point in time, the seedling would have emerged from the soil and be capable of photosynthesizing its own carbohydrate supply.

Lettuce has also been shown to have temporal and spatial localization of endo-β-mannanase (Nonogaki et al., 1999). Much like barley, lettuce endo-β-mannanase activity was not detected before germination and only began to develop after radicle protrusion. The increase in activity of endo-β-mannanase in the endosperm during seedling growth was parallel to the increase in concentration of the enzyme, which indicated that the increase in enzyme activity is due to the accumulation of the enzyme rather than an increase in the activity of the individual enzymes.

Nonogaki et al. (1999) took laterally cut seeds and placed them cut side down on a galactomannan gel to get a tissue print, incubated for an hour then stained the gels. Clear zones of these tissue prints showed zones of enzyme activity. Tissue prints of lettuce seeds showed that the activity initially developed in the endosperm near the embryonic axis and then spread over the endosperm tissue. These results indicated that endo-β-mannanase production in lettuce endosperm was carried out in a spatially and temporally regulated manner (Nonogaki, 1999). In lettuce it was noted there were

three isoforms of endo-β-mannanase working in the endosperm. In future work on barley it would be of interest to detect any isoforms of mannanase and observe their temporal and spatial localization.

Southern hybridization analyses of tomato mannanase indicated that the enzyme was derived from a family of approximately four genes. Expression of the genes, as determined by the presence of mRNA transcripts in Northern hybridization analyses, occurs in the endosperm of germinated seeds and not in hypocotyls, cotyledons, roots or leaves (Bewley et al., 1997). These mannanase localization findings are consistent with the results found in the localization study undertaken in barley (Figures 3.4 and 3.5).

In Figure 3.4 a small decrease in the activity of endo-β-mannanase in Dash at day 4 was found. Further repetition of this experiment would have confirmed whether this decrease was due to natural variation or a true decrease in activity. However, assuming this result was not the product of natural variation or contamination there are a few possible explanations for this activity decrease. The decrease in activity could be representative of a lag phase as the aleurone layer switches from producing one isoform to another. Dirk et al. found that barley (cultivar Himalaya) contains multiple isoforms of endo-β-mannanase which occur in spatially and temporally different sites (Dirk et al., 1995). Another explanation for this decrease is human error. It was not possible to separate, grind and extract enzymes in exactly the same way each time so it is possible that on day 4 the barley seeds were not ground in the mortar long enough or conversely were ground too long, resulting in the enzyme not being released or being mechanically damaged. Repetition of the experiment would have eliminated some of the variation caused by human error and the natural variation among the seeds and given a more accurate indication of enzyme activity changes.

The addition of a protease inhibitor was trialed in section 3.4. The results showed that the presence of a protease inhibitor was in fact inhibiting the action of endo-β-mannanase. An examination of the actions of the three compounds that were used to make up the cocktail (iodoacetate, phenanthroline and phenylmethylsulfonyl fluoride (PMSF)) may shed some light on the biochemical requirements for the barley endo-β-mannanase activity.

Iodoacetate is a chemical which blocks the thiol site on an enzyme or protease. The thiol group (-SH) is the functional group on the amino acid cysteine which is of biological significance. When the thiol groups of two cysteine residues are brought near each other in the course of protein folding, an oxidation reaction can create a cysteine unit with a disulfide bond (-S-S-). Disulfide bonds can contribute to a protein's tertiary structure if the cysteines are part of the same peptide chain, or contribute to the quaternary structure of multi-unit proteins by forming strong covalent bonds between different peptide chains (Witt et al., 2007). Therefore, iodoacetate prevents proteins from correctly folding which in turn reduces or completely removes the substrate binding capacity of the active site. However, unless there is a cysteine residue at or near the active site, iodoacetate will have little effect of a protein that is already folded as its inhibitory function depends on preventing proteins from folding.

Phenanthroline is a metal chelating protease. Some enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Phenanthroline acts by forming a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on a carbonyl group. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Moreau, 2005). As the presence of metal ions in plant proteases is quite widespread, phenanthroline makes a useful addition to a protease inhibitor cocktail.

PMSF is a highly toxic compound used in order to block serine proteases such as trypsin and chymotrypsin. PMSF is a nonspecific protease inhibitor and so will inhibit any proteases which contain serine groups in their active site. PMSF is commonly used as a protease inhibitor in studies on cannabinoids, particularly anandamide, in neural tissue (Laine et al., 2002). However, due to the non-specific nature of this inhibitor, it is useful to add to a protease inhibitor cocktail even though the proteases are of a plant source.

The action of endo-β mannanase is reliant on its ability to donate a hydrogen from a hydroxyl group to the oxygen on the β-(1, 4) bond of the substrate as shown in Figure 1.5 (Bourgault et al., 2005). As a serine protease inhibitor, PMSF targets the –OH nucleophile present in serine residues. Endo-β-mannanase, the polypeptide substrate, binds to the surface of the serine protease enzyme such that a scissile bond

is inserted into the active site of the enzyme, with the carbonyl carbon of this bond positioned near the nucleophilic serine (Rose and Di Cera, 1999). This reaction with the active site hydroxyl of endo-β-mannanase will lead to inhibition of the enzyme. Therefore it is the presence of PMSF in the protease inhibitor cocktail that is probably inhibiting the action of endo-β-mannanase in the extracts. In future work, it would be of interest to try different combinations of protease inhibitors, particularly with the omission of PMSF, and observe if there is a significant change in the level of enzyme activity.

Alternatively, Bourgault et al. (2002) found that the addition of another protein such as BSA or gelatin protected the enzyme from proteolytic degradation. The addition of BSA to the extraction buffer led to a 45% increase in the observed enzyme activity. Another protein, Rubisco, resulted in 105% increase in activity when added to the extraction buffer at a 1:4 dilution. The increase in endo-β-mannanase activity in the presence of these proteins is probably because these proteins are provided at higher concentrations and are therefore acting as an alternative substrate for proteases (Bourgault et al., 2002). In future experiments, it would be highly beneficial to include a protective protein to the extraction buffer either in lieu of or in conjunction with a more effective protease inhibitor cocktail.

4.2. Action of Sugars and Hormones

The level of endo-β-mannanase activity in response to the addition of gibberellic acid (GA) or abscisic acid (ABA) was monitored. There was an increase in the level of enzyme activity when GA was added but a decrease when ABA was added. Further repetition of the assays would rule out any anomalies in these results as well as lessen the variation in results due to natural variation among the seeds. However, this response pattern is analogous to that found in the regulation of amylase production in Lucerne (*Medicago sativa* L.*)* (Kepczynska et al., 2006).

The interaction between GA and ABA is an important factor controlling the germination of a seed. During the germination of cereal grains, the embryo secretes GA to the aleurone layer where it promotes the expression of amylase as well as other hydrolytic enzymes, possibly including endo-β-mannanase. Expression of these genes

is blocked by ABA during seed development in dormant seeds, and in seedlings under unfavorable germination conditions (Gomez-Cadenas et al., 2001).

The promoter sequences, important for the GA induction and ABA suppression of α-amylase genes, have been studied extensively (Skriver et al., 1991). There are three regions: box1 (amylase box), GARE box (gibberellin response element), and pyrimidine box are found in the promoters of all GA-inducible α-amylase genes. The characterization of these promoters has been the basis for the discovery of trans-acting factors involved in the transduction of GA signals in barley aleurone cells (Gomez-Cadenas et al., 2001). In future work, similar characterizations of the barley seed endo-β-mannanase genes would be required for comparison with the regulation of the α-amylase gene.

The sensitivity of seeds to ABA is useful in nature as it promotes reserve accumulation, developmental arrest and seed dormancy. However, in terms of a germination experiment, the presence of ABA is inhibitory and undesirable. When a seed encounters favorable conditions, the production of GA overrides the ABA's action and the seed begins to germinate. These antagonistic interactions between ABA and GA in regulating reserve mobilization in cereal aleurone layers are well documented (Bradford et al., 2003).

In the presence of exogenous sucrose there was very little difference between the seeds imbibed in sucrose solution and those imbibed in dH_2O. There was, however, a more marked difference between experimental and control seeds in the glucose treatment. In the presence of exogenous glucose the level of detectable endo-β-mannanase was low for the first few days before it soon overtook the control (osmotic control containing mannitol). It was also observed that the seeds in the glucose treatments showed signs of stunted germination, possessing smaller roots and shoots than those of control seeds. Previous studies have shown that seeds will germinate even on very high concentrations of glucose or sucrose but the germination can be significantly delayed and seedlings fail to form or extend true leaves, cotyledons or extensive root systems (Gibson, 2005). The observed stunted or delayed germination in barley seeds is similar to those made in studies of germinating *Arabidopsis* seeds which have been exposed to exogenous glucose. A delay in *Arabidopsis* seed germination was observed at a glucose concentration as low as 0.5%

with the delay increasing with increasing glucose concentrations. While intermediate glucose concentrations (1.5–3%) dramatically delay seed germination, similar concentrations of mannitol or sorbitol have very little effect, indicating that the effect of glucose is not simply osmotic (Yuan et al., 2006).

It has been suggested that glucose at intermediate and high concentrations may delay germination by slowing the decline of endogenous ABA concentration (Gibson, 2005). High concentrations of glucose increased the accumulation of ABA in seedlings, and many sugar-insensitive *Arabidopsis* mutants selected for post-germination seedling developmental abnormalities have been found to be allelic to genes involved in ABA biosynthesis or ABA signaling pathways (Gibson, 2005). It remains to be investigated if this mechanism also operates in glucose inhibition of barley seedling growth shown in the present study.

When mannose, a stereoisomer of glucose is used in place of glucose, delayed germination occurs. As mannose is a good substrate for hexokinase (HXN), mannose might delay germination via a HXN-dependent pathway.

3-O-Methylglucose, a glucose analogue, has a similar effect on delaying the timing of germination as glucose and is not a good substrate for HXN. As 3-O-methylglucose has a very similar effect on germination as glucose, and does not inhibit via the HXN pathway, it is thought that delay or inhibition of germination is due to glucose inhibition of a HXN-independent pathway in *Arabidopsis thaliana* (Pego et al., 1999). In future work, these experimental approaches could be applied to examine the glucose-inhibited barley seedling growth.

It is likely that barley early seedling growth is controlled by a similar mechanism as in *Arabidopsis* seeds. Sections 3.5.1 and 3.5.3 show a delayed germination due to the action of glucose as well as almost no germination at all in the presence of ABA. This shows that ABA inhibits germination and that the presence of glucose may be slowing the decline of endogenous ABA and therefore delaying germination. These results are very similar to the results found in the *Arabidopsis* studies carried out by Gibson (2005).

For the purpose of malting, the addition of glucose or ABA to the malting floor would not be advised. The presence of the glucose would delay the germination of the seed, which would in turn delay the production of essential enzymes such as amylases and increase the length of time required to produce the malt.

In section 3.6, it was shown that isolated endosperms of Dash seeds which were incubated in GA contained a higher level of detectable enzyme activity than those of control seeds (imbibed in dH_2O). This is consistent with previous studies on α-amylase production by isolated endosperms of cereals, indicating that the embryo might normally control production in the aleurone layer via the GA signal. For example, when maize endosperms were incubated in the presence of 0.035 μg GA per endosperm, they showed a marked increase in the soluble sugar content when compared to a water-incubated control. The increased sugar content was indicative of an increased level of α–amylase and it was proposed that the presence of exogenous GA, which was normally produced by the embryo, was stimulating the aleurone cells to synthesize hydrolytic enzymes (Ingle and Hageman, 1965). It was also shown that there was a decrease in the soluble sugars, meaning a decrease in the synthesis of hydrolytic enzymes, when there were higher than optimal GA concentrations.

The enzyme extracts from the Fairview endosperms showed less detectable activity in comparison with extracts of isolated Dash endosperms. The inhibition of enzyme synthesis in the presence of too great a concentration of GA may present one answer as to why this occurred. Fairview, the malting barley, may contain higher endogenous levels of GA than the feedstock barley Dash. The endogenous level of GA combined with the added exogenous supply of GA may be pushing the total GA concentration too high and therefore inhibiting the production of enzymes (Riley, 1987).

It is very likely that endo-β-mannanase is secreted from the excised endosperm into the imbibition media. In the presence of GA, the water in which the Fairview endosperms were incubated contained a very high level of enzyme activity. This result lends weight to the hypothesis that GA could stimulate endo-β-mannanase production, although the concentration of GA added would need to depend on the endogenous level of GA in the seed variety: less exogenous GA would be needed in Fairview than in Dash. It also shows that the enzyme is secreted from the aleurone cells to the endosperm and is not produced and used only within the aleurone cells themselves. Previous studies involving cereal grain protoplasts haves shown that barley, wheat and oats make particularly good models for enzyme secretion and hormone regulation as they still retain their sensitivity to GA and ABA (Hillmer et al.,

1993). It would be interesting to apply this barley protoplast approach to study GA-ABA interactions as far as endo-β-mannanase regulation is concerned.

Theoretically we should see very little to no enzyme secretion into the imbibing fluid in the condition containing ABA, as this hormone should be inhibiting germination and therefore inhibiting enzyme synthesis (Kucera et al., 2005). However, in Figure 3.18 we see a very high level of endo-β-mannanase being detected in the imbibing liquid containing ABA. This was most likely due to contamination. There are many fungi (e.g. *A. niger* and *Sclerotium rolfsii* (Gubitz et al., 1996)) and bacteria (e.g. *Bacillus subtilis* (Khanongnuch et al., 1998), *Trichoderma reesei* (Sabini et al., 2000)} which produce large amounts of endo-β-mannanase and are able to secrete this enzyme into the surrounding media. The presence of contaminating fungi or bacteria could account for much of the variation seen in the results throughout this thesis. This could be remedied by surface sterilizing the seeds with a sodium hypochlorite solution before germination (Miché et al., 2001).

Overall these results could be applied to the brewery industry. Already many breweries add GA to the malting floor to stimulate germination. However, it would be useful to assay the malting barley for endogenous GA levels to ascertain the concentration of exogenous GA to add. The endogenous level of GA can be quantified by extracting and chromatographically purifying GA and its precursors (GA_{20} and GA_{19}), then quantifying via gas chromatography selected ion monitoring (GC-SIM) against an internal standard (Zanewich, 1993). This was not done in the course of this thesis due to time constraints.

The addition of too much GA will lead to inhibition of germination (Riley, 1987) and therefore less modification of the endosperm, leading to less free sugar for yeast fermentation. The ability of barley aleurone to secrete hydrolytic enzymes into the surrounding media may also be of use in the malting process. If the malting barley had the embryos removed or the endosperms exposed in some manner, the addition of GA to the floor would increase the rate of production and secretion of endo-β-mannanase. This will lead to a source of exogenous endo-β-mannanase for surrounding seeds. However, for this to be effective, the surrounding seeds would

need to have an exposed section of endosperm. This shows there is very little effect of exogenous enzyme on a whole seed (Figure 3.19).

4.3. Mechanical Strength

There was no significant difference between the seeds with or without an exogenous source of endo-β-mannanase (Figure 3.19). This is not surprising as the husk is designed to protect the seed from external attack. In nature, the husk would protect the seeds from myriad hazards ranging from environmental conditions (rain, snow, UV light) to insect and bacterial attacks (mechanical attack from insects and enzymological attacks from bacteria and fungi) (Hornsey, 1999). The protective layers of many plants serve a similar function to the husk of the barley kernel.

With the rising cost of oil, biofuels are being explored and the USA seeks to replace 75% of fossil fuels with alternative fuels by 2025 (Hahn-Hagerdal et al., 2006). The first step in the production of an ethanol-based biofuel is the breakdown of lignocellulosic bioproducts into sugars. Much like the experiment in which an exogenous supply of endo-β-mannanase was applied to barley seeds, enzyme-catalyzed conversion of cellulose to glucose is slow unless the biomass has been subjected to some form of pretreatment. This pretreatment step is crucial to give high yields and make this process commercially viable (Hahn-Hagerdal et al., 2006).

In keeping with the methods used to treat solid biomass for the production of biofuel, it may be possible to add a pretreatment step to the barley malting stage. Some of the pretreatments used on softwoods and corn stover, common ingredients in the biofuel market, are steaming the biomass with the use of acid catalysts (H_2SO_4 or SO_2). This pretreatment opens pores in the biomass which allow enzymes to more easily enter and carry out their function (Hahn-Hagerdal et al., 2006). While this method would not be applicable in the brewery industry, it gives a starting point to develop a pretreatment process for malting barley which will allow an exogenous enzyme source to work effectively. Alternatively, simply crushing the seeds would also be a useful pre-treatment step. While it would not create quite the porous environment that steaming with an acid catalyst would, it would still allow enzymes to more easily enter the seed and carry out their function.

There is a very clear correlation between the force required to crush the seed and the length of time the seed has been imbibed (Figures 3.19 and 3.20). This trend also correlates well with that of an increase in detectable enzyme activity. It is important to note however that correlation does not mean causality. While there is a strong correlation between an increase in detectable endo-β-mannanase activity and a decrease in the mechanical strength of the seed, it does not necessarily mean that endo-β-mannanase is the sole cause of the result. This increase in endo-β-mannanase activity is in keeping with a documented increase in many other hemicellulases and amylases (Nonogaki et al., 1999). Ideally, we would want to know the exact composition of the barley seed coat and endosperm as well as how the activities of the other enzymes change over time. This would allow us to more easily assign a causative agent to this decrease in mechanical strength. It is most likely that the combined increase and action of many enzymes as well as the physical effect of soaking the seed in water are causing the mechanical strength decrease and we cannot definitively say how much of a contribution endo-β-mannanase is making.

As an endohydrolase of the important structural compound mannan, it is likely that endo-β-mannanase contributes more to the decrease in mechanical strength in plants and seeds which have a high level of mannan in their cell walls (coffee, locust bean gum, guar gum etc) (Regaldo et al., 2000). The endosperm cell walls contain a variety of carbohydrates, such as mannans, xylans and glucans (Wilson et al., 2006). Therefore determining the percentage of mannan in the cell wall of barley kernels would be a good start to determine how much of an effect the addition of endo-β-mannanase is likely to have. In plants or seeds with a relatively high mannan concentration, increasing the endogenous level or increasing the production of endo-β-mannanase is likely to significantly contribute to the decrease in mechanical strength.

In terms of the brewery industry, it would be interesting to know the composition of the cell wall of the variety of barley being used. There is extensive literature available on the composition of the barley cell wall and the methods used to obtain this information (Downie et al, 1994), (Jacobsen, 1971), (Carpita, 1996), (Buckeridge, 2000). Previous studies have shown that barley has a relatively high level of endo-β-mannanase activity when compared to a variety of other seeds (Downie et al., 1994). This is indicative of a reasonably high percentage of mannan

present in the cell walls. Therefore, up-regulating the production of endo-β-mannanase within barley, or subjecting barley to a pretreatment process to allow for an exogenous enzyme source to be effective, may cause a significant decrease in the mechanical strength of the seed.

The result of decreasing the mechanical strength of the seeds via degrading mannan in the cell wall may lead to an increased quality of beer. During malting, the seed is softened due to the action of amylase on the starch endosperm. When the seed reaches a desired 'softness', which is indicative of enzymatic modification of the endosperm, the barley is kilned and milled (Hornsey, 1999). In plants such as lettuce and tomato, it is believed that mannan functions by increasing the hardness of the endosperm (Buckeridge et al., 2000). Therefore, if the desired softness in barley could be more quickly achieved due to the degradation of structural mannan, less starch will be degraded and used by the embryo. This will leave more starch intact for yeast during the fermentation process. With more starch available there will be a higher yield of alcohol per unit weight of malted barley, which will make the process more cost effective to the brewer.

4.4. Spectrophotometric Assay

The development of a spectrophotometric assay was attempted during this research project. While the assay showed promise there were problems in replicating the experiment. The data from this experiment showed a definite increase in absorbance at 350 nm as the enzyme concentration in the solution increased. As the enzyme concentration increased, the percentage of substrate hydrolyzed also increased and, due to the hydrolyzed substrate's inability to bind the dye, the absorbance was increased. It was hypothesized that the bound Congo red dye had a lower absorbance reading than the unbound Congo red dye, so the change in absorbance could be directly related to the amount of substrate hydrolyzed and hence the activity of the enzyme.

The experiment was repeated many times with a largely different absorbance being read for each replicate. While this difference was sometimes a result of adding in a new dilution step or trying a longer incubation, there were often times when the

same conditions produced vastly different results. Despite the different absorbance readings, the general trend of the data was always the same: an increase in absorbance as the enzyme's concentration was increased.

The consistent pattern of increasing absorbance with increasing enzyme concentrations is indicative of the assay being feasible. There are still many optimization steps that need to be trialed. A different range of substrates could be trialed such as guar gum or coffee bean waste. These are both excellent substrates for endo-β-mannanase as they contain a high percentage of galactomannan (Regaldo et al., 2000). It is possible that a locust bean gum, which was optimal for the gel diffusion assay, is not optimal for a spectrophotometric assay. If a new substrate is trialed, the process of trialing incubation times, dye concentrations, buffer volumes, buffer pH, incubation temperatures and other assay related parameters would need to be reassessed. It would also be useful to look at sources of experimental error. The presence of pipetting, measuring, mixing and timing errors may all be contributing to the variation observed in the assays.

The optimum wavelengths that the absorbance spectra suggested were 350 nm and 490 nm. These wavelengths are in the ultraviolet and violet parts of the spectrum so the plastic cuvettes may have interfered with the readings. UV cuvettes were trialed and, while there was a difference in the absorbance read, the variation between replicates remained. Future work will need to concentrate on using the UV cuvettes if new substrate absorbance spectra yield similar wavelengths for the spectrophotometric assay. The data presented in section 3.8 were obtained using normal (non-UV) cuvettes.

One final theory held as to the cause of the variation is the difficulty in accurately drawing a viscous solution up with a pipette. Further trials into different concentrations of substrate, as well as adding in dilutions steps, needs to be carried out.

4.5. Future Work

This research has raised some questions and future work could be carried out based on these results. In the localization assays it would be interesting to continue the time course until there is no endosperm left to observe the decline of the detected enzyme in the various seed parts. This would lend weight to the suggestion that due to programmed cell death of the aleurone cells, there will be a maximum concentration of endo-β-mannanase produced and it will eventually decrease as the endosperm is hydrolyzed and decays. It would also be of interest to trial a larger range of pH for the experiments. The pH range for the commercial enzyme is 3-8 so using each of these pH separately and then testing around the most suitable one – for example, if pH 7 is most suitable, test pH 6.7, 6.8 . . . . 7.4, 7.5. This will yield the optimum pH for barley endo-β-mannanase. Hrmova et al. (2006) found pH 5 to be the optimum pH for carrying out their assays. It would be of interest to repeat the experiments of this thesis at pH 5 to compare with the results obtained by Hrmova. It would also be of interest to ascertain whether the different varieties of barley used in experiments by Hrmova (2006) have the same optimum pH as the Dash and Fairview.

The work of sugars and hormone could be further extrapolated by testing a larger range of concentrations. By testing some very low concentrations and some very high concentrations it may be possible to observe different degrees of inhibition or promotion. In these experiments, it would also be useful to surface sterilize the seeds with a sodium hypochlorite solution before use to prevent fungal or bacterial contamination from skewing results. The presence of fungal and bacterial contamination is most likely the major cause of the variation observed in most of the experiments.

There is still a large area for the improvement and optimization of a spectrophotometric assay for endo-β-mannanase. The initial results showed that this is a promising assay which will provide a faster, more cost-effective and accurate method of detecting endo-β-mannanase in extracts. With more research, this assay has the potential to replace the Congo red dye diffusion assay.

Another area of interest for future work would be to determine the presence, spatial and temporal localization and number of isoforms of endo-β-mannanase present in barley. By comparing and contrasting these isoforms between the two

cultivars it might be possible to explain the differences in endo-β-mannanase production detected between the two cultivars. More barley cultivars would also be incubated in this line of further investigation.

The final, and largest, area of research prompted by these findings would be to use the recently cloned barley endo-β-mannanase gene (Hrmova et al., 2006) and look at the effects of over-expression or down-regulation on a variety of parameters. Previous studies have cloned the mannanase gene of bacteria such as of *Bacillus subtilis* (Ethier et al., 1998) and plants such as tomato (Bewley et al., 1997).

A cDNA encoding a mannanase from the endosperm of germinated tomato seeds has been isolated and characterized by Bewley (1997). The amino acid sequence deduced from the 5'-region of the cDNA exactly matches the sequence of the 65 NH_2-terminal amino acids determined directly from the purified enzyme. Overall, the tomato enzyme exhibits only 28-30% sequence identity with fungal mannanase. However, there are more highly conserved regions, which may represent catalytic and substrate-binding areas in the enzyme (Bewley et al., 1997). While there is only 28-30% sequence homology between tomato seed mannanase and a fungal mannanase, there may be a much greater similarity between two seed mannanase such as lettuce and tomato even though they are of different species. For example Hrmova et al (2006) have recently shown that the barley endo-β-mannanase gene HvMan1 shows 73.6% sequence similarity with the tomato (1,4)-β-D-mannan endohydrolase crystal structure 1RH9. These heterologous endo-β-mannanase cDNAs could also be transferred to barley for expression in the endosperm during germination and their effects on the brewing performance of the grains monitored.

The barley endo-β-mannanase cDNA could be used, with the appropriate gene constructs, in barley transformation, yielding either an over-expression or down-regulation of the enzyme. It would be of interest to monitor the effects of both over-expression and down-regulation on the mechanical strength of the seed and the overall reserve mobilization, particularly endosperm mannan.

Hypothetically, the over-expression of endo-β-mannanase could contribute to a decrease in the mechanical strength of the seed faster than the unmodified seed as there is a larger quantity of the enzyme resulting in a reduction in the time needed to degrade the endosperm walls. The opposite effect would be expected if the gene was down-regulated. Although there have been no documented cases, there is the

possibility that the over-expression of endo-β-mannanase may result in end product (mannose or mannose oligosaccharide) inhibition. The level of endosperm mannan mobilized in the modified cell may be inhibitory to the production of endo-β-mannanase, other enzymes or cellular functions, whereas the normal level in the unmodified cell may not cause this. This would need to be examined more closely by comparing enzyme levels, hormone levels and germinative characteristics of modified barley seeds with those of unmodified barley seeds.

If there is no inhibition of normal germinative processes by increased mobilization of endosperm mannan, there would be an increase in the concentration of free mannose in the seed. Potentially the seedling would be supplied with an abundant alternative source of sugar for growth. This might leave a greater percentage of total endosperm glucose available for fermentation by yeast. Therefore the modified barley seed could be of great potential interest to the brewery industry.

REFERENCES

ANDERSON, P. M., OELKE, E. A., SIMMONS, S. R. (1995) Growth and development guide for spring barley. Minnesota, University of Minnesota.

ARABLE FAMING, from An Encyclopedia of New Zealand, edited by A. H. McLintock, originally published in 1966.
Te Ara - The Encyclopedia of New Zealand, updated 18-Sep-2007

BAMFORTH, C. W. (1998) *Beer: tap into the art and science of brewing,* New York, Insight Books.

BAMFORTH, C. W., BARCLAY, A. H. P. (1993) Malting technology: the uses of malt. Page: 298. Barley: Chemistry and Technology. A.A.C.C .Inc. St. Paul, Minnesota, USA.

BEER GLOBAL INDUSTRY GUIDE (2006) Research and markets. Retrieved 17/02/2008

BERGER, F. (1999) Endosperm development. *Current Opinion in Plant Biology,* 2, 28-32.

BEWLEY, J. D. (1997) Seed germination and dormancy. *The Plant Cell*, 9, 1055-1066.

BEWLEY, D. J., BANIK, M., BOURGAULT, R., FEURTADO, J. A., TOOROP, P., HILHORST, H. W. M. (2000) Endo-β-mannanase activity increases in the skin and outer pericarp of tomato fruits during ripening. *Journal of Experimental Botany,* 51, 529-538.

BEWLEY, D. J., BURTON, R. A., MOROHASHI, Y., FINCHER, G. B. (1997) Molecular cloning of a cDNA encoding a (1→4)-β-mannan endohydrolase from the seeds of germinated tomato (*Lycopersicon esculentum*). *Planta,* 203, 454-459.

BOURGAULT, R., BEWLEY, D. J. (2002) Gel diffusion assays for endo-β-mannanase and pectin methylesterase can underestimate enzyme activity due to proteolytic degradation: A remedy. *Analytical Biochemistry,* 300, 87-93.

BOURGAULT, R., BEWLEY, D. J. (2002) Variation in its C-Terminal amino acids determines whether endo-beta-mannanase is active or inactive in ripening tomato fruits of different cultivars. *Plant Physiology,* 130, 1254-1262.

BOURGAULT, R., OAKLEY, A. J., BEWLEY, D. J., WILCE, M. C. J. (2005) Three-dimensional structure of (1,4)-β-D-mannan mannanohydrolase from tomato fruit. *Protein Science,* 14, 1233-1241.

BRADFORD, K. J., DOWNIE, B. A., GEE, O. H., ALVARADO, V., YANG, H., DAHAL, P. (2003) Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. *Plant Physiology,* 132, 1560-1576.

BUCKERIDGE, M. S., DOS SANTOS, H. P., TINE, M. A. S. (2000) Mobilization of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry,* 38, 141-156.

CARPITA, N. C. (1996) Structure and biogenesis of the cell wall of grasses. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47, 445-476.

DA SILVA, A. E. A., TOOROP, P. E., VAN AELST, A. C., HILHORST, H. W. M. (2004) Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination. *Planta,* 220, 251-261.

DA SILVA, J. M., DA SILVA, A. B., PÁDUA. (2007) Modulated chlorophyll a fluorescence: a tool for teaching photosynthesis. *Journal of Biological Education,* 41, 178-183.

DASKIRAN, M., TEETER, R. G. (2001) Effect of endo-β-D-Mannanase (Hemicell®) Supplementation on Growth Performance of Two-Week-Old Broilers Fed Diets Varying in β-Mannan Content. *2001 Animal Science Research Report,* Oklahoma Agricultural Experiment Station.

DAVIES, G. J., MACKENZIE, L., VARROT, A., DAUTER, M., BRZOZOWSKI, A. M., SCHULEIN, M., WITHERS, S. G. (1998) Snapshots along an enzymatic reaction coordinate: analysis of a retaining α-glycoside hydrolase. *Biochemistry,* 37, 11707-11713.

DIRK, L. M. A., GRIFFEN, A. M., DOWNIE, B., BEWLEY, D. J. (1995) Multiple isozymes of endo-β-mannanase in dry and imbibed seeds. *Phytochemistry,* 40, 1045-1056.

DOAK, M. (2003) Situation and Outlook for New Zealand Agriculture and Forestry. *SONAF 2002/2003.* Christchurch, Ministry of Agriculture and Forestry.

DOONAN, (2004) Protein purification protocols. *Methods in microbiology, 59.* Humana Press.

DOWNIE, B., HILHORST, H. W. M., BEWLEY, D. J. (1994) A new assay for quantifying endo-β-D-mannanase activity using Congo Red dye. *Phytochemistry,* 36, 829-835.

ETHIER, N., TALBOT, G., SYGUSCH, J. (1998) Gene cloning, DNA sequencing, and expression of thermostable beta-mannanase from *Bacillus stearothermophilus*. *Applied Environmental Microbiology,* 64, 4428-4432.

GEPTS, P. (2002). A Comparison between Crop Domestication, Classical Plant Breeding, and Genetic Engineering. *Crop Science,* 42, 780–1790

GIBSON, S. I. (2005) Control of plant development and gene expression by sugar signalling. *Current Opinion in Plant Biology,* 8, 93-102.

GOMEZ-CADENAS, A., ZENTELLA, R., WALKER-SIMMONS, M. K., HO, T.-H. D. (2001) Gibberellin/abscisic acid antagonism in barley aleurone cells: site of action of the protein kinase PKABA1 in relation to gibberellin signalling Molecules. *Plant Cell,* 13, 667-679.

GONG, X. M., BASSEL, G. W., WANG, A. X., GREENWOOD, J. S., BEWLEY, J. D. (2005) The emergence of embryos from hard seeds is related to the structure of the cell walls of the micropylar endosperm, and not to endo-beta-mannanase activity. *Annals of Botany,* 96, 1165-1173.

GRAMENE (2006a) Barley Taxonomy. *Gramene - A resource for comparative grass genomics.* Gramene Project.

GRAMENE (2006b) Doorway to the land of *Hordeum*. *Gramene - A resource for comparative grass genomics.*

GUBITZ, G. M., HAYN, M., SOMMERAUER, M., STEINER, W. (1996) Mannan-degrading enzymes from *Sclerotium rolfsii:* Characterisation and synergism of two endo [beta]-mannanases and a [beta]-mannosidase. *Bioresource Technology,* 58, 127-135.

HAHN-HAGERDAL, B., GALBE, M., GORWA-GRAUSLUND, M. F., LIDEN, G., ZACCHI, G. (2006) Bio-ethanol - the fuel of tomorrow from the residues of today. *Trends in Biotechnology,* 24, 549-556.

HARTMAN, L. F., OPPENHEIM, A. L. (1950) On beer and brewing techniques in ancient Mesopotamia. *Journal of the American Oriental Society: Supplement 10.*

HAMES, B. D. (1998) Gel electrophoresis of proteins: A practical approach. Oxford University Press, Page 13-33

HENRISSAT, B. (2007) Glycosyl hydrolase families: classification and list of entries. IN UNIPROT (Ed.) 51.7 ed. Marseille ExPASy.

HILLMER, S., GILROY, S., JONES, R. L. (1993) Visualizing enzyme secretion from individual barley (*Hordeum vulgare*) aleurone protoplasts. *Plant Physiology,* 102, 279-286.

HOLOPAINEN, U. R. M., WILHELMSON, A., SALMENKALLIO-MARTTILA, M., PELTONEN-SAINIO, P., RAJALA, A., REINIKAINEN, P., KOTAVTA, E., SIMOLIN, H., HOME, S. (2005) Endosperm structure affects the malting quality of barley (*Hordeum vulgare* L.). *Journal of Agricultural and Food Chemistry,* 53, 7279-7287.

HOMRICHHAUSEN, T. M., HEWITT, J. R., NONOGAKI, H. (2003) Endo-beta-mannanase activity is associated with the completion of embryogenesis in imbibed carrot (*Daucus carota* L.) seeds. *Seed Science Research,* 13, 219-227.

HORNSEY, I. S. (1999) *Brewing,* Cambridge, Royal Society of Chemistry.

HRMOVA, M., BURTON, R. A., BIELY, P., LAHNSTEIN, J., FINCHER, G. B. (2006) Hydrolysis of (1,4)-β-D-mannans in barley (*Hordeum vulgare* L.) is mediated by the concerted action of (1,4)-β-D-mannan endohydrolase and β-D-mannosidase. *Biochemical Journal*, 399, 77-90.

INGLE, J. & HAGEMAN, H. R. (1965) Metabolic changes associated with the germination of corn. III. Effects of gibberellic acid on endosperm metabolism. *Plant Physiology,* 40**,** 672-675.

ISHTAR SNOEK, I. S., STEENSMA, H. Y. (2007) Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. *Yeast,* 24, 1-10.

JACKSON, M. E., GERONIAN, K., KNOX, A., MCNAB, J., MCCARTNEY, E. A (2004) A dose-response study with the feed enzyme β-mannanase in broilers provided with corn-soybean meal based diets in the absence of antibiotic growth promoters. *Poultry Science*, 83, 1992-1996.

JACOBSEN, J. V., KNOX, R. B., PYLIOTIS, N. A. (1971) The structure and composition of aleurone grains in the barley aleurone layer. *Planta*, 101, 189-209.

JONES, B. L. (2005) The endogenous endoprotease inhibitors of barley and malt and their roles in malting and brewing. *Journal of Cereal Science,* 42, 271-280.

JUGE, N., LE GAL-COEFFET, M. F., FURNISS, C. S. M., GUNNING, A. P., KRAMHOFT, B., MORRIS, V. J., WILLIAMSON, G., SVENSSON, B. (2002) The starch binding domain of glucoamylase from *Aspergillus niger*: overview of its structure, function, and role in raw-starch hydrolysis. *Biologia,* 57, 239-245.

KEPCZYNSKA, E., ZIELINSKA, S. (2006) Regulation of *Medicago sativa* L. somatic embryos regeneration by gibberellin A(3) and abscisic acid in relation to starch content and alpha-amylase activity. *Plant Growth Regulation,* 49, 209-217.

KHANDEPARKAR, R., BHOSLE, N. B. (2007) Application of thermoalkalophilic xylanase from *Arthrobacter sp* MTCC 5214 in biobleaching of kraft pulp. *Bioresource Technology,* 98**,** 897-903.

KHANONGNUCH, C., ASADA, K., TSURUGA, H., OOI, T., KINOSHITA, S., LUMYONG, S. (1998) Beta-mannanase and xylanase of *Bacillus subtilis* 5H active for bleaching of crude pulp. *Journal of Fermentation and Bioengineering,* 86, 461-466.

KONNING, R. E. (1994) Plant physiology information website. *Eastern Connecticut State University*. Retrieved 14/04/2008.

KONTOS, F., SPYROPOULOS, C. G. (1996) Seed coat inhibits the production of alpha-galactosidase and endo-beta-mannanase in the endosperm of developing carob seeds. *Plant Physiology and Biochemistry,* 34, 787-793.

KUCERA, B., COHN, M. A., LEUBNER-METZGER, G. (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research,* 15, 281-307.

LAINE, K., JARVINEN, K., PATE, D. W., URTTI, A., JARVINEN, T. (2002) Effect of the enzyme inhibitor, phenylmethylsulfonyl fluoride, on the IOP profiles of topical anandamides. *Investigative Ophthalmology and Visual Sciences.,* 43, 393-397.

LE NOURS, J., ANDERSON, L., STOLL, D., STÅLBRAND, H. & LO LEGGIO, L. (2005) The Structure and Characterization of a Modular Endo-β-1,4-mannanase from *Cellulomonas fimi*. *Biochemistry*, 44, 12700-12708

MADAN BABU, M. (2007) TIM Barrel Analysis. Anna University, Centre for Biotechnology.

MARRACCINI, P., ROGERS, J. W., ALLARD, C., ANDRÉ, M. L., CAILLET, V., LACOSTE, N., LAUSANNE, F., MICHAUX, S. (2001) Molecular and biochemical characterization of endo-β-mannanases from germinating coffee (*Coffea arabica*) grains. *Planta*, 231, 296-308.

MCCLEARY, B. V. (1978) A simple assay procedure for β-D-mannanase. *Carbohydrate Research,* 67, 213-221.

MCCLEARY, B. V. (1988) Beta-D-Mannanase. *Methods in Enzymology,* 160, 596-610.

MEGAZYME (2007) Endo-beta-MANNANASE from Aspergillus niger (Lot 008101). Megazyme.

MICHÉ, L., BALANDREAU, J. (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Applied and Environmental Microbiology*, 67, 3046-3052.

MONTIEL, M. D., HERNANDEZ, M., RODRIGUEZ, J., ARIAS, M. E. (2002) Evaluation of an endo-beta-mannanase produced by *Streptomyces ipomoea* CECT 3341 for the biobleaching of pine kraft pulps. *Applied Microbiology and Biotechnology,* 58, 67-72.

MOREAU, T. (2005) Introduction to Proteases. Tours, France.

NAPOLITANO, A., LANZUISE, S., RUOCCO, M., ARLOTTI, G., RANIERI, R., KNUTSEN, S. H., LORITO, M., FOGLIANO, V. (2006) Treatment of cereal products with a tailored preparation of *Trichoderma* enzymes increases the amount of soluble dietary fibre. *Journal of Agricultural and Food Chemistry,* 54, 7863-7869.

NONOGAKI, H., MOROHASHI, Y. (1999) Temporal and spatial pattern of the development of endo-[beta]-mannanase activity in germinating and germinated lettuce seeds. *Journal of Experimental Botany,* 50, 1307.

NZGSTA (2007) New Zealand Grain and Seed Trade Association.

PALMA, K., KERMODE, A. R. (2003) Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (*Hordeum vulgare* L.) aleurone layer cells. *Free Radical Biology and Medicine,* 35, 1261-1270.

PEGO, J. V., WEISBEEK, P. J., SMEEKENS, C. M. (1999) Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. *Plant Physiology,* 119, 1017-1024.

PYNE GOULD GUINNESS (2006) Personal communication

RAMAKRISHMA, P., AMPRIPHALE, D. (2005) The perisperm-endosperm envelope in *Cucumis*: structure, proton diffusion and cell wall hydrolysing activity. *Annals of Botany,* 96, 769-778.

REGALDO, C., GARCÍA-ALMENDÁREZ, B. E., VENEGAS-BARRERA, L. M., TELLÉZ-JUARDO, A., RODRÍGUEZ-SERRANO, G., HUERTA-OCHOA, S., WHITAKER, J. R. (2000) Production, partial purification and properties of β-mannanases obtained by solid substrate fermentation of spent soluble coffee waste and copra paste using *Aspergillus oryzae* and *Aspergillus niger*. *Journal of the Science of Food and Agriculture,* 80, 1343-1350.

RILEY, J. M. (1987) Gibberellic acid for fruit set and seed germination. *California Rare Fruit Growers Journal*, 19, 10-12.

RITCHIE, S., SWANSON, S. J., GILROY, S. (2000) Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: new insights from cell and molecular biology. *Seed Science Research,* 10, 193-212.

ROEHR, M. (2001) *The biotechnology of ethanol: classical and future applications*. Wiley-VCH. Chapter 2, page 18.

ROSE, T., DI CERA, E. (1999) Serine Proteases. *Department of Biochemistry and Molecular Biophysics.* Saint Louis, Washington University School of Medicine.

SAMUEL, D., (1996) Archaeology of ancient Egyptian beer. *Journal of the American Society of Brewing Chemists,* 54, 3-12.

SABINI, E., WILSON, K. S., SIIKA-AHO, M., BOISSET, C., CHANZY, H. (2000) Digestion of single crystals of mannan I by an endo-mannanase from *Trichoderma reesei*. *European Journal of Biochemistry,* 267, 2340-2344.

SCHELLING, K., BORN, K., WEISSTEINER, C., KÜHBAUCH, W. (2003) Relationships between yield and quality parameters of malting barley (Hordeum vulgare L.) and phenological and meteorological data. *Journal of Agronomy and Crop Science,* 189, 113-122

SCANDRETT, C. (1997) Maillard Reactions .101: Theory. *The Brewery Technical Library.* The Brewery

SCHRODER, R., WEGRZYN, T. F., SHARMA, N. N., ATKINSON, R. G. (2006) LeMAN4 endo-beta-mannanase from ripe tomato fruit can act as a mannan transglycosylase or hydrolase. *Planta,* 224, 1091-1102.

SKRIVER, K., OLSEN, F. L., ROGERS, J. C., MUNDY, J. (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proceedings of the National Academy of Science,* 88, 7266-7270.

SRIVASTAVA, L. M., (2002) *Plant growth and development: hormones and the environment*, Academic Press, Chapter 19, page 449-450.

TAIZ, L., ZEIGER, E. (2006) *Plant Physiology,* Sunderland, Sinaeur Associates Inc.

TAMPION, J. (1972) A viscometric assay for beta-mannanase. *Enzymolgia,* 42, 523-525.

UNGER, R. W. (2004) *Beer in the middle ages and the Renaissance*. University of Pennsylvania Press. Chapter 2, page 10.

VARNER, J. E., MENSE, R. M. (1972) Characteristics of the process of enzyme release from secretory plant cells. *Plant Physiology,* 49, 187-189.

WANG, A. X., WANG, X. F., REN, Y. F., GONG, X. M., BEWLEY, J. D. (2005) Endo-beta-mannanase and beta-mannosidase activities in rice grains during and following germination, and the influence of gibberellin and abscisic acid. *Seed Science Research,* 15, 219-227.

WORLD HEALTH ORGANIZATION (1974) Toxological evaluation of some food colours, enzymes, flavours, enhancers, thickening agents, and certain food additives. *International Programme of Chemical Safety.* Geneva, World Health Organization.

WILSON, S. M., BURTON, R. A., DOBLIN, M. S., STONE, B. A., NEWBIGIN, E. J., FINCHER, G. B., BACIC, A. (2006) Temporal and spatial appearance of wall polysaccharides during cellularization of barley (*Hordeum vulgare*) endosperm. *Planta,* 224, 655-667.

WITT, D., KLAJN, P., BARSKI, P., GRZYBOWSKI, B. A. (2007) Applications, properties, and synthesis of w-functionalized n-alkanenthiols and disulfides - the building blocks of self-assembled monolayers. Evanston, Northwestern University.

YUAN, K., WYSOCKA-DILLER, J. (2006) Phytohormone signalling pathways interact with sugars during seed germination and seedling development. *Journal of Experimental Botany,* 57, 3359-3367.

ZANEWICH, K. P., ROOD, S. B. (1993). Distribution of endogenous gibberellins in vegetative and reproductive organs of *Brassica*. *Journal of Plant Growth Regulation*, 12, 41-46.

ZHOU, P., REGENSTEIN, J. M. (2006) Determination of total protein content in gelatin solutions with the Lowry or Biuret assay. *Journal of Food Science,* 71, C474-C479.

APPENDIX

1.0. Congo Red Dye Assay Components

1.1. Citric Acid and Sodium Dibasic Phosphate Buffer

A. 0.1 M solution of citric acid (19.21 g L^{-1})

B. 0.2 M solution of dibasic sodium phosphate ((53.65 g L^{-1}) of $Na_2HPO_4.7H_2O$))

100 mL (pH 3) = 39.8 mL (A) + 10.2 mL (B) + 50 mL dH_2O

100 mL (pH 5) = 24.3 mL (A) + 25.7 mL (B) + 50 mL dH_2O

100 mL (pH 7) = 6.5 mL (A) + 43.6 mL (B) + 50 mL dH_2O (McIlvaine Buffer)

1.2. Locust Bean Gum Plates

0.1% (w/v) Locust Bean Gum in pH 7 Buffer

Plates hold 20 mL of buffer /substrate solution

100 mL (pH 7) = 6.5 mL (A) + 43.6 mL (B) + 50 mL dH_2O + 0.1 g Locust Bean Gum

1.3. Enzyme Extraction Buffer

3 M LiCl in McIlvane Buffer

3 M LiCl = 127.182 g L^{-1}

100 mL = 6.5 mL (A) + 43.6 (B) + 50 mL dH_2O + 12.718 g (LiCl)

1.4. Congo Red Dye Solution

Congo red dye (1% (w/v)) in 0.2 M K_2HPO_4 and 0.0.5% (w/v) NaN_3

K_2HPO_4 = 174.18 g L^{-1}, 0.2 M K_2HPO_4 = 34.8 g L^{-1}
Congo Red dye 1% (w/v/) = 1 g 100 mL^{-1}
NaN_3 0.05% (w/v) = 0.05 g 100 mL^{-1}

500 mL Congo red dye solution = 500 mL 0.2 M K_2HPO_4 + 0.5 g Congo red dye
+ 0.025 g NaN_3

1.5. Washing Solution

Plates washed in 3 M NaCl solution
1 L (3 M NaCl) = 1 L dH_2O + 58.44 g NaCl

2.0. Protein Quantification and Inhibitor Components

2.1. Folin-Lowry Reagents

A. 2% (w/v) Na_2CO_3 in 0.1 M NaOH
B. 1% (w/v) NaK Tartrate in dH_2O
C. 0.5% (w/v) $CuSO_4.5H_20$ in dH_2O
D. 150 mL (D) = 144 mL (A) + 3 mL (B) + 3 mL (C)
E. Phenol reagent = 1:1 (Folin phenol:dH_2O)

2.2. Protease Inhibitor Cocktail

PMSF (1 mL) = 0.038 g PMSF in 1 mL methanol
Iodoacetate (1 mL) = 0.042 g iodoacetate in 1 mL dH_2O
Phenanthroline (1 mL) = 0.0198 g phenanthroline in 1 mL methanol

Cocktail

- 200 μL PMSF
- 200 μL iodoacetate
- 100 μL phenanthroline
- 400 μL dH_2O extraction buffer

3.0. Sugar/Hormone Solutions

3.1. Glucose

10 mM = 0.072 g in 40 mL dH_2O
20 mM = 0.144 g in 40 mL dH_2O
30 mM = 0.216 g in 40 mL dH_2O

30 mM (Manitol) = 0.218 g in 40 mL dH_2O

1% (w/v) Phytagel agar added to solution = 0.4 g in 40 mL

3.2. Sucrose

10 mM = 0.136 g in 40 mL dH_2O

20 mM = 0.272 g in 40 mL dH_2O

30 mM = 0.408 g in 40 mL dH_2O

30 mM (Sorbitol) = 0.218 g in 40 mL dH_2O

1% (w/v) Phytagel agar added to solution = 0.4 g in 40 mL

3.3. GA

83 μM = 1.15 mg in 40 mL dH_2O

167 μM = 2.31 mg in 40 mL dH_2O

245 μM = 3.4 mg in 40 mL dH_2O

1% (w/v) Phytagel agar added to solution = 0.4 g in 40 mL

Control Plate = dH_2O + 1% (w/v) Phytagel agar

3.4. IAA

314 μM = 2.2 mg in 40 mL dH_2O

642 μM = 4.5 mg in 40 mL dH_2O

970 μM = 6.8mg in 40 mL dH_2O

1% (w/v) Phytagel agar added to solution = 0.4 g in 40 mL

Control Plate = dH_2O + 1% (w/v) Phytagel agar

3.5. ABA

142 μM = 1.51 mg in 40 mL dH_2O

286 μM = 3.02 mg in 40 mL dH_2O

505 μM = 4.54 mg in 40 mL dH_2O

1% (w/v) Phytagel agar added to solution = 0.4 g in 40 mL

Control Plate = dH_2O + 1% (w/v) Phytagel agar